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Ionic storm in hypoxic/ischemic stress: Can opioid receptors subside it?

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Abstract

Neurons in the mammalian central nervous system are extremely vulnerable to oxygen deprivation and blood supply insufficiency. Indeed, hypoxic/ischemic stress triggers multiple pathophysiological changes in the brain, forming the basis of hypoxic/ischemic encephalopathy. One of the initial and crucial events induced by hypoxia/ischemia is the disruption of ionic homeostasis characterized by enhanced K^+ efflux and Na^+ , Ca^{2+} and Cl^- influx, which causes neuronal injury or even death. Recent data from our laboratory and those of others have shown that activation of opioid receptors, particularly δ -opioid receptors (DOR), is neuroprotective against hypoxic/ischemic insult. This protective mechanism may be one of the key factors that determine neuronal survival under hypoxic/ischemic condition. An important aspect of the DOR-mediated neuroprotection is its action against hypoxic/ischemic disruption of ionic homeostasis. Specially, DOR signal inhibits Na^+ influx through the membrane and reduces the increase in intracellular Ca^{2+} , thus decreasing the excessive leakage of intracellular K^+ . Such protection is dependent on a PKC-dependent and PKA-independent signaling pathway. Furthermore, our novel exploration shows that DOR attenuates hypoxic/ischemic disruption of ionic homeostasis through the inhibitory regulation of Na^+ channels. In this review, we will first update current information regarding the process and features of hypoxic/ischemic disruption of ionic homeostasis and then discuss the opioid-mediated regulation of ionic homeostasis, especially in hypoxic/ischemic condition, and the underlying mechanisms.

Keywords

opioids; δ -opioid receptor; hypoxia/ischemia; neuroprotection; Na^+ channels; K^+ channels; Ca^{2+} channels; ionic homeostasis

1. Introduction

Neurons in the mammalian central nervous system are extremely vulnerable to hypoxic/ischemic stress due to their high demand for energy from oxidative phosphorylation and extremely limited capacity in glucose storage. Hypoxia/ischemia triggers a series of alterations in neuronal events, such as changes in membrane function, metabolism, intracellular transduction, and even cell morphology, which eventually leads to cell injury or death. Initially, the most important alteration is the disruption of ionic homeostasis, that is characterized by

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enhanced K^+ efflux and influx of Na^+ and Ca^{2+} (Hansen, 1985; Jiang and Haddad, 1991; Xia et al, 1992; Chao et al, 2007a, 2007b, 2008, 2009; Kang et al, 2009).

Accumulating evidence has demonstrated that the disruption of ionic homeostasis is a key trigger for neuronal injury and death. Over the past two decades, there have been vast and growing interests in the importance of Ca^{2+} and Na^+ in neuronal responses to hypoxic/ischemic stress and their roles in neuronal injury. Recently, much light has been shed on the relationship between the disruption of K^+ homeostasis and neuronal death. In comparison with the harmful role of Ca^{2+} and Na^+ overload in hypoxia/ischemia, the role of K^+ efflux appears to be complicated. The enhanced K^+ efflux used to be considered an adaptive mechanism by decreasing neuronal excitability during hypoxia/ischemia. On the other hand, recent evidence suggests that an excessive loss of neuronal K^+ is casually linked to neuronal damage and death in various conditions including hypoxia/ischemia (Yu et al, 1997, 1999, 2003; Huang et al, 2001; Wei et al, 2003; Liu et al, 2003; Zhao et al, 2006; Nistico et al, 2007; Karki et al, 2007). Presently, the process and features of hypoxic/ischemic disruption of ionic homeostasis as well as ionic interactions during the process are not well understood, despite their importance in terms of understanding neuronal responses to hypoxia/ischemia. In fact, such information is largely neglected because much attention in the past was paid to the resultant consequence of disturbed ionic homeostasis under the serious circumstance. Therefore, one of the important purposes of this review is to update the information concerning the process and features of hypoxia/ischemia-induced disruption of ionic homeostasis.

Endogenous opioids mainly play an inhibitory role in cell activities by binding to three major classes (δ , μ , κ) of opioid receptors (DOR, MOR, KOR), which are widely distributed throughout the central nervous system (Xia and Haddad, 1991, 2001; Mansour et al, 1994; Hiller et al, 1996; Law et al, 2000). Opioid receptors belong to classical G protein-coupled receptor superfamily (Raynor et al, 1994; Law et al, 2000;). Recent studies, especially those from our laboratory, have demonstrated that activation of opioid receptors, particularly DOR, is neuroprotective against hypoxic/ischemic insults and DOR activity may be one of the key factors to determine neuronal survival during hypoxic/ischemic as well as excitotoxic stress (Zhang et al, 2000, 2002, 2006; Borlongan et al, 2004, ²⁰⁰⁹; Horiuchi et al, 2004, ²⁰⁰⁸; Lim et al, 2004; Ma et al, 2005; Zhao et al, 2006; Su et al, 2007; Xiong et al, 2007; Iwata et al, 2007; Charron et al, 2008; Govindaswami et al, 2008; Kao et al, 2008; Pamenter and Buck, 2008; Tian et al, 2008a, 2008b; Peng et al, 2009; Zhu et al, 2009; Yang et al, 2009). Early evidence suggested that opioids may alter ionic transportation across the membrane in normoxic conditions (North et al, 1987; Fan et al, 1991, 1993; Piros et al, 1996). Our recent data further indicate that an important aspect of the DOR-mediated neuroprotection is its action against disruption of ionic homeostasis during anoxia or ischemia (Chao et al, 2006, 2007a, 2007b, 2008, 2009; Kang et al, 2009). Therefore, it appears that opioids are involved in the regulation of ionic homeostasis in both rest and hypoxic/ischemic conditions. We will summarize the research progress in this field and discuss the underlying mechanism based on the current information after reviewing hypoxic/ischemic disruption of ionic homeostasis.

This review mainly covers the data available in PubMed in the last 10 years. In some subjects, we also extended our search to the earlier literature for more details.

As commonly used in the literature, the terms of hypoxia, anoxia, ischemia and chemical ischemia are widely used in this review. Their definitions are listed in Table 1 for reader's reference.

2. Ionic homeostasis and neuronal function

Ions are not uniformly distributed in neurons. They differentially transport across the membrane depending on neuronal conditions. In normal conditions, there is a steep

electrochemical gradient across the plasma membrane with the extracellular fluid being abundant in Na^+ (concentrations up to 150 mM) and relatively poor in K^+ (around 3 mM). Conversely, the concentrations of these two ions are reversed inside the cells, with up to 150 mM of K^+ and 4–12 mM of Na^+ . Other main ions (e.g. Ca^{2+} and Cl^-) are also asymmetrically distributed, with high levels in the extracellular space (Hansen, 1985; Panayiotidis et al, 2006).

The maintenance of this steep electrochemical gradient across the membrane is determined by at least two major factors: selective permeability of membrane to ions and active transport of ions. Lipid bilayer membrane functions as a permeability barrier selective to ions, in which some functional proteins (ionic channels/exchangers/transporters) act as the pathways in ionic movement. Active transport reversely moves the ions against the electrochemical gradients by directly/indirectly consuming the energy from hydrolysis of ATP. Electrogenic $\text{Na}^+\text{-K}^+$ ATPase (also called $\text{Na}^+\text{-K}^+$ pump or Na^+ pump), a transmembrane enzyme with the ability to bind and hydrolyze ATP, is the primary active transporter which functions to actively extrude three Na^+ outside the cell, and move two K^+ into the cells in each cycle of activity. Thus, the primary role of $\text{Na}^+\text{-K}^+$ ATPase is to maintain the high intracellular K^+ and low intracellular Na^+ (Jorgensen et al, 2003; Panayiotidis et al, 2006). An inhibition of Na^+ pumps induces an increase in $[\text{Na}^+]_i$ and a fall in $[\text{K}^+]_i$ in neurons (Alvarez-Leefmans et al, 1994). With $[\text{K}^+]_e/[\text{Na}^+]_i$ increase, $\text{Na}^+\text{-K}^+$ ATPase is expected to be activated and brings back $[\text{K}^+]_e/[\text{Na}^+]_i$ to resting levels (Jorgensen et al, 2003; Panayiotidis et al, 2006). Besides the key role of $\text{Na}^+\text{-K}^+$ ATPase, other machinery mechanisms are also involved in the steady-state maintenance of highly asymmetric concentrations of major inorganic cations and anions (Russell, 2000; Kofuji and Newman, 2004; Dipolo and Beaugé 2006).

The concentrations of ions are in dynamic balance under physiological conditions. With neuronal activity, the ionic concentrations fluctuate within a small range, and this fluctuation is often transient. For example, using ion-sensitive microelectrodes, it has been found that repetitive stimulation of the dorsal root nerves causes an increase in $[\text{Na}^+]_i$ of 4–9 mM, a decrease in $[\text{K}^+]_i$ of 8–20 mM and an increase in $[\text{K}^+]_e$ of 2.0–3.5 mM to a so-called ceiling level followed by a post-stimulation K^+ undershoot, and an extracellular pH decrease after an alkaline shift in ganglions/spinal cords (Ballanyi et al 1983; Syková and Chvátal, 1993). By intracellular Na^+ imaging with Na^+ -sensitive fluorescent dye SBFI, spontaneous and transient $[\text{Na}^+]_i$ increase (+5 mM) was observed in 25% of cultured hippocampal neurons (Rose and Ransom, 1997). An action potential-induced dendritic $[\text{Na}^+]_i$ increase was accompanied by a rise in $[\text{Ca}^{2+}]_i$ in hippocampal CA1 neurons (Spruston et al, 1995). Following ionic concentration fluctuation, a rapid increase in activity of ionic pumps (e.g. $\text{Na}^+\text{-K}^+$ ATPase, Ca^{2+} ATPase) and exchangers (e.g. $\text{Na}^+/\text{Ca}^{2+}$ exchangers) as well as spatial ionic buffering mechanisms brings back the ionic gradients or levels towards their previous levels (Russell, 2000; Jorgensen et al, 2003; Kofuji and Newman, 2004; Dipolo and Beaugé 2006; Panayiotidis et al, 2006).

Maintenance of ionic homeostasis is critical to normal functions of neurons. Cells cannot live without an appropriate balance of ions across their cell membrane. The transmembrane ionic gradients provide the critical and necessary conditions not only for the maintenance of membrane potential, generation of action potential, synaptic transmission, electrogenic transport of neurotransmitters and neuronal plasticity, but also for regulating osmotic balance, cell volume, proper pH and ionic microenvironment for the normal functioning of macromolecules including enzymes, Na^+ -coupled transport of nutrients into cells. Therefore, the changes in ionic concentrations or blockade of transmembrane ionic movement can affect a wide variety of neuronal processes (Syková and Chvátal, 1993; Alvarez-Leefmans et al, 1994; Qian and Saggau, 1999; Yu and Salter 1998; D'Ambrosio et al, 1999; Hu et al, 2001; Franceschetti et al, 2003; Rose, 2003; Page and Di Cera 2006; Cui et al, 2009). Since neuronal

function is critically dependent on the maintenance of Na^+ , K^+ , Ca^{2+} and Cl^- transmembrane distribution, deregulated ionic imbalances as well as loss of ion gradients are considered to be key events in brain pathological consequences (Hansen, 1985; Yu, 2003; Franco et al, 2006; Chao et al, 2007a, 2007b, 2008, 2009).

3. Hypoxia/ischemia -induced disruption of ionic homeostasis

Ion gradients are very sensitive to impairment of energy metabolism (Silver et al, 1997; Hertz, 2008). Under normal condition, the ionic concentrations both inside and outside the membrane are relatively constant with fluctuation in a small range, because brain possesses homeostatic mechanisms that maintain constant ionic concentrations in the cerebrospinal fluid and neurons (Hansen, 1985; Kofuji and Newman, 2004). However, such homeostatic mechanisms can be disrupted in certain pathophysiological conditions, particularly in energy depletion. For example, hypoxia, ischemia, or oxygen-glucose deprivation (OGD, simultaneous ischemia) can produce considerably longer or sustained changes in ionic concentrations that are characterized by enhanced K^+ efflux and Na^+ -, Ca^{2+} - and Cl^- influx (Sick et al, 1982; Hansen, 1985; Jiang and Haddad, 1991; Müller and Somjen, 2000a, 2000b; Galeffi et al, 2004; Martinez-Sanchez et al, 2004; Pond et al, 2006; Chao et al, 2006, 2007a, 2007b, 2008, 2009; Kang et al, 2009). This disturbance of ion regulation in the models of the intact brain (Sick et al, 1982; Hansen, 1985; Zetterström et al, 1995; Reid and Paterson, 1996) as well as in vitro brain slices (Müller and Somjen, 2000a, 2000b; Chao et al, 2007a, 2007b, 2008, 2009; Kang et al, 2009) can be distinctly divided into two phases. Phase 1 is characterized by a slow and progressive rise in $[\text{K}^+]_e$, with little changes in other ions. Phase 2 is featured by a rapid, additional large increase in $[\text{K}^+]_e$, accompanied by a negative shift in extracellular DC potential, and substantial decrease in extracellular Na^+ , Ca^{2+} and Cl^- concentrations when $[\text{K}^+]_e$ reaches a threshold level in phase 1 as hypoxia/ischemia continues (Hansen, 1985; Zetterström et al, 1995; Reid and Paterson, 1996; Müller and Somjen, 2000a, 2000b; Chao et al, 2007a, 2007b, 2008, 2009). In the following section, we will discuss the main characteristics of hypoxia/ischemia disruption of several major cations.

3.1 Hypoxia/ischemia-induced intracellular calcium accumulation

Evidence has shown that there is an elevation and accumulation of cytosolic Ca^{2+} during global or focal ischemia (Uematsu et al, 1988; Silver and Execińska, 1990, 1992; Kristián and Siesjö, 1998; Nakamura et al, 1999; Vannucci et al, 2001) and in vitro hypoxia or ischemia (e.g., in the brain slices) (Mitani et al, 1993; Nabetani et al, 1997; Fukuda et al, 1998; Pisani et al, 1998b, 1999; Ballanyi and Kulik, 1998; Connor et al, 1999; Zhang and Lipton, 1999; Tjong et al, 2007), organotypic brain slice cultures (Martinez-Sánchez et al, 2004) and acutely isolated or cultured cells (Friedman and Haddad, 1993; Goldberg and Choi, 1993; Tymianski et al, 1993; Murai et al, 1997; Silver et al, 1997; Chen et al, 1999; Diarra et al, 1999; Smith et al, 2004; Kiedrowski, 2007). During hypoxia/ischemia, most of the brain tissues/neurons show a progressive or transient small increase followed by a large sustained increase in cytosolic Ca^{2+} (Silver and Execińska, 1990, 1992; Mitani et al, 1993; Murai et al, 1997; Ballanyi and Kulik, 1998; Fukuda et al, 1998; Chen et al, 1999; Smith et al, 2004; Martinez-Sánchez et al, 2004). The increase in cytosolic Ca^{2+} varies from several hundred nanomolar to micromolar range in magnitude, with different time required for the onset (Silver and Execińska, 1990; Friedman and Haddad, 1993; Nabetani et al, 1997; Zhang and Lipton 1999; Martinez-Sánchez et al, 2004).

Hypoxia/ischemia has been shown to increase $[\text{Ca}^{2+}]_i$. The onset and magnitude of this increase depend on multiple inherent and environmental factors, including the duration (short vs. long) and severity (mild vs. severe) of insult, the age of animals (young vs. old), the region of the brain (hippocampus, cortex vs. other regions such as thalamus, striatum, dorsal vagal neurons, locus coeruleus, cerebellar granule cells), and even the type of cells (neurons vs. glial cells).

For example, there is a minor or moderate increase in cytosolic Ca^{2+} (decades to hundreds nanomolar) with short or transient hypoxia/ischemia, but a large sustained increase in $[\text{Ca}^{2+}]_i$ (up to 35 μM) in severe and prolonged hypoxia/ischemia (Uematsu, et al, 1988; Silver and Execińska, 1990, 1992; Tymianski et al, 1993; Silver et al, 1997; Chen et al, 1999; Zhang and Lipton, 1999; Diarra et al, 1999; Nakamura et al, 1999; Martinez-Sánchez et al, 2004). Young animals show a much less extension and longer latency (up to five times longer) of Ca^{2+} rise in neurons than those of the mature ones during hypoxia/ischemia (Friedman and Haddad, 1993; Nabetani et al, 1997; Vannucci et al, 2001) because the young cells have a higher resistance to energy failure and adaptation capacity for disturbance of Ca^{2+} homeostasis (Hartmann et al, 1996; Nabetani et al, 1997). As compared to glial cells, neurons show a much faster increase in $[\text{Ca}^{2+}]_i$ during hypoxia/ischemic energy limitation (Goldberg and Choi et al, 1993; Silver et al, 1997; Kahlert and Reiser, 2004). This is because neurons lack glycogen storage (Silver et al, 1997) with less active energy generation and have abundant ion pathways with marked suppression of Ca^{2+} extrusion during ischemic and excitotoxic stress (Kiedrowski et al, 1994; Duffy and Macvicar, 1996).

Moreover, there exist regional differences in hypoxic/ischemic cytosolic Ca^{2+} accumulation. The magnitude of the rapid elevation of $[\text{Ca}^{2+}]_i$ is very large in many, but not all hippocampal CA1 and cortical cells. In contrast to hippocampal CA1 and cortical cells, the change in $[\text{Ca}^{2+}]_i$ is smaller and relatively delayed in thalamic and dorsal vagal neurons during hypoxia/ischemia (Silver and Execińska, 1990; Ballanyi and Kulik, 1998). Even in the various parts of the same brain regions, hypoxia/ischemia-induced Ca^{2+} responses may also be different. For example, neocortical cells show a great increase in $[\text{Ca}^{2+}]_i$ in layer II/III than in layer IV, V, or VI during OGD (Fukuda et al, 1998). In hippocampal CA1 neurons, ischemia induces an increase in $[\text{Ca}^{2+}]_i$ up to 30 μM , and this change decreases rapidly to 73 nM during reperfusion. In contrast, the changes in $[\text{Ca}^{2+}]_i$ in CA3 area are much smaller (3 μM) and slightly slower than in CA1 area (Silver and Execińska, 1992). These notions sufficiently reflect a regional difference in the sensitivity to hypoxia/ischemia. In addition, Pisani et al (1999) reported a very interesting phenomenon that OGD-induced $[\text{Ca}^{2+}]_i$ elevation was more pronounced in dendritic branches rather than in somatic regions in striatal neurons, and a similar result was also found in the hippocampus (Zhang and Lipton, 1999). This interesting phenomenon can be explained by the larger surface area/volume ratios in dendrites than in soma, or by the variability of Ca^{2+} channel expression (Ladewig et al, 2003; Vander Jagt et al, 2008), or by higher Ca^{2+} conductance in dendrites than in soma due to more excitatory synaptic inputs and higher density of NMDA receptors in dendrites (Connor et al, 1988; Wadman and Connor, 1992; Vander Jagt et al, 2008). Finally, not only are the onset and magnitude of Ca^{2+} accumulation different with differences in multiple inherent and experimental factors during hypoxia/ischemia, but also posthypoxic/postischemic changes in $[\text{Ca}^{2+}]_i$ show great varieties. For instance, hypoxic/ischemic cytosolic Ca^{2+} accumulation gradually recovers with reperfusion or reoxygenation after short and/or mild hypoxia/ischemia, but exhibits a further increase during reperfusion period after prolonged and/or severe hypoxia/ischemia (Uematsu et al, 1988; Vannucci et al, 2001). This may reflect damaged Ca^{2+} extrusion mechanisms (Bano et al, 2005; Bano and Nicotera, 2007), sequestering mechanisms of free cytosolic Ca^{2+} into organelles (Bergmann and Keller, 2003; Ladewig et al, 2003), and activation of non-selective cationic channels such as TRPM7 (a member of the melastatin branch of the transient receptor potential channel family), and calcium-permeable acid-sensing ion channels (Aarts et al, 2003; Xiong et al, 2004, 2007; Macdonald et al, 2006; Chinopoulos and Adam-Vizi, 2006; Langeslag et al, 2007) in severe and prolonged insults. Thus, hypoxia/ischemia-induced intracellular Ca^{2+} accumulation is clearly evident, but varies much in neurons. This may partially account for differences in neuronal tolerance to hypoxic/ischemic stress.

The hypoxia/ischemia-induced Ca^{2+} accumulation may originate from two sources: (1) an influx of extracellular Ca^{2+} and (2) the release of Ca^{2+} from intracellular store. At least three

factors are involved in the regulation of Ca^{2+} homeostasis, including (1) Ca^{2+} influx and efflux through the plasma membrane; (2) Ca^{2+} uptake and release from intracellular stores; and (3) intracellular Ca^{2+} buffering. Any disruptions of these three aspects would contribute to hypoxia/ischemia-induced increase in $[\text{Ca}^{2+}]_i$.

3.1.1 Ca^{2+} influx and efflux through the plasma membrane during hypoxia/ischemia—At rest, $[\text{Ca}^{2+}]_o$ concentration is about 1.2–1.5 mM, while the cytosolic free Ca^{2+} concentration is 10,000-fold lower than in extracellular fluid in neurons (Hansen, 1985). Obviously, there is a very large concentration difference between inside and outside of cells. The membrane potential of neurons is relatively stable at 60–90 mV negative inside cell than outside one, and together with the large transmembrane concentration difference, provides a very powerful electrochemical driving force for the influx of Ca^{2+} into cells. Nevertheless, the cell membranes have very low permeability to Ca^{2+} for most cells, including neurons. Neurons, like many other types of cells, also possess two parallel, independent mechanisms to extrude Ca^{2+} , namely plasma membrane Ca^{2+} -ATPase (also called Ca^{2+} pump) and $\text{Na}^+/\text{Ca}^{2+}$ exchangers (Budd, 1998; Dipolo and Beaugé, 2006). The former has high Ca^{2+} affinity but low transport capacity, whereas $\text{Na}^+/\text{Ca}^{2+}$ exchangers have a low affinity, but a higher capacity to transport Ca^{2+} and can operate reversibly. $\text{Na}^+/\text{Ca}^{2+}$ exchangers operate in a forward mode to maintain $[\text{Ca}^{2+}]_i$ at physiological levels by extruding Ca^{2+} out with Na^+ in (in 1:3 stoichiometry) by consuming Na^+ electrochemical gradients generated by the ATP-driven Na^+ pumps under normal conditions. $\text{Na}^+/\text{Ca}^{2+}$ exchangers are very efficient in extruding Ca^{2+} and therefore considered as a “genuine Ca^{2+} pump”. Even a small Ca^{2+} load in nerve terminals can activate $\text{Na}^+/\text{Ca}^{2+}$ exchangers. This Ca^{2+} extrusion is almost completely dependent on extracellular Na^+ in this condition (Budd, 1998; Dipolo and Beaugé, 2006). These two Ca^{2+} extrusion mechanisms, together with less permeability to Ca^{2+} , work in parallel in the plasma membrane to keep $[\text{Ca}^{2+}]_i$ at physiological levels under normal conditions (Budd, 1998; Dipolo and Beaugé, 2006).

As discussed above, neurons have a very powerful electrochemical driving force for the influx of Ca^{2+} into cells and very low permeability to Ca^{2+} at rest. Thus, even very minor changes in Ca^{2+} permeability of the plasma membrane can produce great increases in $[\text{Ca}^{2+}]_i$ levels by the influx of Ca^{2+} . Indeed, such an event can happen under hypoxic/ischemic conditions (Siver and Erecińska, 1990; Friedman and Haddad, 1993; Mitani et al, 1993; Diarra et al, 1999; Nakamura et al, 1999). By using ion-selective microelectrodes, Silver and Erecińska (1990) directly evaluated the profiles of changes in $[\text{Ca}^{2+}]_o$ and $[\text{Ca}^{2+}]_i$ during anoxia and ischemia in different regions of intact rat brains. They found that the major increase in $[\text{Ca}^{2+}]_i$ (rising from ~70 nM to 24–35 μM) was associated with a large reduction in $[\text{Ca}^{2+}]_o$ (dropping from ~1.5 mM to 0.5 mM or less) in many but not all neurons examined in the hippocampus, cortex and thalamus (Siver and Erecińska, 1990). Actually, considering that the extracellular fluid space decreases to approximately half of control during hypoxia/ischemia (Hansen, 1985; Jiang and Haddad, 1991), the drop of $[\text{Ca}^{2+}]_o$ may be greatly underestimated, and the value of $[\text{Ca}^{2+}]_o$ decrease can be approximately 0.1 mM during hypoxia/ischemia (Kristián and Siesjö, 1998). This lends support to the idea that hypoxic/ischemic overload of intracellular Ca^{2+} in neurons is attributable primarily to Ca^{2+} influx from extracellular space (Yao and Haddad, 2004; Martínez-Sánchez et al, 2004). This viewpoint is also supported by the following notion from in vivo and in vitro studies: hypoxic/ischemic increase in $[\text{Ca}^{2+}]_i$ is greatly attenuated by removal of extracellular Ca^{2+} (Mitani et al, 1993; Friedman and Haddad, 1993; Diarra et al, 1999; Nakamura et al, 1999). Obviously, neurons with very high calcium conductance, e.g., hippocampal CA1 and neocortical layer II/III neurons (with high density of NMDA receptors) (Kristián and Siesjö, 1998; Fukuda et al, 1998), can suffer more severe Ca^{2+} overload due to an over-influx of extracellular Ca^{2+} , as shown by Siver and Erecińska (1990, 1992).

The influx of calcium occurs mainly through two pathways: the voltage-gated Ca^{2+} channels and receptor-controlled channels. A variety of reports using microfluorometric techniques have shown that neuronal activity leads to elevation of $[\text{Ca}^{2+}]_i$ due to Ca^{2+} influx via ionotropic receptors and/or voltage-gated Ca^{2+} channels. Plasmalamel $\text{Na}^+/\text{Ca}^{2+}$ exchangers can also greatly contribute to Ca^{2+} entry by operating in a reverse mode under hypoxic/ischemic condition. Nevertheless, for certain cells, these pathways do not necessarily contribute equally to the influx of Ca^{2+} through the plasma membrane. They may function for different phases of $[\text{Ca}^{2+}]_i$ rise depending on inherent and environmental factors including the duration (short vs. long) and severity (mild vs. severe) of insults, the age of animals (immature or young vs. old), the region of the brain, and even the type of cells (neurons vs. glial cells).

Voltage-gated Ca^{2+} channels (VGCCs): The activation of VGCCs is driven by the membrane potential and plays a major role in shaping neuronal excitability. According to their activation properties and pharmacological profiles, VGCCs are divided into T (low-voltage activated) and P/Q, N, L, R (high-voltage activated) types based on the channel subunit compositions (Budde et al, 2002). Some reports indicate that VGCCs are one of major pathways for Ca^{2+} influx and contribute to neurodegeneration following hypoxia/ischemia (Lukyanetz et al, 2003; Yao and Haddad, 2004). During hypoxia/ischemia, neuronal membrane potential can be depolarized (Calabresi et al, 1999; Müller and Somjen, 2000b; Raley-Susman et al, 2001; Xia et al, 2003), which activates VGCCs and results in a remarkable increase in $[\text{Ca}^{2+}]_i$ (Duffy and MacVicar, 1996; Pisani et al, 1998b; Bergmann and Keller, 2003; Lukyanetz et al, 2003; Tjong et al, 2007). Glutamate-induced membrane potential depolarization can also activate VGCCs and result in a remarkable increase in $[\text{Ca}^{2+}]_i$. It seems that high rather than low-voltage activated VGCCs are responsible for Ca^{2+} influx induced by hypoxia/ischemia, since the blockade of high-voltage activated VGCCs can, while the blockade of low-voltage activated VGCCs can not, attenuate hypoxic/ischemic $[\text{Ca}^{2+}]_i$ increase (Pisani et al, 1998b; Zhang and Lipton, 1999; Lopachin et al, 2001; Lukyanetz et al, 2003; Yao and Haddad, 2004; Tjong et al, 2007;). However, there are also reports showing that VGCCs are not significantly involved in hypoxic/ischemic Ca^{2+} influx (Chen et al, 1999; Martinez-Sánchez et al, 2004). This discrepancy can be partially explained by the quick inactivation of VGCCs during a maintained depolarization (Ozawa et al, 1989; Young and Somjen, 1992), which often occurs during hypoxia/ischemia (Young and Somjen, 1992; Calabresi et al, 1999).

Receptor-operated channels: The receptor-operated channels in neurons are activated predominantly by the acidic amino acid transmitters (Siver and Erecińska, 1990). Because VGCCs in cultured hippocampal neurons have been shown to be quickly inactivated during a maintained depolarization (Ozawa et al, 1989), the major component of hypoxic/ischemic Ca^{2+} influx under this condition may be through receptor-operated channels, especially ionotropic glutamate receptor channels (Mitani et al, 1993). Indeed, the contribution of Ca^{2+} -permeable glutamate receptor channels to hypoxic/ischemic Ca^{2+} influx has been proposed in numerous studies (Siver and Erecińska, 1990; Goldberg and Choi, 1993; McDonald et al, 1998; Zhang and Lipton, 1999; Lopachin et al, 2001; Martinez-Sánchez et al, 2004; Yao and Haddad, 2004; Kiedrowski, 2007; Micu et al, 2007; Pamenter and Buck, 2008). During hypoxic/ischemic conditions, glutamate is remarkably released to extracellular space from presynaptic vesicles or by activating reversely operated glutamate- Na^+ transport system due to the reversal of Na^+ gradients (Benveniste et al, 1984; Bouvier et al, 1992; Goldberg and Choi, 1993; Camacho and Massieu, 2006; Micu et al, 2007). The massive accumulation of glutamate in extracellular space is causally linked to Ca^{2+} entry through ionotropic glutamate receptors, because activation of ionotropic glutamate receptors triggers $^{45}\text{Ca}^{2+}$ entry into neurons and subsequently leads to remarkable $[\text{Ca}^{2+}]_i$ rise. This increase is similar to hypoxic/ischemic Ca^{2+} responses (Tymianski et al, 1993; Dugan et al, 1995; McDonald et al, 1998; Kiedrowski, 1999, 2007; Zhang and Lipton, 1999; Shen and Slaughter, 2002; Micu et al, 2006, 2007). Pharmacologically blocking ionotropic glutamate receptors largely eliminates

anoxia/ischemia-induced $[Ca^{2+}]_o$ falls (Macdonald et al, 2006) and $[Ca^{2+}]_i$ rise (Silver and Erecińska, 1990; Goldberg and Choi, 1993; Zhang and Lipton, 1999; Lopachin et al, 2001; Martinez-Sánchez et al, 2004; Yao and Haddad, 2004; Micu et al, 2006). These findings strongly suggest that ionotropic glutamate receptor channels are one of the major components of Ca^{2+} influx induced by severe hypoxia/ischemia.

Ionotropic glutamate receptors can be pharmacologically classified to three subtypes, namely NMDA receptors, AMPA receptors, and kainate receptors (KA receptors). The latter two receptors are also generally called non-NMDA receptors due to their similarity to functional properties (Budd, 1998; Dingledine et al, 1999). Though ionotropic glutamate receptor-mediated Ca^{2+} influx contributes to intracellular Ca^{2+} accumulation, the relative contributions of different receptor subtypes are not equal to hypoxic/ischemic $[Ca^{2+}]_i$ rise, and some results are even contradictory. For example, in hippocampal slices, blockade of NMDA receptors with MK-801 or 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) can partially (Zhang and Lipton, 1999; Martinez-Sánchez et al, 2004) or completely (Lopachin et al, 2001) prevent ischemic Ca^{2+} accumulation. Broad-spectrum NMDA receptor antagonists (MK-801, 7-chlorokynurenic acid, D-AP5) abolish ischemia-induced Ca^{2+} increases in myelin (Micu et al, 2006). On the other hand, blocking AMPA/KA receptors with CNQX or NBQX completely prevents Ca^{2+} accumulation (Lopachin et al, 2001; Micu et al, 2006), or reduces approximately 50% of the sustained $[Ca^{2+}]_i$ rise (Zhang and Lipton, 1999), or has no effect on ischemic $[Ca^{2+}]_i$ rise (Martinez-Sánchez et al, 2004). By contrast, blockade of either NMDA receptors or AMPA/KA receptors with MK-801 and CNQX respectively does not attenuate the increase in $[Ca^{2+}]_i$ during anoxia/ischemia in acutely isolated hippocampal CA1 neurons and cultured cerebellar granular cells (Friedman and Haddad, 1993; Chen et al, 1999). In the in vitro cortex, blocking NMDA receptors strongly suppresses intracellular Ca^{2+} accumulation induced by OGD (Goldberg and Choi, 1993; Fukuda et al, 1998), whereas blockade of AMPA/KA receptors with CNQX either greatly attenuates OGD-induced $[Ca^{2+}]_i$ rise (Fukuda et al, 1998) or has no effects on $[Ca^{2+}]_i$ rise (Goldberg and Choi, 1993). These discrepancy outcomes can be attributed to many factors, especially the differences in (1) tissue/cell preparation (acutely isolated/cultured neurons vs. brain slices which keep relatively intact microenvironments and neuronal connections) (Friedman and Haddad, 1993; Chen et al, 1999; Zhang and Lipton, 1999; Lopachin et al, 2001; Martinez-Sánchez et al, 2004); (2) determination of $[Ca^{2+}]_i$ (cryoultramicrotomy and electron probe X-ray microanalysis by Lopachin et al. vs. microfluorometric imaging by others); (3) drugs used (e.g., MK-801 vs. CPP and AP5 for NMDA receptors; CNQX vs. NBQX for AMPA/KA receptors); and (4) drug doses used (higher vs. lower concentrations). Besides, the following issues should also be considered:

1. *Ca^{2+} permeability of receptor subtypes.* NMDA receptors have a relatively higher permeability to Ca^{2+} than that of AMPA/KA receptors (Mayer and Westbrook, 1987; Budd, 1998). Activation of these subtypes to the same extension could result in different $[Ca^{2+}]_i$ rise.
2. *Sensitivity to extracellular pH changes.* In contrast to NMDA and KA receptors, AMPA receptor-mediated $[Ca^{2+}]_i$ rise is more sensitive to extracellular acidity (McDonald et al, 1998) which often occurs during ischemia/hypoxia (Silver and Erecińska, 1992; Lipton 1999; Sheldon and Church, 2002, 2004; Sheldon et al, 2004). Lowering extracellular pH significantly reduces AMPA receptor-mediated peak elevations in intracellular free Ca^{2+} , but slows the recovery of intracellular homeostasis (McDonald et al, 1998).
3. *Rapid receptor desensitization.* AMPA/KA receptors are easily desensitized after activation (half decay times c.a. 30 ms vs. 250 ms for NMDA receptors in hippocampal cells) (Vyklíček Jr et al, 1990; Moudy et al, 1994; Fedele and Raiteri,

1996; Wilding and Huettner, 1997). Therefore, AMPA/KA receptors may not be a major contributor to glutamate-induced Ca^{2+} influx, but could participate in a Na^{+} -dependent depolarization and Ca^{2+} influx through voltage-activated Ca^{2+} channels and $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers (Goldman et al, 1994; Budd, 1998; Kiedrowski, 2007) and Ca^{2+} release from intra-axonal Ca^{2+} store during ischemia (Ouardouz et al, 2006).

Finally, the distribution of receptor subtypes may contribute to the differences in glutamate-induced Ca^{2+} influx. The distributions of ionotropic glutamate receptors are heterogeneous in pre-/post-synaptic membrane as well as dendrite and soma (Schiller et al, 1997; Dodt et al, 1998; Yin et al, 1999; Frick et al, 2001; Nicholson et al, 2006; Groom et al, 2006; Micu et al, 2006). Therefore, different receptor subtypes in different neurons of different regions possibly mediate different magnitudes of Ca^{2+} influx during hypoxia/ischemia. In support of this hypothesis are the findings that hypoxic/ischemic Ca^{2+} entry is largely attributed to glutamate-operated channels in many (though not all) neurons examined in the hippocampus, cortex and thalamus (Siver and Erecińska, 1990) and OGD-induced $[\text{Ca}^{2+}]_i$ elevation is more pronounced in dendritic branches rather than in somatic regions (Pisani et al, 1999; Zhang and Lipton, 1999). This assumption is also supported by the observation that AMPA/KA receptor antagonist NBQX completely blocks the ischemia Ca^{2+} increase in oligodendroglial cell bodies, but only modestly reduces the Ca^{2+} increase in myelin, whereas the Ca^{2+} increases in myelin is abolished by broad-spectrum NMDA receptor antagonists (MK-801, 7-chlorokynurenic acid, D-AP5) (Micu et al, 2006).

Although there is a well established idea that ionotropic glutamate receptor-mediated Ca^{2+} influx is one of major sources of hypoxia/ischemia-induced increase in $[\text{Ca}^{2+}]_i$, we should keep in mind that ischemia and hypoxia are not the same stimulus for activation of ionotropic glutamate receptors in terms of increase in $[\text{Ca}^{2+}]_i$. In addition, glutamate is not equal to hypoxic or ischemic stress. For example, although anoxia and glutamate induce a similar increase in $[\text{Ca}^{2+}]_i$ in acutely isolated neurons (Friedman and Haddad, 1993), they are actually different in the nature of the stress. In isolated hippocampal CA1 neurons, there are very large extracellular spaces and no synaptic input which provide a microenvironment in the absence of glutamate accumulation in the bathing fluid. Anoxia indeed induces a significant increase in $[\text{Ca}^{2+}]_i$. This increase could be completely eliminated by removal of extracellular Ca^{2+} or by replacement of it with EGTA, but could not be attenuated by glutamate receptor blockers (Friedman and Haddad, 1993). The authors thus argue that glutamate release is not at the basis of the anoxia-induced increase in intracellular Ca^{2+} (Friedman and Haddad, 1993). Chen et al (1999) observed similar results in cultured cerebellar granule cells. Taking all the results together, these findings suggest a complicated and delicate Ca^{2+} response to hypoxia/ischemia and diversity of Ca^{2+} signaling in stress.

Plasmalemmal $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX): In some cases, pronounced $[\text{Ca}^{2+}]_i$ elevation under ischemia-like conditions greatly depends on marked elevation of intracellular Na^{+} , which results in the reversal of the operation of NCX (Itoh et al, 1998; Kiedrowski, 1999, 2007; Rojas et al, 2007; Luo et al, 2008). NCX operates in a forward mode to maintain $[\text{Ca}^{2+}]_i$ at physiological levels by extruding 1 Ca^{2+} out with 3 Na^{+} in by consuming Na^{+} electrochemical gradient generated by the ATP-driven Na^{+} pumps under normal conditions (Blaustein and Lederer, 1999). It can operate in either reverse or forward mode depending on the changes in transmembrane potential and Na^{+} and Ca^{2+} gradients. The roles of NCX following cerebral ischemia are complicated and may function as a double-edged sword (either beneficial or detrimental) (Jeffs et al, 2007). Both forward and reverse $\text{Na}^{+}/\text{Ca}^{2+}$ exchange can take place concurrently in the same cell (Yu and Choi, 1997). During hypoxia/ischemia, massive Na^{+} enters into neurons (Friedman and Haddad, 1994; Calabresi et al, 1999; Banasiak et al, 2004; Martínez-Sánchez, et al, 2004; Sheldon et al, 2004; Nikolaeva et al, 2005). It has been demonstrated that hypoxia-induced membrane depolarization (Calabresi et al, 1999; Müller

and Somjen, 2000b; Raley-Susman et al, 2001; Xia et al, 2003) and large increase in $[Na^+]_i$ (Friedman and Haddad, 1994; Calabresi et al, 1999; Banasiak et al, 2004; Martinez-Sánchez, et al, 2004; Sheldon et al, 2004; Nikolaeva et al, 2005) favor NCX to become reversed and Ca^{2+} entry/ Na^+ extrusion mode (Stys et al, 1992; Czyz and Kiedrowski 2002; Kiedrowski, 2007), though Ca^{2+} extrusion/ Na^+ entry mode may also concomitantly run (Jeon et al, 2008; Molinaro et al, 2008; Cuomo et al, 2008). This can lead to further enhancement of the Ca^{2+} overload during hypoxic/ischemic stress (Stys and Lopachin, 1998; Tanaka et al, 2002; Kiedrowski, 2007; Luo et al, 2008). In addition, some reports show that part of this major Ca^{2+} extrusion system (NCX) is cleaved by calpains in the ischemic brain, which jeopardizes Ca^{2+} extrusion and is also attributed to a second uncontrolled, irreversible Ca^{2+} increase that leads to neuronal demise (Bano et al, 2005; Bano and Nicotera, 2007; Luo et al, 2008). Furthermore, activation of the reverse NCX-induced increase in $[Ca^{2+}]_i$ can trigger a great amount of Ca^{2+} release from endoplasmic reticulum (Ca^{2+} - Ca^{2+} release) (Rojas et al, 2007; Chen et al, 2008). Therefore, reverse operation and cleavage of the plasmalemmal NCX are also responsible for hypoxic/ischemic $[Ca^{2+}]_i$ rises with reverse operation of exchangers contributing to $[Ca^{2+}]_i$ rise in the earlier phase of hypoxia/ischemia. The cleavage of NCX accounts for a second uncontrolled, irreversible Ca^{2+} increase in the later phase of severe and prolonged hypoxia/ischemia and during reoxygenation/reperfusion period.

Other possible pathways: Despite the substantial influx of extracellular Ca^{2+} , the inability of Ca^{2+} extrusion mechanisms such as Ca^{2+} pumps, the reverse operation of plasmalemmal NCX (Bano et al, 2005; Bano and Nicotera, 2007; Lou et al, 2008), may also contribute to intracellular Ca^{2+} accumulation. This is because it is very difficult, if not impossible, for plasmalemmal Ca^{2+} pumps to remove the overloaded intracellular Ca^{2+} efficiently to extracellular spaces due to the decrease or failure in ATP generation during hypoxia/ischemia.

Hypoxia/ischemia evokes a significant fall in pH_i , which activates Na^+/H^+ exchangers (NHE) to extrude excessive intracellular H^+ in an electroneutral exchange of intracellular H^+ for extracellular Na^+ (Silver and Erecińska, 1990; Diarra et al, 1999; Sheldon and Church, 2002; Pedersen et al, 2006). This leads to $[Na^+]_i$ rise immediately after (not during) hypoxia/ischemia (Sheldon et al, 2004; Luo et al, 2005). Hypoxia/ischemia also activates electroneutral $Na^+-K^+-2Cl^-$ cotransporters (NKCC) leading to intracellular Na^+ overload (Sheldon et al, 2004; Lenart et al, 2004; Kintner et al, 2007; Chen et al, 2008). In addition, released glutamate, through stimulation of glutamate transporters, results in Na^+ influx (Kirischuk et al, 2007; Rojas et al, 2007). As a result, $[Ca^{2+}]_i$ increases significantly due to the operation of plasmalemmal NCX in a reverse mode (Zhang and Lipton, 1999; Sheldon et al, 2004; Martinez-Sánchez et al, 2004; Luo et al, 2005, 2008; Nikolaeva et al, 2005; Pedersen et al, 2006; Rojas et al, 2007; Kiedrowski, 2007; Chen et al, 2007). The increase in $[Ca^{2+}]_i$, in turn, triggers endoplasmic reticulum to release Ca^{2+} into cytosol (a typical Ca^{2+} - Ca^{2+} release) (Rojas et al, 2007; Chen et al, 2008). Therefore, NHE, NKCC, and glutamate transporters can be additional contributors to secondary intracellular Ca^{2+} elevation by indirectly activating reverse NCX operation and Ca^{2+} - Ca^{2+} release from endoplasmic reticulum.

Reactive oxygen species (ROS) generated during hypoxia/ischemia induce Ca^{2+} influx (Chen et al, 1999) by activating non-selective cation channels (Chinopoulos and Adam-Vizi, 2006). At least two non-selective cation channels, i.e., TRPM7, and calcium-permeable acid-sensing ion channels (ASICs), are proposed to be involved in Ca^{2+} influx and neuronal death in hypoxic/ischemic stress (Aarts et al, 2003; Xiong et al, 2004, 2007; Gao et al, 2005; Macdonald et al, 2006; Langeslag et al, 2007; Pignataro et al, 2007; Simard et al, 2007; Fang et al, 2008). Giving the factors of a vast amount of ROS generation during severe/prolonged hypoxia/ischemia (Lafon-Cazal et al, 1993; Dugan et al, 1995; Perez Valazquez et al, 1997; Carriedo et al, 2000), ROS-sensitive, non-selective cation channels-mediated Ca^{2+} influx composes a major contributor to a second uncontrolled $[Ca^{2+}]_i$ rise. The increase in $[Ca^{2+}]_i$ through ROS-

sensitive, non-selective cation channels often occurs in the late phase of severe hypoxia/ischemia and posthypoxic/postischemic reperfusion period, and triggers the delayed neuronal death (Aarts et al, 2003; Macdonald et al, 2006; Chinopoulos and Adam-Vizi, 2006). This is because during the period of postischemic reperfusion, Ca^{2+} signaling through voltage-dependent Ca^{2+} channels is attenuated (Connor et al, 1999), and glutamate receptor channels are down-regulated in expression (Pellegrini-Giampietro et al, 1992). Then, non-selective cation channel-mediated delayed Ca^{2+} overload cannot be arrested by blocking Ca^{2+} channels and NMDA channels in this period (Tymianski et al, 1993). Taken together, Ca^{2+} entry through non-selective cation channels is a major route for hypoxic/ischemic $[\text{Ca}^{2+}]_i$ accumulation, especially a second uncontrolled $[\text{Ca}^{2+}]_i$ increase, in the late phase of severe hypoxia/ischemia and posthypoxic/postischemic reperfusion period.

3.1.2 Ca^{2+} uptake and release from intracellular stores during hypoxia/ischemia

—Intracellular Ca^{2+} stores is a contributor to cytosolic Ca^{2+} rise in hypoxic/ischemic neurons (Dubinsky and Rothman et al, 1991; Mitani et al, 1993; Murai et al, 1997; Grøndahl et al, 1998; Chen et al, 1999; Zhang and Lipton, 1999; Bergmann and Keller, 2003; Nikolaeva et al, 2005). Measurements with Ca^{2+} -sensitive electrodes have revealed 50–200% increases in $[\text{Ca}^{2+}]_i$ with no accompanying decrease in $[\text{Ca}^{2+}]_o$ in several brain regions within the first minute after oxygen depletion and prior to the reduction in ATP (Siver and Erecińska, 1990). When extracellular Ca^{2+} is removed, synaptic transmission and neuronal activities are blocked (to eliminate Ca^{2+} influx due to possible activation of Ca^{2+} -permeable glutamate receptor channels and voltage-dependent Ca^{2+} channels). In this circumstance, chemical ischemia or OGD still induces an increase in $[\text{Ca}^{2+}]_i$, though relatively less than that in normal $[\text{Ca}^{2+}]_o$ in neurons (Dubinsky and Rothman et al, 1991; Mitani et al, 1993; Duffy and MacVicar, 1996; Grøndahl et al, 1998; Chen et al, 1999; Bergmann and Keller, 2003; Nikolaeva et al, 2005; Misonou et al, 2005). In hippocampal slices, a combination of the removal of extracellular Ca^{2+} and thapsigargin-induced depletion of intracellular Ca^{2+} store, greatly decreases ischemic $[\text{Ca}^{2+}]_i$ increase (from 25 μM to 630 nM) (Grøndahl et al, 1998). These results strongly suggest the involvement of intracellular Ca^{2+} stores in hypoxia/ischemia-induced increase in $[\text{Ca}^{2+}]_i$. The most important intracellular stores are endoplasmic reticulum and mitochondria.

Endoplasmic reticulum (ER): The primary intracellular Ca^{2+} storage/release organelle in most cells is the ER that has two major families of Ca^{2+} release channels: the inositol triphosphatase (IP3) receptors and ryanodine receptors (Paschen, 2001; Verkhratsky, 2005). In these two Ca^{2+} release channels, IP3 receptors are activated by inositol 1,4,5-trisphosphate that is generated from phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC), while ryanodine receptors are activated either by Ca^{2+} itself or by depolarization via coupling with voltage-gated Ca^{2+} channels. Most recent evidence shows that, ryanodine receptors are activated by cyclic ADP ribose (Aley et al, 2006). Endoplasmic reticular Ca^{2+} ATPase, which is similar to plasmalemmal Ca^{2+} ATPase, pumps back cytosolic Ca^{2+} in an energy-requiring process against a steep concentration gradient (Paschen, 2001). ER Ca^{2+} is in high micromolar levels or even millimolar range, and is divided into a number of subcompartments (Paschen, 2001).

Considerable evidence suggests that ER is involved in hypoxia/ischemia-induced cytosolic Ca^{2+} accumulation in the central nervous system (Dubinsky and Rothman et al, 1991; Mitani et al, 1993; Murai et al, 1997; Wang et al, 2002; Meini et al, 2003; Ouardouz et al, 2003, 2006; Smith et al, 2004; Nikolaeva et al, 2005; Verkhratsky, 2005; Aley et al, 2006). Blockade of Ca^{2+} -induced Ca^{2+} release from ryanodine receptor channels with dantrolene reduces hypoxia/ischemia-induced increase in $[\text{Ca}^{2+}]_i$ in hippocampal neurons as well as neuronal cell lines in the absence of extracellular Ca^{2+} (Dubinsky and Rothman et al, 1991; Mitani et al, 1993; Wang et al, 2002). Application of ryanodine results in a significant decrease in hypoxic/ischemic $[\text{Ca}^{2+}]_i$ rise in 0 external Ca^{2+} perfusion in rat striatal slices and optic nerves (Meini

et al, 2003; Nikolaeva et al, 2005). In dorsal columns, ischemia-induced axoplasmic Ca^{2+} rise can be blocked by ryanodine or blockers of L-type Ca^{2+} channel voltage sensors (nimodipine, diltiazem) but not blocker of channel pores (Cd^{2+}) in Ca^{2+} free perfusate (Ouardouz et al, 2003). Immunoprecipitation and immunohistochemistry reveal a colocalization of Ca^{2+} channels and ryanodine receptor clusters and an association between L-type Ca^{2+} channels and ryanodine receptors (Ouardouz et al, 2003). These findings suggest that Ca^{2+} release from ryanodine receptor channels can also be evoked by depolarization sensed by L-type Ca^{2+} channels during ischemia, similar to “excitation-contraction coupling” in skeletal muscle (Ouardouz et al, 2003, 2006). Most recently, cyclic ADP ribose-activated ryanodine receptors has been proposed to be a contributor to hypoxia-induced $[\text{Ca}^{2+}]_i$ increase in cultured cortical astrocytes (Aley et al, 2006). All these observations are in support of the contribution of ryanodine receptor channels to hypoxic/ischemic $[\text{Ca}^{2+}]_i$ rise.

IP3 receptor channels also contribute to hypoxic/ischemic Ca^{2+} response. For instance, depletion of IP3-sensitive Ca^{2+} store with thapsigargin (Murai et al, 1997; Grøndahl et al, 1998), or IP3-generating agonist, bradykinin (Smith et al, 2004), reduces hypoxic/ischemic $[\text{Ca}^{2+}]_i$ rise in the absence of external Ca^{2+} in hippocampal and locus coeruleus neurons as well as cortical astrocytes. In optic nerves, application of U73122 (an irreversible antagonist of PLC), neomycin (rendering PLC substrates unrecognizable), or heparin (inhibiting IP3-induced release) reduces ischemic peak Ca^{2+} response by 50% in 0 external Ca^{2+} condition (Nikolaeva et al, 2005). It has been reported that activation of G-protein coupled metabotropic glutamate receptors (mGluR) leads to the formation of IP3. Therefore, Ca^{2+} release from IP3 receptor channels can be induced by great glutamate release during hypoxic/ischemic stress (Benveniste et al, 1984; Bouvier et al, 1992; Goldberg and Choi, 1993; Camacho and Massieu, 2006).

The possible mechanisms of Ca^{2+} release from ER during hypoxia/ischemia can be as follows: released glutamate binds to both mGluR and ionotropic glutamate receptors, inducing IP3-sensitive Ca^{2+} release from ER and Ca^{2+} influx from extracellular space through ionotropic glutamate receptor channels and VGCCs, which subsequently results in $[\text{Ca}^{2+}]_i$ rise. Elevated cytosolic Ca^{2+} stimulates more Ca^{2+} release from ryanodine receptor channels. Therefore, ER-based Ca^{2+} release contributes not only to the initial progressive increase in $[\text{Ca}^{2+}]_i$, but also to the large $[\text{Ca}^{2+}]_i$ increase at the later phase of hypoxic/ischemic stress. Due to the depletion of ATP during hypoxia/ischemia, endoplasmic reticular Ca^{2+} ATPase might be inhibited, and therefore cannot efficiently pump back cytosolic Ca^{2+} against a steep concentration gradient into ER, which also aggravates hypoxic/ischemic Ca^{2+} accumulation.

Mitochondria: Mitochondria are capable of storage and release of intracellular Ca^{2+} . Isolated mitochondria have been shown to be capable of accumulating over 3 $\mu\text{mol Ca}^{2+}/\text{mg}$ protein (equivalent to 3 mM at 1 mg/ml protein in the presence of ATP and Pi), most of which exist in the form of osmotically inactive $\text{Ca}_3(\text{PO}_4)_2$ in matrix to prevent a rise in the matrix osmolarity. Thus, mitochondria can function as a Ca^{2+} sink (Budd, 1998). Under physiological conditions, Ca^{2+} uptake by mitochondria is mediated by a specific Ca^{2+} uniport system, which operates strictly according to the Ca^{2+} electrochemical equilibrium. Thus, the theoretical Ca^{2+} concentration in matrix is up to 0.1 M. Ca^{2+} efflux from mitochondria is mediated primarily by the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchangers. In addition, under certain conditions, the Ca^{2+} uniporters (reverse mode) and permeability transition pores (PTP) may serve as pathways of Ca^{2+} release (Budd et al, 1998; Montero et al, 2001; Wang and Thayer, 2002; Chinopoulos and Adam-Vizi, 2006; Hernández-SanMiguel et al, 2006).

It has been shown that an initial small increase in $[\text{Ca}^{2+}]_i$ is attributed to Ca^{2+} release from mitochondria in cerebellar granule cells and brainstem motoneurons during chemical ischemia (Chen et al, 1999; Bergmann and Keller, 2003). In hippocampal neurons, chemical ischemia

induces a large and sustained increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} , which is also attributed to Ca^{2+} release from mitochondria (Misonou et al, 2005). Based on the work by Zhang and Lipton (1999), mitochondrial Ca^{2+} release is responsible for approximately 50% of ischemic increase in $[Ca^{2+}]_i$ in hippocampal slices. Similar results are also observed in optical axon during ischemia (Nikolaeva et al, 2005). These results suggest that mitochondria are an important contributor to hypoxia/ischemia-induced Ca^{2+} accumulation.

By using electron probe x-ray microanalysis, Taylor et al (1999) reported that despite Ca^{2+} release from mitochondria during OGD, cytosolic Ca^{2+} levels increase substantially in hippocampal CA1 cells during 42 min of OGD. They also showed that total Ca^{2+} contents in mitochondria increased significantly at 5 min and then decreased after 12 min of ischemia, but still kept in a significant higher level during all the periods of OGD. The functional and morphological impairment of brain mitochondria is causally linked with Ca^{2+} accumulation in matrix during hypoxic stress (Schild et al, 2003). These observations suggest that mitochondria also take up Ca^{2+} during ischemia. Actually, there may be a “set-point” for mitochondrial response to cytosolic $[Ca^{2+}]$ (the value is 0.5–1 μM in neurons), at which Ca^{2+} uptake and efflux are equal but opposite in mitochondria (Nicholls, 2005). Therefore, hypoxia/ischemia, as a stimulus, may stimulate light Ca^{2+} release from mitochondria before the significantly elevated cytosolic $[Ca^{2+}]$ more than the “set-point”, as has been shown in cerebellar granule cells and brainstem motoneurons (Chen et al, 1999; Bergmann and Keller, 2003). When the cytosolic Ca^{2+} elevation is more than the “set-point”, the activities of mitochondrial Ca^{2+} uniporters greatly increase (Nicholls, 2005), then, large quantities of Ca^{2+} are taken up, and accumulate in the mitochondrial matrix. However, the rise in mitochondrial Ca^{2+} can cause the depolarization of the inner membrane, and stimulate the generation of factors, such as ROS and free fatty acids, resulting in the open of PTP and subsequent large Ca^{2+} efflux to cytosol. This Ca^{2+} efflux from mitochondria is responsible for delayed Ca^{2+} deregulation (Bahar et al, 2000; Pokhilko et al, 2006; Hajnóczky et al, 2006; Chinopoulos and Adam-Vizi, 2006). Therefore, the results of Taylor et al (1999) and Schild et al (2003) are not surprising.

Several pathways are responsible for hypoxia/ischemia-induced Ca^{2+} release from mitochondria. Besides PTP, mitochondrial $2Na^+/Ca^{2+}$ exchangers directly modulate Ca^{2+} efflux (Hernández-SanMiguel et al, 2006). It is known that intracellular Na^+ can promote the release of Ca^{2+} from mitochondria by coupling via the mitochondrial $2Na^+/Ca^{2+}$ exchangers (Ritter et al, 2003). Na^+ entry during ischemia activates Ca^{2+} release from mitochondria by the inner membrane $2Na^+/Ca^{2+}$ exchangers, therefore blockade of mitochondrial $2Na^+/Ca^{2+}$ exchangers with CGP37157 greatly reduces ischemia-induced mitochondrial Ca^{2+} release and the subsequent increase in cytosolic $[Ca^{2+}]$ in hippocampal neurons and optic axon (Zhang and Lipton, 1999; Nikolaeva, 2005). In addition, reverse Ca^{2+} uniporters are another pathway for Ca^{2+} release from mitochondria. As a result of depolarization of inner membrane during hypoxia/ischemia (Bahar et al, 2000; Schild et al, 2003), Ca^{2+} uniporters can operate in a reverse mode to cause a rapid release of Ca^{2+} from mitochondria (Jurkowitz et al, 1983; Montero et al, 2001). Among these pathways, $2Na^+/Ca^{2+}$ exchangers are the most important one in hypoxic/ischemic Ca^{2+} release from mitochondria (Zhang and Lipton, 1999; Nikolaeva, 2005).

3.1.3 Intracellular Ca^{2+} buffering during hypoxia/ischemia—There is a fair amount of calcium inside cells, but most of them are either bound to specific proteins or sequestered in organelles, principally ER and mitochondria. Strictly, some intracellular organelles such as ER, mitochondria, microsoma, synaptic vesicles and neurosecretory granules, together with specific Ca^{2+} binding proteins, should be included in the intracellular Ca^{2+} buffering system. We have specifically discussed the roles of ER and mitochondria in $[Ca^{2+}]_i$ rise during hypoxia/

ischemia, here we put special emphasis on high-affinity Ca^{2+} binding cytosolic proteins, most of which share the typical EF-hand structure of their binding sites (Ikura and Ames, 2006).

A number of cytosolic Ca^{2+} binding proteins have been found (Ikura and Ames, 2006). Among them, is calmodulin, which is highly concentrated in the brain (30–50 μM) with a considerable fraction (up to 100–200 μM) of the Ca^{2+} buffering capacity, acts primarily as cytosolic Ca^{2+} buffering except its functioning as other signaling messenger (Budd, 1998; Burgoyne, 2007). Under normal conditions, when Ca^{2+} enters neurons or releases from internal stores, Ca^{2+} binding cytosolic proteins promptly bind some of Ca^{2+} during its diffusion to keep Ca^{2+} in a proper localization and level, and therefore serve as a modulator of Ca^{2+} signaling (Burgoyne, 2007). Although the process of Ca^{2+} binding to cytosolic Ca^{2+} binding proteins is rapid (several msec), the binding is very limited in capacity, and is easily saturated with higher $[\text{Ca}^{2+}]_i$ (Budd, 1998). Therefore, these Ca^{2+} binding proteins may play a very minor role in the regulation of $[\text{Ca}^{2+}]_i$ overload during hypoxia/ischemia.

3.2 Hypoxia/ischemia-induced increase in Na^+ influx

Because of the large transmembrane electrochemical gradients of Na^+ , a great amount of Na^+ entry can occur in certain conditions, e.g., hypoxic/ischemic stress. During hypoxia, extracellular Na^+ (150–155 mM) sharply drops to about 60 mM in the hippocampus (Fung et al, 1999; Müller et al, 2000a, 2000b) and 40 mM in hypoglossal neurons (Jiang et al, 1992). Meanwhile, hypoxia/ischemia induces an increase in $[\text{Na}^+]_i$ from baseline level (about 8 mM) to 20–50 mM depending on the duration and severity of insults in nerve cells of various regions, including the cortex (Friedman and Haddad, 1994; Lynch III et al, 1995; Pisani et al, 1998a; Banasiak et al, 2004; Lenart et al, 2004; Luo et al, 2005), hippocampus (Taylor et al, 1999; Lopachin et al, 2001; Raley-Susman et al, 2001; Sheldon et al, 2004; Martinez-Sánchez et al, 2004), cerebellum (Chen et al, 1999), substantia nigra (Guatteo et al, 1998b), striatal neurons (Calabresi et al, 1999), hypoglossal cells (Jiang et al, 1992), optic nerve (Stys and Lopachin, 1998; Nikolaeva et al, 2005) and spinal cord (Rose et al, 1998). It is worthy to note that the drop of $[\text{Na}^+]_o$ may be over-estimated, and the increase in $[\text{Na}^+]_i$ may be under-estimated provided that the changes in volume of extracellular spaces and cells are considered. Indeed, when extracellular fluid spaces decrease to 30–50% of the control level, cells may swell by 10–30% during hypoxia/ischemia (Hansen, 1985; Jiang and Haddad, 1991; Kristián and Siesjö, 1998; Lenart et al, 2004). Therefore, the actual changes should be about 30–50 mM of $[\text{Na}^+]_o$ drops and corresponding increases in $[\text{Na}^+]_i$. Despite the real values of changes in $[\text{Na}^+]_o$ and $[\text{Na}^+]_i$, these results strongly indicate a significant increase in $[\text{Na}^+]_i$ due to Na^+ entry from extracellular spaces during hypoxia/ischemia. During reoxygenation or reperfusion period, though Na^+ extrusion mechanisms (e.g. Na^+ pumps and reversely operated NCX) are rebuilt or running, there is still Na^+ influx (Sheldon et al, 2004). Therefore, $[\text{Na}^+]_i$ gradually recovers with varied durations or keeps sustained increase for a period and then recovers (Lenart et al, 2004; Luo et al, 2005), depending on the duration and severity of insults.

Then the question arises: how does hypoxia/ischemia induce the extracellular Na^+ to enter and increase in $[\text{Na}^+]_i$? Several potential routes for the increase in $[\text{Na}^+]_i$ during hypoxia/ischemia have been proposed, but with very conflicting results. These routes include the failure of Na^+-K^+ ATPase due to the decrease in ATP, the influx through ionic channels (e.g. voltage-gated Na^+ channels, ligand-gated Na^+ channels and non-selective cation channels), and entry through membrane exchangers/transporters (e.g. NCX, NHE, NKCC etc). The following section will discuss these potential pathways for Na^+ entry.

Na^+-K^+ ATPase—The primary role of Na^+-K^+ ATPase is to maintain the high intracellular K^+ and low intracellular Na^+ (Jorgensen et al, 2003; Panayiotidis et al, 2006). Inhibition of Na^+ pumps induces the entry of extracellular Na^+ following the powerful electrochemical

gradients and subsequent increase in $[Na^+]_i$ in neurons (Alvarez-Leefmans et al, 1994). Since ATP is greatly decreased during hypoxia/ischemia (Silver et al, 1997; Raley-Susman et al, 2001; Martinez-Sánchez et al, 2004; Sheldon and Church, 2004), the first thing one might think is that the failure of Na^+ pumps is responsible for the increase in $[Na^+]_i$ (Friedman and Haddad, 1994; Silver et al, 1997; Rose et al, 1998; Stys, 1998; Calabresi et al, 1999; Lenart et al, 2004). However, this is not the case at least in dopamine neurons of rat substantia nigra pars compacta and rat cerebellar granule cells because supply of ATP (10 mM) via the recording pipette did not prevent the hypoxic increase in $[Na^+]_i$ in dopamine neurons of substantia nigra despite a great decrease in hypoxia-induced outward current (Guatteo et al, 1998a). Also, blockade of Na^+-K^+ ATPase with strophanthidin (100 μ M) did not induce an increase in $[Na^+]_i$ comparable to that produced by chemical ischemia in cerebellar granule cells (Chen et al, 1999). Therefore, the failure of Na^+ pumps may at most partially be responsible for increase in $[Na^+]_i$.

Voltage-gated sodium channels (VGSCs)—VGSCs play a critical role in neuronal functions, and are involved in many pathophysiological conditions such as pain (Cummins et al, 2007). No matter whether the membrane potential is hyperpolarized or depolarized at the initial time, hypoxia/ischemia leads to membrane depolarization with stress prolonged (Calabresi et al, 1999; Müller and Somjen, 2000a, 2000b; Raley-Susman et al, 2001, Xia et al, 2003). As a result, VGSCs are activated, leading to the entry of Na^+ and subsequent increase in $[Na^+]_i$. Based on the experimental data, Dronne et al (2006) recently established a mathematical model of ion movements during stroke, in which the involvement of VGSCs was extrapolated. In fact, previous studies already provided hints for this possibility. For example, some researchers made measurements with ion-selective microelectrodes and revealed that TTX treatment partially or completely prevented the hippocampal slices from anoxia-induced $[Na^+]_o$ drop (Fung et al, 1999; Müller and Somjen, 2000a). The authors concluded that activation of TTX-sensitive Na^+ channels is an important component of anoxic disruption of ion homeostasis, and attributed it partially to Na^+ influx (Fung et al, 1999). Data yielded from other measurements (e.g., flame photometer, electron probe X-ray microanalysis, Na^+ -sensitive fluorescent dye) also showed that blockade of VGSCs with TTX or lidocaine significantly reduced hypoxia/ischemia-induced excessive intraneuronal Na^+ accumulation in all compartments including cytosol in the hippocampus in vitro (Lopachin et al, 2001; Raley-Susman et al, 2001). Similar results were seen in neocortical neurons (Lynch III, et al, 1995; Banasiak et al, 2004), optic nerves (Stys and Lopachin, 1998; Nikolaeva et al, 2005), and isolated, in situ perfused rat brains (Xie et al, 1994). All these reports support the idea that VGSCs are a major, though not sole, source of Na^+ influx during hypoxia/ischemia. In consistent with this idea, several groups observed that blockade of VGSCs with Na^+ channel blockers such as TTX and lidocaine attenuated/postponed anoxic depolarization, and prevented hypoxia/ischemia-induced neuronal injury and death (Probert et al, 1997; Takahashi et al, 1999; Müller and Somjen, 2000a; Breder et al, 2000; Raley-Susman et al, 2001; Banasiak et al, 2004).

There are, however, some reports disproving the idea of VGSCs as an important pathway of hypoxia/ischemia-induced Na^+ influx. Under conditions of voltage clamp with the membrane potential hold in -60 mV, brief hypoxia still causes a reversible elevation in $[Na^+]_i$ in dopamine neurons of substantia nigra pars. This elevation in $[Na^+]_i$ obviously is not mediated by VGSCs because VGSCs can not be activated under such a condition (membrane potential in -60 mV) (Guatteo et al, 1998b). TTX (1 μ M) or lidocaine (250 μ M) failed to affect the magnitude of anoxia/OGD-induced increase in $[Na^+]_i$ in hippocampal and cortical neurons (Pisani et al, 1998a; Sheldon et al, 2004). Similar results were also observed in cerebellar granule cells and spinal cord astrocytes (Rose et al, 1998; Chen et al, 1999). In striatal spiny neurons, OGD-induced membrane depolarization and increase in $[Na^+]_i$ can be significantly reduced by lowering external Na^+ , but not by blockade of VGSCs by either TTX or saxitoxin, which seems

to suggest that the ischemia-induced increase in $[Na^+]_i$ is not due to Na^+ entry through TTX-sensitive Na^+ channels (Calabresi et al, 1999).

The reasons for these conflicting results can be attributed to differences in regions, insult duration (acute vs. prolonged) and intensity (e.g. mild hypoxia vs. severe ischemia). Moreover, observations made on isolated cells may be very different from those on brain slices that have more intact microenvironment. In the above mentioned work, for example, most of the studies disproving VGSCs as an important pathway of hypoxia/ischemia-induced Na^+ influx were conducted in cultured cells (Rose et al, 1998; Chen et al, 1999; Sheldon et al, 2004).

Our recent observations made in the cortical slices directly or indirectly indicate that anoxia induces Na^+ influx through VGSCs (Chao et al, 2008, 2009, data to be published; Kang et al, 2009), which prompts us to believe that VGSCs are a major route for Na^+ entry during hypoxia/ischemia.

Ligand-gated Na^+ channels—Hypoxia/ischemia induces massive release of glutamate into synaptic cleft (Benveniste et al, 1984; Bouvier et al, 1992; Goldberg and Choi, 1993; Camacho and Massieu, 2006). A common feature of AMPA/KA channels and NMDA channels is Na^+ permeability (Mayer and Westbrook, 1987). Therefore, glutamate may increase $[Na^+]_i$ by activation of glutamate receptors, and induce neuronal injury (Goldman et al, 1994; Agostinho et al, 1996; Hasbani et al, 1998; Houzen et al, 1998; Kiedrowski, 2007; Rojas et al, 2007; Kirischuk et al, 2007). Thus, it is conceivable that elevation of $[Na^+]_i$ during hypoxia/ischemia is at least partially mediated by glutamate receptor channels. Indeed, some studies fairly support this viewpoint (Lynch III, et al, 1995; Zhang and Lipton, 1999; Lopachin et al, 2001; Kiedrowski, 2007). Nevertheless, most studies indicate that activation of glutamate-gated receptor channels does not contribute to hypoxic/ischemic Na^+ influx in neurons and glial cells in various brain regions (Guatteo et al, 1998b; Pisani et al, 1998a; Sheldon et al, 2004; Silver et al, 1997; Calabresi et al, 1999; Müller and Somjen, 2000b; Martinez-Sánchez et al, 2004). This is based on the fact that blockade of NMDA receptors, AMPA/KA receptors, or even metabotropic glutamate receptors has little effect on hypoxia/ischemia-induced increase in $[Na^+]_i$. Therefore, ligand-gated Na^+ channels, especially glutamate receptor channels are not the major routes for Na^+ influx during hypoxia/ischemia.

Non-selective cation channels (NSCCs)—NSCCs superfamily consists of a large number of cation channels, such as transient receptor potential (TRP) channels, ASICs, and Ca^{2+} -activated, ATP-sensitive nonselective cation channels (NC_{Ca-ATP}), which are mostly permeable to both monovalent and divalent cations, and are easily activated by Ca^{2+} and/or ROS that increase in response to hypoxia/ischemia (Chen and Simard, 2001; Krishtal, 2003; Gao et al, 2005; Chinopoulos and Adam-Vizi, 2006; Simard et al, 2006, 2007; Nilius et al, 2007). There is evidence showing that NSCCs activation plays a critical role in hypoxic/ischemic neuronal death (Chen and Simard, 2001; Aarts et al, 2003; Xiong et al, 2004, 2007; Gao et al, 2005; Simard et al, 2006). In rat cerebellar granule cells, chemical ischemia-induced large $[Na^+]_i$ increase can not be attenuated by blocking TTX-sensitive Na^+ channels, NCX, NHE or NKCC, but can be attenuated by blocking ROS-activated NSCCs (Chen et al, 1999). Another recent study reveals that Na^+ influx during and after anoxia can be attenuated by blockade of NSCCs with Gd^{3+} and trolox (antioxidant) (Sheldon et al, 2004). Blockade of NC_{Ca-ATP} channels is highly effective in preventing Na^+ influx, and reduces cerebral edema, infarct volume and mortality by 50% after ischemic stroke (Chen and Simard, 2001; Simard et al, 2006). Marked accumulation of arachidonic acid seen during brain ischemia also induces cytosolic Na^+ and Ca^{2+} overload via a non-selective cation conductance, resulting in mitochondrial $[Na^+]$ and $[Ca^{2+}]$ overload (Fang et al, 2008). Therefore, NSCCs activation contributes to hypoxia/ischemia-induced increase in $[Na^+]_i$.

Plasmalemmal Na⁺/Ca²⁺ exchanger (NCX)—Though reverse operation of NCX has been demonstrated to be responsible for ischemic [Ca²⁺]_i rise (Stys and Lopachin, 1998; Tanaka et al, 2002; Martinez-Sánchez et al, 2004; Kiedrowski, 2007), both forward and reverse Na⁺/Ca²⁺ exchange can take place concurrently in the same cell (Yu and Choi, 1997). Therefore, it is possible for NCX to contribute to hypoxic/ischemic Na⁺ influx. In their mathematical model of ion movement in neurons, Dronne et al (2006) extrapolated the involvement of NCX in Na⁺ entry into neurons during severe ischemia by simulating the dynamics of Na⁺. The Data from some NCX knockout/knockdown models suggest that Ca²⁺ extrusion/Na⁺ entry mode of NCX is possibly running during hypoxia because NCX knockout/knockdown aggravates Ca²⁺ overload in neurons (Jeon et al, 2008; Molinaro et al, 2008; Cuomo et al, 2008). However, it seems that NCX does not contribute to [Na⁺]_i rise in a major way in rat cortical (Pisani et al, 1998a) and cerebellar granule (Chen et al, 1999) neurons during chemical ischemia, and in the hippocampal neurons in brief anoxic (Sheldon et al, 2004), although it contributes to Na⁺ influx immediately after anoxia in hippocampal neurons (Sheldon et al, 2004). Therefore, NCX is one of the major routes in hypoxia/ischemia-induced [Ca²⁺]_i rise (as discussed above) rather than Na⁺ influx during severe hypoxia/ischemia (particularly in the phase of the delayed uncontrolled [Ca²⁺]_i rise).

Na⁺/H⁺ exchanger (NHE)—The NHE family of ion exchangers includes six isoforms (NHE1–NHE6) that function in an electroneutral exchange of intracellular H⁺ for extracellular Na⁺ (Pedersen et al, 2006). The type 1 NHE (NHE1) is the most abundant NHE isoform in the rat brain (Ma and Haddad, 1997), and may be an important pathway for Na⁺ entry in neurons during hypoxia/ischemia since oxygen deprivation or blood supply insufficiency evokes a significant fall in pHi (Silver and Erecińska, 1990; Diarra et al, 1999; Sheldon and Church, 2002, 2004; Sheldon et al, 2004), and activates NHE to extrude excessive intracellular H⁺ (Diarra et al, 1999; Sheldon and Church, 2002; Horikawa et al., 2001; Yao et al., 2003; Pedersen et al, 2006). However, it has been demonstrated that NHE is inhibited during hypoxia/ischemia but activated immediately after reoxygenation/reperfusion (Diarra et al, 1999; Sheldon and Church, 2002, 2004; Sheldon et al, 2004). Therefore, NHE contributes to Na⁺ influx in the period immediately after anoxia rather than during anoxia in neurons (Chen et al, 1999; Sheldon et al, 2004). This viewpoint is further supported by the results from genetic ablation of NHE1 or blockade of NHE1 with HOE 642 in cultured cortical neurons (Luo et al, 2005). In this study, OGD causes a two-fold increase in [Na⁺]_i, and during 60 min reperfusion [Na⁺]_i increases sevenfold. Genetic ablation of NHE1 or blockade of NHE1 has no effects on OGD-mediated initial [Na⁺]_i rise but reduces the second phase of [Na⁺]_i by 40–50% (Luo et al, 2005). Therefore, NHE activation after hypoxia/ischemia has a significant impact on the loss of Na⁺ homeostasis and neuronal cell death (Luo et al, 2005, 2007; Xue et al, 2008; Wang et al, 2008).

Na⁺-K⁺-2Cl[−] cotransporter (NKCC)—NKCC belongs to the cation-dependent Cl[−] transporter family and transports Na⁺, K⁺, and Cl[−] into cells under physiological condition (Lenart et al, 2004). It has been shown that an OGD-induced increase of [Na⁺]_i can be significantly attenuated by inhibition of NKCC with bumetanide or genetic ablation of NKCC in cultured cortical astrocytes (Lenart et al, 2004; Kintner et al, 2007; Chen et al, 2008). Since inward NKCC may be limited by high [Na⁺]_i and presumably [Cl[−]]_i immediately after anoxia, NKCC-mediated Na⁺ entry seems to be ascribed to [Na⁺]_i rise during anoxia rather than immediately after anoxia in cultured hippocampal neurons. This is partially supported by the observation that 100 μM bumetanide causes a 40% reduction in the rise in [Na⁺]_i in 11–14 DIV neurons during anoxia but fails to significantly reduce increase in [Na⁺]_i after anoxia (Sheldon et al, 2004). In cultured cerebellar granule cells, however, chemical ischemia-induced increase in [Na⁺]_i can not be attenuated by bumetanide. Thus, NKCC is not accounted for ischemia-induced Na⁺ influx in this kind of neurons (Chen, et al, 1999). Taken together, NKCC

may be one of the routes for hypoxia/ischemia-induced Na^+ influx in neurons in the early phase of stress.

Glutamate transporter—Extracellular glutamate after its synaptic release should be rapidly cleaned for the normal neuronal functioning. Glutamate transporters are rapidly activated by extracellular glutamate to terminate possible sustained excitation by the clearance of glutamate on time to maintain normal neuronal function and transmission (Camacho and Massieu, 2006; Tzingounis and Wadiche, 2007). Downregulation of glutamate transporters significantly decreases glutamate uptake (Rosa et al, 2007), and induces a marked increase in extracellular glutamate concentrations in the rat striatum following microsphere embolism (Han et al, 2008). The clearance of glutamate by its transporters is highly dependent on transmembrane Na^+ gradients generated by membrane Na^+/K^+ ATPase. One glutamate molecule is transported into cytoplasm together with two Na^+ in (Bouvie et al, 1992; Camacho and Massieu, 2006). Thus $[\text{Na}^+]_i$ increases with glutamate transporter activation (Kirischuk et al, 2007; Rojas et al, 2007). During the first minutes of ischemia (before energy exhaustion and Na^+ gradient collapse), glutamate transporters operate normally to eliminate released glutamate (Camacho and Massieu, 2006; Colleoni et al, 2008) and therefore may partially account for the limited $[\text{Na}^+]_i$ increase in the early phase of hypoxia/ischemia. However, with hypoxia/ischemia being prolonged and $[\text{Na}^+]_i$ being largely increased (Friedman and Haddad, 1994; Calabresi et al, 1999; Banasiak et al, 2004; Martinez-Sánchez, et al, 2004; Sheldon et al, 2004; Nikolaeva et al, 2005), glutamate transporters are reversely operated (Rossi et al, 2000; Li and Stys, 2001; Mitani and Tanaka, 2003; Camacho and Massieu, 2006; Chen et al, 2007; Micu et al, 2007) to release massive glutamate and move intracellular Na^+ out.

3.3 Hypoxia/ischemia-induced K^+ efflux

It is known that extracellular K^+ ($[\text{K}^+]_e$) increases in the brain during hypoxia/ischemia (Hansen, 1985; Silver and Erecińska, 1990; Jiang and Haddad, 1991; Xia et al, 1992; Xie et al, 1994; Zetterström et al, 1995; Reid and Paterson, 1996; Gido et al, 1997; Taylor et al, 1999; Müller and Somjen, 2000a, 2000b; Lopachin et al, 2001; Raley-susman et al, 2001; Chao et al, 2006, 2007a, 2007b, 2008, 2009). Indeed, the increase in extracellular K^+ is a typical response of the brain tissues to hypoxia or ischemia. Unlike the changes in Ca^{2+} and Na^+ during hypoxia/ischemia, the changes in K^+ are characterized by a two-phase response to hypoxia/ischemia (Hansen, 1985; Jiang and Haddad, 1991; Silver and Erecińska, 1990; Xie et al, 1994; Reid and Paterson, 1996; Müller and Somjen, 2000a, 2000b; Chao et al, 2007a, 2007b, 2008, 2009) (Figure 1). The first phase shows a relatively slow increase in $[\text{K}^+]_e$, starting after 20–60 sec of hypoxia/ischemia, at this time Na^+ and Ca^{2+} remain unchanged. Over several minutes of hypoxia/ischemia, $[\text{K}^+]_e$ increase reaches a threshold level (no more than the so-called “ K^+ ceiling” level). Phase 2 is characterized by a rapid, abrupt and large increase in $[\text{K}^+]_e$, accompanied by a negative shift in extracellular DC potential and a sudden change in Na^+ and Ca^{2+} . Within about 20 seconds of onset of phase 2, $[\text{K}^+]_e$ reaches a peak value (range from 25–100 mM depending on animal ages, brain regions, neuronal types, severity and duration of insult, etc), then gradually decreases. On return to normoxia, $[\text{K}^+]_e$ falls rapidly, and often undershoots to below the basal levels before steadily returning to the pre-hypoxia/ischemia level. The increase in $[\text{K}^+]_e$ in the intact brain is more profound than in vitro brain tissues/cells (Silver and Erecińska, 1990; Xie et al, 1994; Zetterström et al, 1995). The hypoxic/ischemic increase in $[\text{K}^+]_e$ is also observed in the brain of some anoxia-tolerant species such as turtles and frogs. However, the magnitude is very small (increase by several mM) and the latency is very long (up to 4 hours delay) in comparison with mammalian brain (Sick et al, 1982, 1987; Doll et al, 1991; Xia et al, 1992; Knickerbocker and Lutz, 2001).

Hypoxic/ischemic increase in extracellular K^+ can be caused by increased K^+ efflux, decreased K^+ influx or both. In addition, neurons along with glial cells could contribute to this net $[\text{K}^+]_e$

the increase. Glial cells are less sensitive to identical insults than neurons (e.g., a decrease in neuronal ion gradients after 10 min of energy limitation is comparable to the reduction seen in glial cells after 16 h) (Silver et al, 1997; Hertz, 2008), and have high capacity of K^+ uptake and buffer (Kofuji and Newman, 2004; Leis et al, 2005). These properties determine that glial cells contribute less in hypoxia/ischemia-induced increase in $[K^+]_e$ than neurons. The main contribution of glial cells to K^+ redistribution during hypoxia/ischemia is buffering the increased extracellular K^+ . Only when the capacity of K^+ uptake and buffering is severely damaged with prolonged ischemia, glial cells begin to contribute to extracellular K^+ rise (Leis et al, 2005). The increase in $[K^+]_e$ seen in adult brain tissue during anoxia is, to a great extent, secondary to neuronal loss of intracellular K^+ (Jiang and Haddad, 1991).

Hypoxic/ischemic increase in $[K^+]_e$ is originated by the loss of intracellular K^+ . Cytoplasm contains considerable amount of K^+ , and therefore, is the main source of K^+ efflux. For some time, intramitochondrial K^+ concentration is assumed to be lower than the cytosolic concentration (Debska et al, 2001), the most of direct measurements suggest an intramitochondrial K^+ concentration of 180 mM or greater (vs. approximate 100–150 mM in cytosol) (Garlid, 1996; Taylor et al, 1999; Lopachin et al, 2001). Therefore, mitochondria can also contribute to increase in extracellular K^+ by releasing K^+ into cytosol and then efflux into extracellular space during ischemia (Liu, et al, 2003).

The loss of cellular K^+ reflects an imbalance between the rate of active K^+ uptake via the Na^+-K^+ ATPase and the rate of passive K^+ efflux. Many mechanisms have been proposed for intracellular K^+ loss during and after cerebral hypoxia/ischemia, including inhibition of Na^+-K^+ ATPase, increased leakage of K^+ due to an increase in excitability and action potential generation in neurons, and activation of K^+ channels (e.g. voltage-gated K^+ channels, ATP-sensitive K^+ channels, Ca^{2+} -activated K^+ channels and Na^+ -activated K^+ channels, as well as ionotropic glutamate receptor channels) (Hansen et al, 1985; Jiang and Haddad, 1991; Haddad and Jiang, 1993; Zetterström et al, 1995; Reid and Paterson 1996; Silver et al, 1997; Erdemli et al, 1998; Yu et al, 1999; Xiao et al, 2001; Müller and Somjen, 2000a; Lopachin et al, 2001; Yuan et al, 2003; Chao et al, 2007a, 2008). However, there is also study showing that moderate hypoxia blocks a voltage-gated, dendrotoxin-sensitive homomeric K^+ channel type in sensory neurons (Gruss et al, 2006).

Inhibition of Na^+/K^+ ATPase—Normal running of Na^+/K^+ ATPase constructs a large transmembrane electrochemical gradient of Na^+ and K^+ ions. Blockade of Na^+/K^+ ATPase with ouabain induces great intracellular K^+ loss (Alvarez-Leefmans et al, 1994; Xiao et al, 2002). ATP is greatly decreased during hypoxia/ischemia (Silver et al, 1997; Raley-Susman et al, 2001; Martinez-Sánchez et al, 2004; Sheldon and Church, 2004), which leads to failure of Na^+/K^+ ATPase. Therefore one might argue that anoxic/ischemic $[K^+]_e$ rise may be a result of failure of Na^+-K^+ pump. However, after $[K^+]_e$ rise in a steady-state level by Na^+-K^+ pump inhibition, anoxia/ischemia still induces a further significant increase in $[K^+]_e$ in the brain neurons, suggesting that mechanisms other than Na^+-K^+ ATPase are responsible for the anoxia/ischemia-induced extracellular K^+ accumulation (Jiang and Haddad, 1991; Reid and Paterson 1996). It appears that the undershoot in $[K^+]_e$ during reoxygenation from hypoxia is most likely due to activation of Na^+-K^+ ATPase and as a result of compensatory over-transportation of K^+ from outside into inside of the membrane by Na^+-K^+ pumps in brain cells (Reid and Paterson 1996).

Activation of K^+ channels—Many studies demonstrate the activation of various kinds of K^+ channels and enhanced outward K^+ current in neurons by hypoxia/ischemia, including K_{ATP} channels, voltage-gated K^+ channels, two pore domain K^+ channels, etc. (Jiang and Haddad, 1993; Guatteo et al, 1998a; Erdemli et al, 1998; Chi and Xu, 2000,²⁰⁰¹; Kulik et al, 2002; Müller et al, 2002; Plant et al, 2002; Park et al, 2003; Chao et al, 2007a). This suggests

a K^+ channel-mediated cellular K^+ loss and K^+ efflux. Because hypoxia/ischemia causes a great increase in $[Ca^{2+}]_i$ and $[Na^+]_i$ as discussed above, a high cytosolic concentration of Ca^{2+} and Na^+ would be expected to activate the Ca^{2+} -activated K^+ channels (BK channels) and Na^+ -activated K^+ channels (K_{Na}) to induce K^+ efflux (Murai et al, 1997; Sun et al, 2003; Yuan et al, 2003; Bhattacharjee and Kaczmarek, 2005; Chao et al, 2007a, 2008). Indeed, blockade of K^+ channels decreases hypoxia/ischemia-induced K^+ efflux and $[K^+]_e$ rise. K^+ efflux through K^+ channels is responsible for at least 35% increase in $[K^+]_e$ induced by anoxia/ischemia in the hippocampus, because quinine, a completely non-selective K^+ channel blocker, attenuates the anoxia-induced rise in $[K^+]_e$ by 35% (Zetterström et al, 1995). In addition, blockade of plasmalemmal and mitochondrial K_{ATP} channels (Jiang and Haddad, 1991; Reid and Paterson, 1996; Liu et al, 2003; Chao et al, 2007a) and voltage-gated K^+ channels (Zetterström et al, 1995) also attenuates hypoxic/ischemic $[K^+]_e$ rise in the brain. Although BK channels are reported to be inhibited by O_2 deprivation (Liu et al, 1999; Gao and Fung, 2002; Lewis et al, 2003), our recent works with K^+ electrode recording show that blockade of BK channels with paxilline (10 μM) attenuates anoxic $[K^+]_e$ rise by 22% in cortical slices (Chao et al, 2007a). We also observed that anoxia-induced Na^+ entry through voltage-gated Na^+ channel and NMDA receptor channels is responsible for Na^+ -based K^+ efflux (Chao et al, 2008, 2009), which may be mediated by K_{Na} channels (Yuan et al, 2003; Bhattacharjee and Kaczmarek, 2005; Ruffin et al, 2008). All these findings suggest that K^+ channel-mediated K^+ efflux is one of major routes for hypoxic/ischemic increase in $[K^+]_e$.

Ionotropic glutamate receptor channels and increased leakage of K^+ —Ionotropic glutamate receptor-gated channels are permeable to Ca^{2+} , Na^+ , and K^+ (Mayer and Westbrook, 1987). However, in comparison with ionotropic receptor channel-mediated Ca^{2+} and Na^+ entry, ionotropic glutamate receptor channel-mediated K^+ efflux has received little attention. The ionotropic glutamate receptor (NMDA, AMPA, and KA)-mediated neuronal K^+ loss may be another reason for hypoxia/ischemia-induced $[K^+]_e$ increase (Croning et al, 1993; Lopachin et al, 2001; Chao et al, 2009). Blockade of ionotropic glutamate receptors with NMDA receptor blockers (CPP, dizocilpine, (+)MK-801), or AMPA receptor blocker CNQX partially decreases OGD and hypoxia-induced intraneuronal K^+ loss in the hippocampus and cortex (Croning et al, 1993; Lopachin et al, 2001; Chao et al, 2009). Activation of NMDA, AMPA, and KA receptors of mouse cortical neurons, even in a condition of decreased inward cation influx by lowering extracellular Na^+ and Ca^{2+} , induces a large outward K^+ current, which causes loss of approximately 50% to 80% intraneuronal K^+ and great shrinkage of cell body and consequently neuronal apoptosis (Yu et al, 1999; Xiao et al, 2001).

Increased leakage of K^+ due to an increase in excitability and action potential generation in neurons may also be another reason for hypoxia/ischemia-induced increase in $[K^+]_e$. It has been shown that in hippocampal slices, when neuronal excitability is decreased and spontaneous and evoked impulse firing is blocked by TTX or lidocaine, hypoxia-induced sharp $[K^+]_e$ increase is significantly dampened (Müller and Somjen, 2000a; Raley-Susman et al, 2001). Similar observations were found in hypoglossal neurons and isolated, in situ perfused rat brain (Jiang and Haddad, 1991; Xie et al, 1994). More recently, we made a very interesting observation in cortical slices that lowering external Na^+ concentration with substitution of impermeable N-methyl-D-glucamine (NMDG⁺) greatly attenuates anoxic $[K^+]_e$ rise, whereas lowering Na^+ concentration with substitution of permeable Li^+ tends to potentiate the anoxic disruption of K^+ homeostasis (Chao et al, 2008). Because NMDG⁺ is a membrane impermeable organic cation, lowering external Na^+ with NMDG⁺ replacement may reduce Na^+ influx and neuronal excitability because of a reduction of driving force for inward sodium current during anoxia (Calabresi et al, 1999; Sheldon et al, 2004). In contrast, Li^+ , unlike NMDG⁺, is permeable cross membrane by multiple transport ways, particularly by Na^+ channels through which Li^+ passes nearly as well as Na^+ itself (Hemsworth et al, 1997; Nikolakopoulos et al, 1998; Franceschetti et al, 2003; Montezinho et al, 2004). Li^+ entry gradually depolarizes

membrane potential (Grafe et al, 1983; Kiedrowski, 1999; Franceschetti et al, 2003), broadens action potential (Mayer et al, 1984; Colino et al, 1998), positively shifts afterpotential (Franceschetti et al, 2003; Liu and Leung, 2004). In addition, it progressively potentiates the high-frequency firing (Franceschetti et al, 2003), and presynaptically enhances excitatory synaptic transmission (Grafe et al, 1983; Evans et al, 1990; Colino et al, 1998). Therefore, our observations (Chao et al, 2008) support the above idea, namely, increased leakage of K^+ due to an increase in excitability and action potential generation in neurons may also contribute to hypoxia/ischemia-induced increase in $[K^+]_e$. Moreover, if an increase in excitability by glutamate receptor activation is considered, $[K^+]_e$ rise can be contributed by increased leakage of K^+ induced by glutamate besides ionotropic glutamate receptor channel-mediated K^+ efflux.

4. Role of opioid systems in ionic homeostasis under normal conditions

Ionic homeostasis is well maintained by cells through rapid changes in activities of ionic pumps (e.g. Na^+-K^+ ATPase, Ca^{2+} ATPase), exchangers (e.g. NCX) and spatial ionic buffering mechanisms under physiological states, which can be regulated by multiple factors, such as neurotransmitters including opioids. Endogenous opioids are generally regarded as neurotransmitters/modulators and mainly play an inhibitory role in cellular activities with three major classes of receptors (DOR, MOR, KOR). They are widely distributed throughout the central nervous system (Mansour et al, 1987, 1994; Xia and Haddad, 1991, 2001; Hiller et al, 1996). Opioid receptors are members of the G protein-coupled receptor superfamily (Raynor et al, 1994; Law et al, 2000), and may functionally couple with ionic channels, including K^+ channels, Ca^{2+} channels, and Na^+ channels etc. (North et al, 1987; Ikeda et al, 1995; Nah et al, 1997; Toselli et al, 1997; Su et al 1998; Witkowski and Szulczyk, 2006; Kang et al, 2008, 2009). Almost all previous studies regarding the regulation of ionic homeostasis by opioids were performed under normoxic condition and focused on the activity of intracellular Ca^{2+} . Until our recent work, few studies focused on the regulation of K^+ and Na^+ homeostasis despite the functional coupling of opioids with K^+ channels and Na^+ channels (North et al, 1987; Ikeda et al, 1995; Witkowski and Szulczyk, 2006; Chattopadhyay et al, 2008; Kang et al, 2008, 2009). Table 2 summarizes the studies of the effects of DOR agonists/antagonists on ionic homeostasis.

4.1 Role of opioids in regulation of Ca^{2+} homeostasis

Substantial evidence indicates that opioid system can affect Ca^{2+} homeostasis. Overall, the predominant effect of opioids on Ca^{2+} entry is inhibitory, which seems to match with classical recognition of opioid system as an inhibitory regulator in the brain. However, some studies show either mobilization of intracellular Ca^{2+} or stimulation of Ca^{2+} entry with opioid activation.

Opioid elevation of intracellular Ca^{2+} —Most of the results about opioid-induced elevation of intracellular Ca^{2+} come from the studies in neuronal-like cell lines (e.g., NG108-15, SK-N-SH, NMB, SH-SY5Y, Neuro2a, CHO cell lines) (Jin et al, 1992; Fields et al, 1995; Smart and Lambert, 1996; Spencer et al, 1997; Connor et al, 1997; Yoon et al, 1998, 1999; Harrison et al, 1999; Morikawa et al, 1998, 1999). Though these cells may not be identical to native brain cells, the data yielded from them provide initial clues for understanding the roles of opioid system in regulation of $[Ca^{2+}]_i$ and the relevant physiological implications (e.g., regulation of transmitter release) in early research. Opioid-induced increase in $[Ca^{2+}]_i$ has also been reported in astrocytes (Hauser et al, 1998; Thorlin et al, 1998) and dorsal root ganglion neurons (Tang et al, 1996; Przewlocki et al, 1999; Bao et al, 2003) (primary nerve cells vs. cell lines). Opioid-induced $[Ca^{2+}]_i$ rise may be caused by either mobilization of intracellular Ca^{2+} stores or stimulation of Ca^{2+} entry across membrane. An excellent review

was presented by Samways and Henderson (2006) for opioid-induced elevation of intracellular Ca^{2+} under normal condition.

In some studies, it was found that opioid-induced increase in $[\text{Ca}^{2+}]_i$ did not require extracellular Ca^{2+} , suggesting the mobilization of intracellular Ca^{2+} store in opioid-induced elevation of $[\text{Ca}^{2+}]_i$. Indeed, brief exposure (30–90s) of NG108-15 cells to DOR agonists in nanomolar concentrations induces Ca^{2+} release from intracellular stores via a process that appears to require phospholipase C (PLC) activation and consequent IP_3 formation (Jin et al, 1992; Smart and Lambert, 1996; Yoon et al, 1998, 1999). This Ca^{2+} mobilization appears to mainly occur in undifferentiated NG108-15 cells (Jin et al, 1992; Smart and Lambert, 1996). DOR activation-induced intracellular Ca^{2+} mobilization is also observed in transfected Neuro2a cells and CHO837 cells (Spencer et al, 1997; Harrison et al, 1999). In SH-SY5Y cells, DOR activation induced intracellular Ca^{2+} mobilization requires the presence of muscarinic agonist (Connor et al, 1997), reflecting a synergetic action between opioid and Gq-coupled receptors in $[\text{Ca}^{2+}]_i$ elevation (Samways and Henderson, 2006). Activation of MOR and KOR was also found to mobilize Ca^{2+} from IP_3 -sensitive intracellular Ca^{2+} store (Spencer et al, 1997). These observations suggest that the mobilization of intracellular Ca^{2+} store may be a general feature of opioid receptor function.

Contrast to the mobilization of intracellular Ca^{2+} stores, stimulation of Ca^{2+} entry also contributes to opioid-induced $[\text{Ca}^{2+}]_i$ elevation. For example, despite the contribution of mobilization of intracellular Ca^{2+} stores to DOR activation-induced $[\text{Ca}^{2+}]_i$ elevation in undifferentiated NG108-15 cells, DOR activation-induced $[\text{Ca}^{2+}]_i$ elevation was abolished in differentiated NG108-15 cells in the absence of extracellular Ca^{2+} (Jin et al, 1992). A similar observation was reported in SK-N-SH and NMB cells with 30–90s opiates application (Fields et al, 1995). In cultured cortical astroglial cells, DOR activation induced a biphasic Ca^{2+} response, with an initial peak of rapid increase in $[\text{Ca}^{2+}]_i$ followed by a sustained level of elevated $[\text{Ca}^{2+}]_i$ or oscillations in $[\text{Ca}^{2+}]_i$ (Thorlin et al, 1998). Removal of extracellular Ca^{2+} did not affect the first peak in $[\text{Ca}^{2+}]_i$, but abolished the sustained level of elevated $[\text{Ca}^{2+}]_i$ or oscillations in $[\text{Ca}^{2+}]_i$. This suggested that the first peak in $[\text{Ca}^{2+}]_i$ was due to mobilization of intracellular Ca^{2+} store, whereas the second part of responses was a result of Ca^{2+} influx through membrane channels (Thorlin et al, 1998). In dorsal root ganglion neurons, activation of three opioid receptor subtypes (DOR, MOR, KOR)-induced $[\text{Ca}^{2+}]_i$ rise can be greatly attenuated by removal of extracellular Ca^{2+} or blockade of dihydropyridine-sensitive Ca^{2+} channels (Tang et al, 1997; Bao et al, 2003). These results suggest a contribution of opioid-induced Ca^{2+} entry. This Ca^{2+} entry may be mediated by dihydropyridine-sensitive, voltage-gated Ca^{2+} channels (Jin et al, 1992; Tang et al, 1997).

It should be keep in mind that only a small number of cell types, but not all cells, show $[\text{Ca}^{2+}]_i$ elevation with opioid activation. Most of the knowledge regarding $[\text{Ca}^{2+}]_i$ elevation with opioid activation comes from the studies in neuronal-like cell lines, but not in “real” neurons. Though these cells may have similar characteristics in some degree as native brain cells, they are not totally identical to each other. In fact, the majority of nerve cells showed an inhibition of Ca^{2+} entry with opioid activation (see the following section), and only a very small amount of nerve cells studied showed an increase in $[\text{Ca}^{2+}]_i$ when stimulated by opioid activation. The opioid-induced elevation of intracellular Ca^{2+} is related to the excitatory effect of opioids on some presynaptic neurons and consequent regulation of neurotransmitter release (Shen and Crain, 1990; Moore et al, 1994; Przewlocki et al, 1999; Bao et al, 2003; Samways and Henderson, 2006).

Opioid inhibition of Ca^{2+} entry—Though some studies showed an excitatory effect of opioids on Ca^{2+} entry, the predominant effect of opioids is inhibitory on Ca^{2+} entry. A major piece of supporting evidence is the blockade of Ca^{2+} entry through voltage-gated Ca^{2+} channels

by opioids. In contrast to DOR activation-induced elevation in $[Ca^{2+}]_i$ in cultured cortical astroglial cells, cultured cortical neurons show a decrease in $[Ca^{2+}]_i$ by inhibition of L-type Ca^{2+} channels with DOR activation in the same culture (Thorlin et al, 1998). DOR activation inhibits diverse calcium currents (e.g. L-, N-, P- and Q- type Ca^{2+} channel currents) which link to Ca^{2+} influx through different high-voltage-activated channel types in neurons as well as neuroblastoma cell lines (Nah et al, 1997; Toselli et al, 1997, 1999; Adams and Trequattrini, 1998; Morikawa et al, 1998, 1999; Acosta and López, 1999; Pirois et al, 2000). This decreases $[Ca^{2+}]_i$ level in resting state as well as depolarization-induced Ca^{2+} uptake in neuron-like cell lines (Jin et al, 1992; Wang et al, 1993) and cultured cortical neurons (Vlaskovska et al, 1997; Thorlin et al, 1998). DOR inhibition greatly potentiates NMDA receptor activities and NMDA-mediated increase in $[Ca^{2+}]_i$ in neurons (Pamenter and Buck, 2008). Similar results are also observed with MOR and KOR activation (Vlaskovska et al, 1997; Yang et al, 2004; Ortiz-Maranda et al, 2003; Fields et al, 1995). For example, activation of MOR or KOR also inhibits Ca^{2+} channel currents in both neurons and neuronal cell lines (Stefani et al, 1994; Nah et al, 1997; Su et al, 1998; Adams and Trequattrini, 1998; Toselli et al, 1999; Spadoni et al, 2004; Komai and McDowell, 2007; Rola et al, 2008), and reduces Ca^{2+} influx and depolarization-induced Ca^{2+} uptake in neurons (Vlaskovska et al, 1997; Ortiz-Maranda et al, 2003; Yang et al, 2004). Obviously, depolarization activates voltage-gated Ca^{2+} channels, which results in the Ca^{2+} entry, while opioids inhibit $[Ca^{2+}]_i$ rise by directly decreasing Ca^{2+} influx through NMDA channels and voltage-gated Ca^{2+} channels, especially dihydropyridine-sensitive Ca^{2+} channels (Jin et al, 1992; Wang et al, 1993; Yang et al, 2004; Ortiz-Maranda et al, 2003; Pamenter and Buck, 2008). Therefore, activation of opioid receptors inhibits the release of neurotransmitters from presynapses and excitatory postsynaptic potentials/currents and neuronal activities (Tanaka and North, 1994; Wang and Mokha, 1996; Ostermeier et al, 2000; Pirois et al, 2000; Ortiz-Maranda et al, 2003; Yang et al, 2004).

4.2 Role of opioids in regulation of Na^+ homeostasis

There is lack of direct evidence regarding the role of opioids in regulation of Na^+ homeostasis under normoxia. However, there is indirect information suggesting an interaction between opioids and Na^+ channels. For example, we observed that DOR down-regulation (Zhao et al, 2005) is associated with an up-regulation of voltage-gated Na^+ channels (Xia et al, 2003). In a recent study, SNC80, a putative DOR agonist, was applied to acutely isolated dentate granule cells of rat hippocampus, and the properties of voltage-gated Na^+ currents were investigated by whole cell patch-clamp recording. The authors observed that SNC80 (1–1000 μM) reduced the maximal Na^+ current amplitude in a dose-dependent manner and selectively prolonged the time course of recovery from slow inactivation without effects on fast inactivation processes (Remy et al, 2004). However, the authors concluded that this effect is opioid receptor independent because the effects of SNC80 were not mimicked by another DOR agonist DPDPE (10 μM), and were not inhibited by high concentrations of opioid receptor antagonists, naloxone (50–300 μM) and naltrindole (10 and 100 μM) (Remy et al, 2004). This study may be complicated by several factors. For example, our autoradiographic work showed that the hippocampus, including the dentate gyrus, has a low density of DOR binding sites, while the cortex has a very high density (Xia and Haddad, 1991; 2001). Applying low concentrations of DOR agonists to hippocampal neurons may induce an insignificant effect because of a scarcity in the density of DOR. On the other hand, applying DOR at high concentrations may lead to non-selective or adverse effects because of the stimulation of multiple and non-specific membrane proteins. Furthermore, some experimental procedures such as mechanical trituration and chemical digestion (e.g., Pronase) (Remy et al, 2004) may affect the function of DOR and Na^+ channels and their interactions, causing complex changes in the effects of DOR agonists/antagonists on Na^+ channel currents. In addition, high concentrations of DOR ligands, either SNC80, naloxone or naltrindole, may stimulate a variety of membrane proteins and induce a complicated effect on cells since no “pure” DOR agonists/antagonists exist at

present. For instance, high concentrations of SCN80 can activate μ -opioid receptors (Knapp et al, 1996) which may inhibit the voltage-dependent Na^+ current in the cortex (Witkowski and Szulczyk, 2006). Also, naltrindole at high concentrations generates adverse effects to neurons (Ma et al, 2005; Chao et al, 2007b). To avoid these pitfalls and explore a “pure” interaction between DOR and Na^+ channels, we recently took the advantages of *Xenopus* oocytes with co-expressed DOR and Na^+ channels to make novel and exciting observations. We made the following observations: (1) Nav1.2 expression induced TTX-sensitive inward currents; (2) DOR expression reduced the inward currents; (3) Activation of DOR reduced the amplitude of the current and rightly shifted the activation curve of the current in the oocytes with both Nav1.2 and DOR, but not in oocytes with Nav1.2 alone; (4) The DOR agonist-induced inhibition of Nav1.2 currents was in a dose-dependent manner and saturable; and (5) The selective DOR agonist had no effect on naive oocytes. These data represent the first demonstration that activation of DOR inhibits Na^+ channel function by decreasing the amplitude of Na^+ currents and increasing its threshold of activation (Kang et al, 2008,2009).

Besides DOR action on Na^+ channels, MOR and KOR also act to modulate Na^+ channel activities. For example, in acutely isolated cortical neurons, the application of 1 μM of [D-Ala2, N-Me-Phe4, Gly5-OL]-enkephalin (DAGO), a specific MOR agonist, caused a decrease in the Na^+ current amplitude to approximately 79% of the controls. Moreover, DAGO decreased the maximum current activation rate, prolonged its time-dependent inactivation, and shifted the half inactivation voltage from -63.4 mV to -71.5 mV and prolonged the time constant of recovery from inactivation from 5.4 ms to 7.4 ms (Witkowski and Szulczyk, 2006). This inhibition involved protein kinase A (PKA) and C (PKC) mechanisms (Witkowski and Szulczyk, 2006). DAGO also inhibits TTX-resistant voltage-dependent Na^+ current in dorsal root ganglion neurons (Gold and Levine, 1996), while U50488, a KOR agonist, decreases voltage-activated Na^+ currents in colon sensory neurons (Su et al, 2002). Spiradoline, another KOR agonist, was reported to reduce peak Na^+ current in a rapid, reversible, and concentration-dependent manner in myocytes (Pugsley et al, 1998).

4.3 Role of opioids in regulation of K^+ homeostasis

Several early studies have demonstrated a coupling of opioid receptor functions with K^+ channels (North et al, 1987; Wild et al, 1991; Ikeda et al, 1995). For example, activation of DOR increases inward rectifier potassium currents (I_{Kir}) and hyperpolarization-activated cation (I_{h}) currents (Ikeda et al, 1995; Svoboda and Lupica, 1998; Piro et al, 2000; Shi et al, 2000). DOR agonist, under low concentrations (nanomolar) decreases, whereas under high concentrations (micromolar) increases, voltage-gated K^+ currents in neurons and neuron-like cell lines (Fan et al, 1991, 1993; Fan and Crain, 1995; Jose et al, 2007). The dual regulation of voltage-gated K^+ currents as well as enhancement of I_{Kir} were also seen in neurons with MOR and KOR activation (Wimpey and Chavkin, 1991; Fan et al, 1991, 1993; Fan and Crain, 1995; Svoboda and Lupica, 1998; Chen et al, 2001; Barral et al, 2003). In contrast to the substantial studies of opioid roles in K^+ channel activities, little is known about the role of opioids in regulation of K^+ homeostasis under normal condition. Recently, we assessed the effect of DOR and MOR activation/inhibition on K^+ homeostasis in mouse cortical slices by directly measuring the extracellular K^+ activities with K^+ -sensitive microelectrode (Chao et al, 2006, 2007a, 2007b, 2008; 2009). Our results show that during 20 min of perfusion of DOR (DADLE, UFP 512) or MOR (DAGO) agonists and DOR antagonist (naltrindole) before hypoxia/ischemia, no obvious changes in $[\text{K}^+]_{\text{e}}$ are observed in the cortical slices. This suggests that there is little effect of DOR and MOR on K^+ homeostasis (vs. the activities of K^+ channels), at least for extracellular K^+ concentration, under normoxic conditions (vs. hypoxia/ischemia, see next section) (Chao et al, 2006, 2007a, 2007b, 2008; 2009), though DOR has been shown to regulate the activities of K^+ channels.

5. Effect of DOR activation on hypoxic/ischemic disruption of ionic homeostasis

There is no information on this subject in the literature. Our recent studies are the first demonstration of DOR interaction with ionic homeostasis under stress (Chao et al, 2006, 2007a, 2007b, 2008, 2009). In this section, we will summarize the major progress in this field and present a clear, though preliminary, picture on the critical role of DOR in the regulation of ionic homeostasis in hypoxic/ischemic conditions.

5.1 DOR induced neuroprotection from hypoxic/ischemic injury

Some clues from studies of our laboratory and those of others suggest the involvement of DOR in neuroprotection against hypoxic/ischemic stress. Those include the following:

Firstly, turtle brain has a much higher density of DOR than rat brain, and is more resistant to hypoxic/ischemic stress as compared to rat brain (Xia and Haddad, 1991, 2001; Xia et al, 1992). Secondly, rat cortex shows higher density of DOR than subcortical regions (Xia and Haddad, 1991), and is more tolerant to hypoxia than subcortical regions (i.e. thalamus, hippocampus and brain stem) (Lin et al, 1990; O'Reilly et al, 1995; Dunn et al, 1999; Buresh et al, 1999). Thirdly, distribution of DOR is heterogeneous in the cortex with the outer and inner layers having higher density than in the middle layer. Correspondingly, the outer and inner layers (layer 2 and 5) of the cortex are more tolerant to hypoxia/ischemia than the middle layer (layer 3) (Kornblum et al, 1987; Nedergaard, 1988; Lin et al, 1990; Georges et al, 1998). In addition, mammalian hibernation, a distinct energy-conserving state, is associated with depletion of energy stores, intracellular acidosis and hypoxia, similar to those which occur during ischemia and this hypoxia-tolerant state can be triggered by DOR activation (Borlongan et al, 2004; 2009). Moreover, endogenous enkephalin levels are greatly increased in the brain during hypoxia (Yan et al, 1995; Armstead, 1998; Chen and Du, 2000). This increase may be one of strategies to counteract hypoxic stress by increasing activity of DOR. Of all the three major opioid receptors (DOR, MOR, KOR), DOR has been proven more sensitive than others to stressful stimuli, e.g., hypoxia and ischemia. (Mayfield et al, 1996; Boutin et al, 1998, 1999, 2003, 2007; Ma et al. 2005; Zhang et al, 2006; Peng et al, 2009). Prolonged exposure to hypoxia (9% O₂) continuously for seven days selectively decreases DOR, but not MOR and KOR expression in the mouse brain homogenates (Mayfield et al, 1996). DOR is also significantly decreased as early as 6 h post transient focal cerebral ischemia (v.s. that of MOR after 24 h and no changes in KOR) (Boutin et al, 1998, 1999, 2003, 2007). All these notions suggest a crucial role of DOR expression in neuronal regulation under hypoxic/ischemic condition.

However, it was unclear until our recent work as for the precise role of opioid receptors in neuroprotection against hypoxic/ischemic injury. Some earlier *in vivo* works (Hosobuchi et al, 1982; Adams et al, 1986; Skarphedinsson and Thoren, 1988; Olinger et al, 1990; Chen et al, 2001) showed that opioid receptor inhibition with opioid antagonists (e.g., naloxone that binds to DOR, MOR and KOR) protected the brain from ischemia-induced injury, which implied that opioid receptor activation are neurotoxic. In sharp contrast, some studies suggested that opioid receptor activation protected the brain from ischemia or extended animal survival time in hypoxia (Hayward et al, 1993; Mayfield and D'Alecy, 1994; Endoh et al, 1999; Summers et al, 2003; Oeltgen et al, 2006). Such major controversy diminished the enthusiasm of researchers in the past. The major controversies may result from the methodological limitations since a majority of these earlier studies mainly based on the application of low-selective ligands at high concentrations via intravenous injection. A high dose of opioid ligands can cross-react with various opioid receptor subtypes or induce non-opioid effects, which may have complicated the interpretation of the results observed. Besides, systemic administration of

opioids may induce multiple effects on various organs, e.g., heart (Schultz et al, 1998; Peart and Gross, 2006), blood vessels (Sun et al, 1989), liver and gastroenteric tissue (Tubbs et al, 2002; Yamanouchi et al, 2003), which makes the results more complex.

To ascertain the role of opioid receptors in neuronal response to hypoxic/ischemic 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. It may yield more precise information to target certain type(s) of opioid receptors in specific nerve cells by using specific opioid agonists/antagonists. There have been, however, few studies on it in the past. One reported that treatment with morphine (preferential MOR-ligand), U-50488H (KOR ligand) or naloxone provided no significant protection on the hippocampus subjected to ischemia (Iwai et al., 1992), suggesting that MOR activation does not induce neuroprotection in the hippocampal neurons. Another study showed that a variety of MOR- ligands at high concentrations (0.1–3 mM) reduced neuroexcitotoxicity in cortical neurons, which could not be blocked by naloxone, suggesting a “non-opioid effect” (Choi and Viseskul, 1988). Since there is a high density of DOR in the cortex, the application of MOR agonists in the cortical neurons may not detect the role of DOR in neuroprotection in the cortex as well as other regions.

To clarify the important issues about the role of opioid in hypoxic/ischemic neuronal injury, more specific techniques and ligands are needed in the studies. Our serial studies since 1997 have proven that activation of DOR is neuroprotective and predisposes neurons to survive under severe stress such as glutamate-, hypoxia- and ischemia- stress, while inhibition of DOR by administration of DOR antagonists causes cell death and further augments neuronal injury during severe hypoxia (Zhang et al., 1999; 2000, 2001, 2002, 2006; Zhao et al., 2002, ²⁰⁰⁵; Hong et al., 2004; Ma et al., 2004, ²⁰⁰⁵; Chao et al, 2006, 2007a, 2007b, 2008, 2009; Kang et al, 2009). In fact, our novel data have clarified several controversies that existed in the literature. For example, the reason that μ -ligands had little protective effect on hippocampal neurons (Iwai et al, 1992) is likely because of low density of DOR in the hippocampus,, while the cause of high-dose μ -ligand induced reduction of neuroexcitotoxicity in cortical neurons (Choi and Viseskul, 1988) might be attributed to activation of DOR that are highly expressed in the cortex. Indeed, recent studies from other independent laboratories demonstrated that DOR is neuroprotective against hypoxic/ischemic stress (Borlongan et al., 2004, 2009; Horiuchi et al., 2004, 2008; Lim et al, 2004; Zhao et al., 2006; Su et al., 2007;Iwato et al, 2007; Xiong et al, 2007; Charron et al, 2008; Kao et al, 2008; Govindaswami et al, 2008; Pamenter and Buck, 2008; Tian et al, 2008a, 2008b; Peng et al, 2009; Zhu et al, 2009) (Table 3). For example, in a rodent model of stroke, up-regulation of DOR expression, and peripherally/centrally administration of DOR agonists (DADLE, DPDPE, TAN-67, Deltorphin-D_{variant}, BW373U86) significantly reduce the infarct volume, attenuate neurological deficits and neuronal loss, and increase neuron survival in post-ischemia in the brain, particularly in the cortex and hippocampus, which can be abolished by DOR antagonist naltrindole (Borlongan et al., 2004, 2009; Su et al., 2007;Iwato et al, 2007; Xiong et al, 2007; Charron et al, 2008; Kao et al, 2008; Govindaswami et al, 2008; Tian et al, 2008a, 2008b). DOR antagonist (naltrindole) alone significantly worsens ischemic neuronal injury (Iwato et al, 2007; Tian et al, 2008b), suggesting a “tonic” role of endogenous DOR system in neuroprotection. Intrathecal treatment with DOR agonist SNC80 can attenuate hind-limb motor dysfunction and neuronal and the ventral white matter injury following spinal cord ischemia in rats (Horiuchi et al., 2004, 2008). In vitro studies also reveal that activation of DOR significantly attenuates neuronal damage and death induced by hypoxia/ischemia (Lim et al, 2004; Zhao et al., 2006; Peng et al, 2009; Zhu et al, 2009). It is very likely that an up-regulation of DOR system, either in function or in expression, may have a therapeutic potential in management of hypoxic/ischemic injury.

Our data suggest that DOR may play an important role in protecting neurons against environmental stress such as hypoxic/ischemic insults. Indeed, there are recent studies demonstrating that hypoxic preconditioning increases the level of DOR and provide the protection against neuronal damages induced by severe hypoxia in cultured cortical neurons (Ma et al, 2005; Zhang et al, 2006) and by elevated intraocular pressure in rat retinas (Peng et al, 2009).

5.2 DOR-mediated regulation of ionic homeostasis during hypoxia/ischemia

In the mechanistic exploration, we noticed that an important aspect of DOR-mediated neuroprotection may be its early action against disruption of ionic homeostasis, particularly K^+ homeostasis during anoxia or ischemia. As discussed earlier, K^+ is the most abundant ion inside cells, and excessive K^+ efflux is believed to be a crucial factor leading to neuronal death under pathophysiological conditions such as anoxic/ischemic and excitotoxic stress (Yu et al, 1997, 1999; Liu et al, 2003; Wei et al, 2003; Yu, 2003; Karki et al, 2007; Hertz, 2008).

In certain circumstances, activation of K^+ channels protects neurons from hypoxia (Ballanyi, 2004; Buck and Pamerter, 2006; Huang et al, 2006; Sun et al, 2007). For example, although K_{ATP} channels are normally inhibited by physiological levels of ATP, a decrease in cytosolic ATP concentration, such as under hypoxic condition (Silver et al, 1997; Raley-Susman et al, 2001; Martinez-Sánchez et al, 2004; Sheldon and Church, 2004), opens K_{ATP} channels, leading to membrane hyperpolarization (Ballanyi, 2004; Miki and Seino, 2005; Huang et al, 2006). Activation of BK channels shapes neuronal excitability and can be an adaptive mechanism in some central neurons because these K^+ channels can dampen or limit the depolarization by K^+ efflux, and reduce neurotransmitter release. BK channels can also be activated by the decrease in ATP and the increase in $[Ca^{2+}]_i$ (Murai et al, 1997; Misonou et al, 2005; Chao et al, 2007a). In addition, K_{Na} channels are sensitive to intracellular Na^+ , and are inhibited by intracellular ATP, decreased pH in low $[Na^+]_i$ and activated by higher $[Na^+]_i$ even in the presence of decreased pH, a situation often seen in hypoxia/ischemia (Yuan et al, 2003; Bhattacharjee et al, 2003; Bhattacharjee and Kaczmarek, 2005; Ruffin et al, 2008). In certain neurons, K_{Na} channels also appear to be activated by Na^+ influx accompanying a single spike. Two genes encoding these channels, i.e., Slick and Slack, are expressed throughout the brain (Yuan et al, 2003; Bhattacharjee et al, 2003, 2005). K_{Na} channels may contribute to K^+ efflux and play a role in adaptation of firing rate and slowing after-hyperpolarization that follows repetitive firing (Lawrence and Rodrigo, 2001; Bhattacharjee and Kaczmarek, 2005). Under the conditions of decreased ATP concentration as well as increased $[Na^+]_i$ and $[Ca^{2+}]_i$ (often occur in hypoxia/ischemia), these channels (K_{ATP} , BK, and K_{Na} etc) can be activated to limit depolarization in certain degree. As it is well known, limitation of depolarization can reduce neuronal excitability and energy consumption and potentially protect nerve cells against hypoxic/ischemic damage.

However, these adaptive mechanisms (K_{ATP} , BK and K_{Na} channels etc) are beneficial to neurons in counteracting hypoxic/ischemic stress only in a very limited range of K^+ efflux. We would like to stress that excessive intracellular K^+ loss is not protective, but harmful to neurons. The loss of ionic homeostasis is the initial and key step in hypoxic/ischemic neuronal injury. Excessive K^+ fluxes play important roles in the neuronal death that occurs in various conditions, including during hypoxia/ischemia (Bortner et al, 1997; Yu et al, 1997, 1999; D'Ambrosio et al, 1999; Wei et al, 2003; Liu et al, 2003; Yu, 2003; Lauritzen et al, 2003; Remillard and Yuan, 2004; Karki et al, 2007; Hertz, 2008). In fact, blockade of K^+ efflux attenuates hypoxia-, ischemia- and glutamate-induced neuronal death (Huang et al, 2001; Wei et al, 2003; Liu et al, 2003; Zhao et al, 2006; Nistico et al, 2007). The reduced expression of K_{ATP} channels protects dopaminergic neurons from degeneration in Parkinson's disease (Liss and Roeper 2001). These studies suggest that inhibition of massive K^+ fluxes and maintaining

cellular K^+ homeostasis is beneficial to neurons under severe hypoxic/ischemic stress, and may be of therapeutic benefit in the treatment of stroke and related neurodegenerative conditions (Wei et al, 2003; Liu et al, 2003; Zhao et al, 2006; Nistico et al, 2007).

Indeed, some anoxia-tolerant species such as turtles, have extraordinary abilities to counteract hypoxia/ischemia-induced disruption of ionic homeostasis in their brain. Even up to 4 hours of anoxia could not induce the abrupt rise in $[K^+]_e$ in turtle brain, while it is very evident in the rat cortex within several minutes of anoxia (Sick et al, 1982; Xia et al, 1992). Although the mechanisms underlying hypoxia/ischemia tolerance are very complex in the turtle, the uniquely high density of DOR in the turtle brain (Xia and Haddad, 1991, 2001) may be one of the potential mechanisms. To explore the possible role of opioid receptors in the regulation of ionic homeostasis during hypoxia/ischemia, we recently made a series of experiments by direct measurement of extracellular K^+ activities with K^+ -sensitive microelectrodes in mouse cortical slices (Chao et al, 2007a, 2007b, 2008, 2009). We also established a mouse model with neuron-specific and tetracycline-regulated up-regulation of DOR in the cortex, and obtained some interesting results with this model regarding the effects of opioids on disruption of ionic homeostasis during hypoxia/ischemia (Chao et al, 2006).

In our serial studies, cortical slices were used to assess the effects of DOR activation on K^+ homeostasis because the cortex has a much higher density of DOR (Xia and Haddad, 1991, 2001). Anoxia was induced by switching from the control superfusate to one continuously aerated with 95% N_2 and 5% CO_2 . Ischemia was mimicked by OGD, i.e., exposing the slices to D-glucose-deficient ACSF (D-glucose was substituted by equal molar mannitol) continuously aerated with 95% N_2 and 5% CO_2 . Each slice was subjected to a single period of anoxia or ischemia that continued for about 1.5 min after the onset of anoxic depolarization (as assessed by a rapid increase in $[K^+]_e$ and negative shift of extracellular DC potential), or for a period of 20 min if anoxic depolarization did not occur. DOR agonists/antagonists were applied to the slices starting 20 min. before anoxia/ischemia and lasting for the whole period of anoxic/ischemic stress. In some experiments, we focused just on the mechanistic investigation by analyzing anoxia/ischemia-induced disruption of ionic homeostasis as well as changes in extracellular DC potential since we (Zhang et al., 2000, 2001, 2002, 2006; Zhao et al., 2002, 2005; Hong et al., 2004; Ma et al., 2004, 2005) and others (Borlongan et al., 2004, 2009; Horiuchi et al., 2004, 2008; Lim et al, 2004; Zhao et al., 2006; Su et al., 2007; Iwata et al, 2007; Xiong et al, 2007; Charron et al, 2008; Kao et al, 2008; Govindaswami et al, 2008; Pamenter and Buck, 2008; Tian et al, 2008a, 2008b; Peng et al, 2009; Zhu et al, 2009) demonstrated that activation of DOR is neuroprotective and predisposes neurons to survive under severe stress such as glutamate-, hypoxia- and ischemia-stress.

We showed that DADLE (1–10 μM), an agonist of DOR, concentration-dependently attenuated the anoxia- and OGD-induced disruption of K^+ homeostasis and decrease in extracellular DC potential in the cortical slices. This attenuation was manifested by a prolonged latency of the response to anoxia/ischemia, largely attenuated maximal increase in $[K^+]_e$, and reduced rate of rise of $[K^+]_e$ from latency to peak (Chao et al, 2007a, 2007b). The effect of DADLE was completely blocked by 1 μM naltrindole, a potent DOR antagonist, suggesting that DOR activation is involved in the effect (Chao et al, 2007a). We previously observed that naltrindole, if administered at 10 μM in neuronal culture, increased hypoxic injury in cortical neurons (Zhang et al, 2002). At the same concentration in the cortical slices, naltrindole induced an extremely larger increase in $[K^+]_e$ during anoxia (Chao et al, 2007b). It is very likely that excessive inhibition of DOR may cause neuronal injury through severe disruption of K^+ homeostasis. Application of UFP 512, a more specific, potent and naturally distinct DOR agonist (Balboni et al, 2002) to cortical slices had similar effects on anoxic disruption of K^+ homeostasis (Figure 2), as seen with DADLE (Chao et al, 2007b). Since almost all DOR agonists, including DADLE and UFP 512, may bind to MOR in a low affinity (Chang and

Cuatrecasas, 1979; James and Goldstein, 1984; Knapp and Yamamura, 1992; Balboni et al, 2002), we asked whether MOR also plays a role in the DOR agonists' effect on the anoxia-induced disruption of K^+ homeostasis. To distinguish the effects of MOR from DOR on anoxia-induced disruption of K^+ homeostasis, we examined the effect of a specific MOR agonist, DAGO, in the cortex. We found that perfusion of DAGO (10 μ M) did not produce any appreciable change in anoxia-induced response latency, increase in peak $[K^+]_e$, the rate of rise of $[K^+]_e$ to peak, and the undershoot of $[K^+]_e$ during reoxygenation in cortical slices. Therefore, activation of MOR had no effects on anoxic disruption of K^+ homeostasis (Chao et al, 2007a). The lack of a neuroprotective effect of MOR against anoxic disruption of K^+ homeostasis may not be surprising since some studies showed that MOR activation actually aggravate the neurotoxic effects of hypoxia/hypoglycemia, exacerbate incomplete forebrain ischemia, induce neuronal apoptosis, and inhibit cell survival (Hauser et al, 1994; Yin et al, 1999; Kofke et al, 1999; Hu et al, 2002; Ammon-Treiber et al, 2005; Harburg et al, 2007; Kolodziej et al, 2008). Blocking MOR activation has been shown to be beneficial in cerebral ischemia/reperfusion injury (Liao et al, 2003; Chen et al, 2005). These results therefore strongly confirm that DOR, but not MOR, activation is protective against anoxia-induced disruption of K^+ homeostasis in the cortex (Chao et al, 2007a, 2007b) (Figure 3).

In the transgenic model with DOR over-expression in the cortex, we found that anoxia-induced disruption of K^+ homeostasis was significantly less in the cortex of transgenic mice than that of the wild-type counterparts. In the transgenic mice, DOR activation with DADLE further attenuated the anoxic response of disruption of K^+ homeostasis in the cortex. Prolonged hypoxia (10% O_2 , up to 5 days) rendered the frontoparietal cortex of the wide-type mice more vulnerable to the subsequent anoxia-induced disruption of K^+ homeostasis, while the transgenic over-expression of DOR in the cortex of transgenic mice significantly attenuated this vulnerability. These findings suggest that transgenic over-expression of DOR protects the cortex from anoxic disruption of ionic homeostasis, which supports our hypothesis that DOR is an inherent neuroprotector in the cortex (Chao et al, 2006).

6. Mechanisms of opioid-mediated regulation of ionic homeostasis

There were no information available on the mechanisms underlying opioid-mediated regulation of ionic homeostasis under hypoxic/ischemic conditions in the past. We have recently shed the first light on mechanisms of DOR-mediated regulation of ionic homeostasis under hypoxic/ischemic condition through our serial studies (Chao et al, 2007a, 2007b, 2008, 2009; Kang et al, 2008, 2009). We found that DOR signal may modulate the function of several membrane channel proteins and involve a PKC-dependent and PKA-independent pathway in the downstream of G proteins.

Ionic mechanisms

The precise mechanisms underlying the DOR regulation of K^+ homeostasis could be very complex. As discussed in Section 2, the increase in extracellular K^+ during anoxia could be caused by multiple pathways in neurons. Therefore, it is possible that activation of DOR attenuates anoxic disruption of K^+ homeostasis by directly or indirectly decreasing neuronal K^+ loss and/or increasing its influx in multiple routes and levels.

We found that the attenuation of DOR activation against anoxic disruption of K^+ homeostasis in the cortex is closely related to an inhibition of anoxia-induced increase in Ca^{2+} entry-BK channel activities (Chao et al, 2007a). Our data indicate that the absence of extracellular Ca^{2+} greatly attenuates the anoxia-induced disruption of K^+ homeostasis. Blockade of either BK channels or K_{ATP} channels decreases anoxia-induced increase in peak $[K^+]_e$ with prolonged response latency (Chao et al, 2007a). These results suggest a key role of extracellular Ca^{2+} in anoxic disruption of K^+ homeostasis, and an important role of BK and K_{ATP} channels

in hypoxic K^+ leakage. DOR activation by DADLE (10 μ M) did not further enhance the effect of a Ca^{2+} free perfusion on anoxic disruption of K^+ homeostasis, suggesting that the effect of DOR activation is mediated at least partially by Ca^{2+} entry. DOR activation also did not further reduce the anoxic disruption of K^+ homeostasis in the presence of a BK channel blocker, paxilline. In contrast, the blockade of K_{ATP} channels does not affect the DOR protection from anoxic disruption of K^+ homeostasis. These results indicate that inhibition of hypoxia-induced increase in Ca^{2+} entry-BK channel activities with DOR activation is one of the mechanisms for the attenuation of anoxic disruption of K^+ homeostasis by DOR activation in the cortex (Chao et al, 2007a).

Besides inhibition of anoxia-induced increase in Ca^{2+} entry-BK channel activities, DOR may stabilize anoxic disruption of K^+ homeostasis in the cortex by acting on Na^+ based neuronal factor(s) since Ca^{2+} -based component of K^+ leakage is not the sole factor for anoxic disruption of K^+ homeostasis (Chao et al, 2008, 2009). Our results suggested that there is a concentration-dependent attenuation of anoxic disruption of K^+ homeostasis in response to lowering Na^+ concentration with substitution of NMDG⁺, a membrane impermeable organic cation and that lowering Na^+ concentration with substitution of NMDG⁺ attenuates anoxic disruption of K^+ homeostasis. However, lowering Na^+ concentration with substitution of Li^+ (membrane permeable and can enhance neuronal excitability) tends to potentiate the anoxic disruption of K^+ homeostasis. Our data also revealed that DOR-induced protection against the anoxic K^+ responses is largely abolished by low Na^+ perfusion either with impermeable NMDG⁺ or permeable Li^+ , substitution. Moreover, our recent study showed that DOR activation inhibits Na^+ currents in DOR and Nav1.2 channel-cotransfected *Xenopus* oocytes (Kang et al, 2008, 2009). Therefore, we conclude that DOR-induced protection against the anoxic K^+ responses in cortical slices is at least partially due to Na^+ channel inhibition. Furthermore, we used specific blockers and opener of Na^+ channels/receptors/exchangers to explore which pathway (s) mediating the Na^+ influx is the target of DOR signals on Na^+ -based anoxic K^+ efflux (Chao et al, 2009). We found that the DOR attenuation of anoxic disruption of K^+ homeostasis is largely dependent on the inhibition of anoxic Na^+ influx mediated by voltage-gated Na^+ channels. Also, DOR activation inhibits Na^+ influx mediated by ionotropic glutamate NMDA receptors, but not those by non-NMDA receptors though both of them play a role in anoxic disruption of K^+ homeostasis. Our results also indicated that DOR activation has little effect on the NCX-based ionic responses to anoxia (Chao et al, 2009). Therefore, we conclude that in the cortex, (1) DOR activation attenuates anoxic disruption of K^+ homeostasis by restricting Na^+ entry through voltage-gated Na^+ channels and NMDA receptor channels, and (2) Non-NMDA receptor channels and NCX, though involved in anoxic disruption of K^+ homeostasis in certain degrees, are less likely the targets of DOR signals. Indeed, our recent observations demonstrate that DOR activation directly attenuates Na^+ influx induced by anoxia in the cortical slices (Kang et al, 2009; Chao et al, data to be published), which may, in turn, attenuates Na^+ -trigger massive K^+ leakage (Chao et al, 2008, 2009).

The relationship between Na^+ and Ca^{2+} in terms of their role in DOR stabilization of hypoxic/ischemic disruption of K^+ homeostasis is not well understood. In the subsequent Na^+ -triggered activities, it is unclear which cellular activity(s) is affected by DOR activation during the attenuation of hypoxic/ischemic disruption of K^+ homeostasis. However, we have a clue showing that DOR activation may also reduce Ca^{2+} influx for the attenuation of anoxic disruption of K^+ homeostasis (Chao et al, 2007a). Indeed, anoxia/ischemia triggers a great amount of Na^+ and Ca^{2+} influx, while DOR activation inhibits both Ca^{2+} current (Toselli et al, 1997, 1999; Adams and Trequattrini, 1998; Acosta and López, 1999) and Na^+ current (Ma et al, 2004; Kang et al, 2008, 2009). Since Na^+ influx plays a prominent role in the ischemia-induced depolarization (Calabresi et al, 1999), and triggers cytosolic [Ca^{2+}] elevations in neurons (Friedman and Haddad, 1993; Zhang and Lipton, 1999; Nikolaeva et al, 2005; Kiedrowski, 2007), the anoxia-induced increase in Na^+ influx may serve as an up-stream signal

for the elevation of cytosolic Ca^{2+} in the cortex. DOR activation may inhibit the ionic reactions in series, though we cannot rule out the independent actions of DOR on Na^+ and Ca^{2+} in the stabilization of ionic homeostasis in hypoxia/ischemia.

Protein kinase mechanisms

Our data demonstrate that protein kinases play an important role in signal transduction of the DOR-mediated regulation of K^+ homeostasis (Chao et al, 2007b). PKA and PKC are important signaling molecules in a variety of cellular functions, including modulation of neurotransmitter release, regulation of ion channels and enzymes, control of growth and differentiation, and modification of neuronal plasticity (Majewski and Iannazzo, 1998; Leenders and Sheng, 2005). In addition to the involvement in normal physiological events, these two kinases have also been shown to play an important role in pathophysiological events such as response to hypoxia/ischemia (Tanaka, 2001; Selvatici et al, 2002; Raval et al, 2003; Libien et al, 2005;). Our studies and those of others have shown that DOR regulates PKA and PKC activities under certain conditions (Lou and Pei, 1997; Yao et al, 2003; Ma et al, 2005), suggesting the involvement of these protein kinases in DOR signaling. In our work (Chao et al, 2007b), we could not demonstrate the involvement of PKA in the DOR protection against anoxic increase in $[\text{K}^+]_e$ since blocking PKA with N-(2-[p-Bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide dihydrochloride did not result in any change in the DOR effect. In sharp contrast, PKC inhibition with chelerythrine reversed, and PKC activation by phorbol 12-myristate 13-acetate mimicked, the protective effect of DOR activation. Interestingly, we previously observed that DOR-mediated neuroprotection by hypoxic preconditioning could be blocked by PKC inhibition, but not by PKA inhibition (Ma et al, 2005). Collectively, these data suggest that the effect of DOR activation on anoxic disruption of K^+ homeostasis is mainly via a PKC-dependent, but PKA-independent, pathway. PKC signaling resulting from DOR activation may decrease anoxia-induced glutamate release (Selvatici et al, 2002) and in turn reduce K^+ efflux (Yu et al, 1999; Lopachin et al, 2001; Xiao et al, 2001). Phosphorylation of K^+ channel by PKC activation with DOR reduces potassium conductance of neurons (Grega et al, 1987), which may be attributed to the blockade of voltage-dependent, rapidly inactivating K^+ efflux (Chaki et al, 1994). The interaction between DOR and PKA may be important in signaling other cellular events and require further investigation. Since DOR belongs to a family of G-protein-coupled receptors that rely on G proteins and G-protein-dependent cytoplasmic second messengers involving protein kinases (Quock et al, 1999; Law et al, 2000; Chao et al, 2007b; Pamenter and Buck, 2008; Audet et al, 2008; Snook et al, 2008), and voltage-gated Na^+ channels, NMDA receptors and Ca^{2+} channels are important targets modulated by metabotropic receptors via G protein/protein kinases (Chen et al, 2006; Szabo et al, 2009; Calin-Jageman and Lee, 2008; Kim et al, 2009), Therefore, G protein/protein kinases are very likely the key mediators linking DOR signaling directly or indirectly to these channels in the regulation of ionic homeostasis in stress.

Decreased excitability and glutamate release

In addition, DOR-induced protection against the anoxic disruption of K^+ homeostasis may involve other mechanisms, for example, DOR activation-induced decrease in neuronal excitability and consequent reduced K^+ leakage, DOR regulation in ionotropic glutamate receptor channel-mediated K^+ influx. Indeed, electrophysiological studies in vivo and in vitro have demonstrated that activation of DOR depresses both spontaneous and stimulus-evoked action potential discharges as well as the amplitudes of stimulus-evoked excitatory postsynaptic potentials/currents (EPSPs/EPSCs) of neocortical neurons (Stanzione et al, 1989; Tanaka and North, 1994; Ostermeier et al, 2000). This depression at least partially reduces increased K^+ leakage induced by an increase in excitability and action potential generation in cortical neurons. In the hippocampal slices, hypoxia-induced sharp $[\text{K}^+]_e$ increase is significantly dampened by decreased neuronal excitability and cease of spontaneous and

evoked impulse firings with perfusion of TTX or lidocaine (Müller and Somjen, 2000a, 2000b; Raley-Susman et al, 2001). Another potential mechanism underlying the DOR attenuation of anoxic disruption of K^+ homeostasis involves DOR regulation of glutamate signaling. Immunocytochemical studies reveals that DOR is localized at presynaptic terminals in a variety of neurons, including mammalian cortical neurons (Bausch et al, 1995; Svingos et al, 1995). DOR activation can prevent the release of glutamate from presynaptic vesicles, thereby reduce glutamate excitability (Tanaka and North, 1994; Ostermeier et al, 2000), and suppress NMDA receptor activities in neurons (Wang and Mokha, 1996; Pamenter and Buck, 2008). All these changes with DOR activation would decrease hyperexcitability-induced K^+ leakage through ionotropic glutamate receptors in neurons.

The DOR attenuation of anoxic disruption of K^+ homeostasis might involve multiple strategies involved in ionic homeostasis in the neurons although the precise mechanisms are still not well understood yet at present. Based on the today's information, the possible mechanisms of DOR-mediated regulation of K^+ homeostasis during hypoxia/ischemia are schematically summarized in Figure 4.

7. Implications of DOR protection from disruption of ionic homeostasis in hypoxic/ischemic neurons

Neuronal injury and death often occur as an outcome of hypoxic/ischemic stress (Zhang et al, 2004; Perez-Pinzon, 2007; Chen et al, 2009). Cell death was first classified as necrosis and apoptosis based on histologic criteria. Necrosis is generally a form of cell death that rapidly occurs in response to severe insults such as anoxia and cell trauma, characterized by swelling of cell body and mitochondria, followed by vacuolization of cytoplasm, random breakdown of DNA, rupture of cell membrane and cell lysis. In contrast, cells undergoing apoptosis share similar characteristic morphologies such as cell shrinkage, nuclear/chromatin condensation, internucleosomal cleavage of DNA, membrane blebbing, and formation of apoptotic bodies, which is controlled by internally encoded death program executed by activation of endogenous proteases and endonucleases (Majno and Joris, 1995; Kroemer et al, 1995; Banasiak et al, 2000). Distinguishing between apoptosis and necrosis is not always straightforward. It is sometimes very difficult to put individual cell as apoptotic or necrotic using morphological or even biochemical parameters in the brain because necrosis, apoptosis, even hybrid death always occur concurrently in stress (Tan et al, 1998; Xiao et al, 2002; Wei et al, 2004). Substantial evidence indicated that increased $[Na^+]_i$ and $[Ca^{2+}]_i$, and intracellular K^+ depletion induce both necrosis and apoptosis in various cell types (Yu et al, 1997, 1999; Kristián and Siesjö, 1998; Warny and Kelly, 1999; Banasiak et al, 2000, 2004; Koike et al, 2000; Barros et al, 2001, 2002; Yu, 2003; Liu et al, 2003; Yamashima, 2004; Wozniak et al, 2006; Petrat et al, 2006; Zhao et al, 2006; Mongin, 2007; Fang et al, 2007).

Excessive disruption of ionic homeostasis is the initial and key step in hypoxic/ischemic neuronal injury and death (see reviews by Banasiak et al, 2000 and Yu et al, 2003). Large Na^+ influx induces cellular injury manifested by acutely functional and morphological changes, e.g., loss of electrophysiological response to stimulus, cell swelling, bleb formation and membrane injury (Friedman and Haddad, 1994; Hasbani et al, 1998; Itoh et al, 1998; Calabresi et al, 1999; Shi et al, 2005; Petrat et al, 2006). Removal of extracellular Na^+ or blockade of Na^+ entry therefore prevents hypoxic/ischemic neuronal damage and death (Friedman and Haddad, 1994; Lynch et al, 1995; Probert et al, 1997; Breder et al, 2000; Raley-Susman et al, 2001; Banasiak et al, 2004). In contrast, an increased level of intracellular Ca^{2+} is not necessary for anoxic/excitotoxic morphological changes as Na^+ does (Friedman and Haddad, 1994; Hasbani et al, 1998). However, Ca^{2+} entry and overload, particularly uncontrolled one in the later phase of hypoxic/ischemic stress, can induce severe damage to nerve cells and lead to delayed neuronal death (Friedman and Haddad, 1994; Hasbani et al, 1998; Nakamura et al,

1999; Aarts et al, 2003; Xiong et al, 2004, 2007; Bano et al, 2005; Gao et al, 2005; Bano and Nicotera, 2007). K^+ efflux is beneficial to neurons in counteracting hypoxic/ischemic stress in a very limited range acting as an adaptive mechanism, and is harmful to neurons in excessive intracellular K^+ loss. K^+ depletion has been shown to be actively implicated in the changes in morphological features of necrosis in monocytic cells (Warny and Kelly, 1999). However, in most cases, excessive K^+ efflux from neurons may induce cell shrinkage, cytochrome c release and degradation of DNA and nuclear proteins - typical features of apoptosis (Bortner et al, 1997; Yu et al, 1997, 1999; D'Ambrosio et al, 1999; Xiao et al, 2001; ²⁰⁰²; Lauritzen et al, 2003; Yu, 2003; Remillard and Yuan, 2004; Karki et al, 2007). The subsequent cellular K^+ uptake occurs mainly into astrocytes, which involves activation of $Na^+-K^+-ATPase$ and NKCC (Waltz, 2000, 2004). Activation of NKCC leading to intracellular Na^+ overload (Kintner et al, 2007; Chen et al, 2008) increases $[Ca^{2+}]_i$ via the operation of plasmalemmal NCX in a reverse mode (Luo et al, 2005, 2008; Zhang and Lipton, 1999; Nikolaeva et al, 2005; Kiedrowski, 2007; Chen et al, 2007). Activation of NKCC also leads to intracellular accumulation of Na^+ , K^+ and Cl^- , osmotically driven water uptake and cell swelling, contributing to ischemia-induced brain edema (Pond et al, 2006; Hertz, 2008; Salter and Fern, 2008). Therefore, blockade of K^+ efflux or the presence of high extracellular K^+ to prevent excessive K^+ efflux has been shown to prevent neuronal death occurs in various conditions including hypoxia/ischemia (Yu et al, 1997, 1999; Huang et al, 2001; Wei et al, 2003; Liu et al, 2003; Lauritzen et al, 2003; Zhao et al, 2006; Karki et al, 2007; Nistico et al, 2007). These findings suggest that inhibition of excessive K^+ flux and maintaining cellular K^+ homeostasis may be of therapeutic benefit in the treatment of stroke and related neurodegenerative conditions (Wei et al, 2003; Liu et al, 2003; Zhao et al, 2006; Karki et al, 2007; Nistico et al, 2007).

In general, among the major cations, $[Na^+]_i$ rise mainly contributes to hypoxia/ischemia-induced rapid cell swelling and necrosis, $[Ca^{2+}]_i$ increase is mainly responsible for the delayed neuronal death, and K^+ efflux is beneficial to neurons in counteracting hypoxic stress in a limited range, and is harmful to neurons in excessive intracellular K^+ loss during hypoxic/ischemic stress. No matter how these cations cause the neuronal injury and death, obviously, stabilization of hypoxic/ischemic disruption of ionic homeostasis may be of therapeutic benefit in the treatment of stroke and related neurodegenerative conditions (Wei et al, 2003; Liu et al, 2003; Karki et al, 2007; Zhao et al, 2006; Nistico et al, 2007). In this aspect, the DOR protection against disruption of ionic homeostasis, particularly disruption of Na^+ and K^+ homeostasis, seems to be most significant in prevention against hypoxia/ischemia-induced neuronal injury.

We and others have demonstrated the DOR-induced neuroprotection not only in hypoxia/ischemia (Zhang et al, 2002, 2006; Borlongan et al, 2004, 2009; Horiuchi et al, 2004, ²⁰⁰⁸; Lim et al, 2004; Ma et al, 2005; Zhao et al, 2006; Su et al, 2007; Iwata et al, 2007; Xiong et al, 2007; Charron et al, 2008; Kao et al, 2008; Govindaswami et al, 2008; Pamenter and Buck, 2008; Tian et al, 2008a, 2008b; Peng et al, 2009; Zhu et al, 2009; Yang et al, 2009), but also in other conditions including cytotoxic and oxidative stress (Zhang et al, 2000; Borlongan et al, 2001; Hayashi et al, 2002; Wallace et al, 2006; Narita et al, 2006), and even in drug and ethanol consumption (Margolis et al, 2008). DOR-mediated neuroprotection is at least partially due to its action against the disruption of ionic homeostasis during hypoxia or ischemia. Of course, this does not mean that DOR stabilization of disruption of ionic homeostasis is the only mechanism mediating the neuroprotection. Since neuronal responses to stress vary depending on the duration and severity of stress and survival time of neurons following hypoxia/ischemia (Nieber, 1999; Lipton, 1999), DOR-mediated regulation may occur at various levels to protect neurons from short- or long-term stress. As have been shown, DOR signals maintain ionic homeostasis in the initial stage of hypoxia (Chao et al, 2006, 2007a, 2007b, 2008, 2009; Kang et al, 2009), while during prolonged hypoxia DOR then signals up-regulation of the expression and function of benefit-signaling molecules and down-regulates those involved in cell injury or death (Ma et al, 2005; Peng et al, 2009). Nevertheless, the stabilization of ionic homeostasis

in the initial stage of hypoxia/ischemia is clearly an important aspect of DOR-mediated neuroprotection, which may have significant impacts on clinical strategies against hypoxic/ischemic injury in the brain.

8. Summary and Perspective

We have demonstrated that DOR is neuroprotective against hypoxic/ischemic stress with direct evidence. This viewpoint is also supported by several lines of new evidence from many independent laboratories. In the mechanistic exploration, we found that DOR activation and/or expression reduce hypoxic/ischemic disruption of ionic homeostasis. Since one of the initial and critical events induced by hypoxia/ischemia is the disruption of ionic homeostasis characterized by enhanced K^+ efflux and Na^+ -, Ca^{2+} - and Cl^- influx, and since such a disruption of ionic homeostasis triggers neuronal injury and death, the DOR's action against hypoxic/ischemic disruption of Na^+ , Ca^{2+} and K^+ homeostasis very likely plays an important role in the DOR-induced neuroprotection from hypoxic/ischemic stress. Based on our recent work and current literatures, we have drawn an initial picture of how DOR protects neurons against hypoxia/ischemia through the regulation of ionic homeostasis. In brief, DOR signals may inhibit Na^+ influx and reduce the increase in intracellular Ca^{2+} , thus decreasing the excessive K^+ leakage, which may involve a PKC-dependent and PKA-independent signaling pathway.

However, it is far away from the full understanding of the DOR-mediated regulation of the important channel proteins at membrane and intracellular levels. A number of important issues need further investigation.

1. Is there any direct interactions between DOR and Na^+ , Ca^{2+} and K^+ channels on the membrane? DOR belongs to a family of G protein-coupled metabotropic receptors, and functionally couples to ionic channels, including K^+ channels, Ca^{2+} channels, and Na^+ channels etc. (North et al, 1987; Ikeda et al, 1995; Su et al 1998; Nah et al, 1997; Toselli et al, 1997; Kang et al, 2008, 2009). DOR effects are mediated by G proteins and G-protein-dependent cytoplasmic second messengers involving protein kinases (Quock et al, 1999; Law et al, 2000). Na^+ , Ca^{2+} and K^+ channels are important targets modulated by metabotropic receptors via G protein/protein kinases (Chen et al, 2006; Dai et al, 2009). Little is known as to whether and how DOR activation directly acts on the target channels to change channels' activities, i.e., protein-protein interaction, (e.g. DOR activation releases some molecules or changes dimensional configuration, thus directly acting on the neighbor channel proteins to alter the activities of voltage sensor domain or pore forming domain) or if DOR regulates the target channels' function mainly by its downstream signaling molecules.
2. Do DOR activation and/or expression chemically or mechanically alter the function and expression of these channels in the neighborhood? DOR down-regulation (Zhao et al, 2005) is associated with an up-regulation of voltage-gated Na^+ channels (Xia et al, 2003), and DOR activation attenuates hypoxic dysregulation of Na^+ channels (Xia et al, 2001; Ma et al, 2004). However, no direct information is available as to whether and how DOR activation/expression regulates K^+ and Ca^{2+} and Na^+ channel expression.
3. Are there any other signal pathways, besides PKC, involved in the DOR-mediated regulation of ionic homeostasis? DOR's signaling pathways involve a lot of signal molecules, including PKC, PKA, MAPK etc. (Quock et al, 1999; Law et al, 2000, Ma et al, 2005, Chattopadhyay et al, 2008). The effect of DOR activation on anoxia-induced disruption of K^+ homeostasis is mainly via a PKC-dependent, but PKA-independent, pathway. It is unknown, whether other signal pathways involve in the DOR-mediated regulation of ionic homeostasis.

4. What is the major difference between DOR and other opioid receptors in terms of the molecular mechanisms underlying the regulation of ionic homeostasis? DOR, but not MOR, modulates K^+ homeostasis in cortical neurons during hypoxic stress (Chao et al, 2007a). This difference between DOR and MOR may be due to differential distribution of DOR and MOR in the cortex (Mansour et al, 1987, 1994; Svingos et al, 1995; Xia et al, 2001). However, the cloned DOR and MOR, and even KOR are highly homologous, and all these receptors interact with G protein and possess many common or similar signal molecules (Quock et al, 1999; Law et al, 2000) with similar effect on ionic channels in some cases (Toselli et al, 1997; Stefani et al, 1994). The difference in the regulation of ionic homeostasis between different opioid receptors would have the bases on different key molecular mechanisms, especially linking to certain key formations of receptors. However, it is unknown about this issue.
5. What chemical group(s) of the DOR molecule plays the most important role in the regulation? The cloned DOR has 372 amino acids, and possesses a common three-dimensional structure that spans the cell membrane seven times, forming three extracellular loops and three intracellular loops. The amino terminus is extracellular, whereas the carboxyl terminus is intracellular forming a cytoplasmic tail and may interact with receptor-associated G proteins (Quock et al, 1999). It is unclear what chemical group(s) of the DOR molecule plays the most important role in the regulation of ionic homeostasis and neuroprotection at present.
6. Is it possible to directly activate a key “point” of signal pathways to mimic DOR neuroprotection without opioid side-effects? Most of opioid compounds, including agonists of δ -opioid receptors, have side-effects in clinic (e.g. addiction). If the key “point” of their signal pathways is known, we may mimic DOR neuroprotection without side-effects by changing the activities of the key “point” of their signal pathways.

Clearly, further investigations into the mechanism may greatly improve our understanding of neuronal adaptation to hypoxic/ischemic stress and may potentially lead to novel clues for better solution of hypoxic/ischemic injury in pharmacological ways.

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Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentrations
$[Ca^{2+}]_o$	extracellular Ca^{2+} concentrations
$[K^+]_e$	extracellular K^+ concentrations
$[K^+]_i$	intracellular K^+ concentrations
$[Na^+]_i$	intracellular Na^+ concentrations
$[Na^+]_o$	extracellular Na^+ concentrations
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ASICs	calcium-permeable acid-sensing ion channels
BK channels	Ca^{2+} -activated K^+ channels
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione

CPP	3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid;
DAGO	[D-Ala2, N-Me-Phe4, Gly5-OL]-enkephalin
DIV	day <i>in vitro</i>
DOR	δ -opioid receptor
EPSPs/EPSCs	excitatory postsynaptic potentials/currents
ER	endoplasmic reticulum
IP3	inositol triphosphahate
KA	kainate
K _{ATP} channels	ATP-sensitive K ⁺ channels
K _{Na} channels	Na ⁺ -activated K ⁺ channels
KOR	κ -opioid receptor
MOR	μ -opioid receptor
NBQX	1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxaline-7-sulfonamide
NC _{Ca-ATP}	Ca ²⁺ -activated, ATP-sensitive nonselective cation channels
NCX	Na ⁺ /Ca ²⁺ exchangers
NHE	Na ⁺ /H ⁺ exchangers
NKCC	Na ⁺ -K ⁺ -2Cl ⁻ cotransporters
NMDA	N-methyl-D-aspartate
NMDG ⁺	N-methyl-D-glucamine
OGD	oxygen-glucose deprivation;
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTP	permeability transition pore
ROS	Reactive oxygen species
TRP	transient receptor potential
TTX	tetrodotoxin
VGCCs	voltage-gated Ca ²⁺ channels;
VGSCs	voltage-gated sodium channels

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Figure 1. Anoxia induced changes in extracellular potassium concentration ($[K^+]_e$) and extracellular DC potential (ΔDC) in mouse cortical slice

Note that anoxia largely increased the concentration of extracellular potassium (upper trace) and decreased DC potential (lower trace). The changes in K^+ were distinctly characterized by a two-phase response to hypoxia/ischemia. The first phase shows a relatively slow increase in $[K^+]_e$ starting after 20–60 sec of hypoxia/ischemia. Over several minutes of anoxia, $[K^+]_e$ increase reached a threshold level (no more than the so-called “ K^+ ceiling” level), phase 2 was characterized by a rapid, abrupt and large increase in $[K^+]_e$, accompanied by a negative shift in extracellular DC potential. Within about 20 seconds of the onset of phase 2, $[K^+]_e$ reached a peak value, then gradually decreased. Upon return to normoxia, $[K^+]_e$ fell rapidly, and often undershot to below the basal levels before steadily returning to the pre-anoxia level.

Figure 2. Effect of DOR activation by UFP 512 on anoxic disruption of K^+ homeostasis
 Trace recordings of A: Control (Cont), B: UFP 512 (1 μ M) (UFP 512-1.0), and C: UFP 512 (10 μ M) (UFP 512-10). D–F are statistical results of each recording parameter. The following parameters were derived to assess K^+ homeostasis: (1) the latency of anoxia-induced $[K^+]_e$ 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 $[K^+]_e$ ($[K^+]_{max}$), which was the peak change in extracellular K^+ concentration induced by anoxia; (3) the undershooting of $[K^+]_e$ (Undershoot), which referred to the minimal value of $[K^+]_e$ during reoxygenation. ** $p < 0.01$, *** $p < 0.001$ as compared with the control; ## $p < 0.01$ in comparison to UFP 512-1.0. Note that UFP 512 at 1 μ M (UFP 512-1.0) significantly

attenuated anoxic increase in $[K^+]_{max}$, with a significantly prolonged latency to anoxia and a significant delay of peak increase in $[K^+]_e$ (n=27). Increasing concentration of UFP 512 up to 10 μM (UFP 512-10) did not further attenuate the anoxia-induced disruption of K^+ homeostasis except for the response latency that was prolonged (n=15).

Figure 3. DOR, but not MOR, activation is protective against anoxia-induced disruption of K^+ homeostasis in the cortex

Trace recordings of A: Control, B: DADLE (1.0 μ M) (DADLE1.0), C: DADLE (10 μ M) (DADLE10), D: UFP 512 (1 μ M), and E: DAGO (10 μ M). Note that anoxia-induced increase in $[K^+]_{max}$ was significantly attenuated by DOR activation with two naturally distinct DOR agonists, DADLE and UFP 512. Whereas MOR activation by DAGO did not produce any significant change in anoxia-induced increase in $[K^+]_{max}$. These results, therefore, confirm DOR, but not MOR, activation is protective of cortical neurons against anoxia-induced disruption of K^+ homeostasis.

Figure 4. Paradigm showing mechanisms of DOR attenuation of anoxic/ischemic disruption of K⁺ homeostasis

Our data showed that the absence of extracellular Ca²⁺ and blockade of Ca²⁺-activated K⁺ channels (BK) greatly attenuated the anoxia-induced disruption of K⁺ homeostasis, suggesting that the attenuation of DOR activation against anoxic disruption of K⁺ homeostasis in the cortex is likely attributed to an inhibition of hypoxia-induced increase in Ca²⁺ entry-BK channel activities. Besides, DOR-induced protection against the anoxic K⁺ responses was largely abolished by low Na⁺ perfusion with either impermeable N-methyl-D-glucamine or permeable Li⁺, substitution, blockade of voltage-gated Na⁺ channels and NMDA receptor channels. Whereas Non-NMDA receptor channels and Na⁺/Ca²⁺ exchangers, though involved in anoxic disruption of K⁺ homeostasis in certain degrees, are less likely the targets of DOR signals. PKC-dependent, but PKA-independent, pathway is also involved in the attenuation of anoxic disruption of K⁺ homeostasis in the cortex because we could not demonstrate the involvement of PKA in the DOR protection against anoxic increase in extracellular K⁺ because blocking PKA with H89 did not result in any changes in the DOR effect. In sharp contrast, PKC inhibition with chelerythrine reversed, and PKC activation by PMA mimicked, the protective effect of DOR activation.

Table 1

Definition of some key terms in this review

Terminology	Definition
Hypoxia	A state of low oxygen supply in the body, organ, or cell, which can be caused by a decrease in partial pressure of oxygen, inadequate oxygen transport, or the inability of the tissues to use oxygen.
Anoxia	An absence of oxygen in the body, organ, or cell.
Ischemia	Insufficient blood flow to provide adequate oxygen and glucose, which, in turn, leads to tissue hypoxia or anoxia and lack of glucose supply. In <i>in vitro</i> study, oxygen-glucose deprivation (OGD) model is usually used to mimic ischemic condition.
Chemical Ischemia	A state of energy limitation of cells by inhibiting both oxidative and glycolytic metabolism with chemicals (e.g. NaN_3 and KCN for oxidative and 2-deoxyglucose and/or glucose-free for glycolytic). The outcomes are similar to OGD regarding the availability of oxygen and glucose to cells.

Note: Though the definitions are easy to make by words, it is relatively difficult to distinguish them among various cell/tissue states regarding the availability and amount of oxygen and/or glucose in a given cell/tissue. In this review, like a common practice in the literature, these terms are alternately used (e.g., hypoxia vs. anoxia depending on the severity of oxygen deprivation) or generally used to reflect a state of insufficient oxygen and/or blood flow (e.g., hypoxia/ischemia or hypoxic/ischemic stress).

Table 2

Effects of DOR activation/inhibition on ionic homeostasis

Condition	Preparation	Observation	References
Normoxia		Ca²⁺	
	Cultured neuronal cell line	Elevation in [Ca ²⁺] _i Brief exposure (30–90s) to DOR agonists in nanomolar concentrations induces Ca ²⁺ release from intracellular stores via a process that appears to activate phospholipase C (PLC) and consequent IP3 formation, which mainly occur in undifferentiated cells.	Yoon et al, 1998, 1999; Smart and Lambert, 1996; Jin et al, 1992; Spencer et al, 1997; Harrison et al, 1999; Connor et al, 1997;
		DOR activation-induced [Ca ²⁺] _i elevation is dependent on the presence of extracellular Ca ²⁺ in differentiated cells line.	Jin et al, 1992; Fields et al, 1995;
	Cultured cortical astrocytes of the rat	Exposure to DOR agonist causes significant increase in [Ca ²⁺] _i or induces a biphasic Ca ²⁺ response, with an initial peak of rapid increase in [Ca ²⁺] _i followed by a sustained level of elevated [Ca ²⁺] _i or oscillations in [Ca ²⁺] _i , and the first peak in [Ca ²⁺] _i is due to mobilization of intracellular Ca ²⁺ store, whereas the second part of response is a result of Ca ²⁺ influx through membrane channels	Hauser et al, 1998; Thorlin et al, 1998;
	Cultured dorsal root ganglion neurons of the mouse	Activation of three opioid receptor subtypes (DOR, MOR, KOR)-induced [Ca ²⁺] _i rise can be greatly attenuated by removal of extracellular Ca ²⁺ or blockade of dihydropyridine-sensitive Ca ²⁺ channels. Inhibition of Ca ²⁺ entry/[Ca ²⁺] _i	Tang et al, 1996; Bao et al, 2003.
	Primary cerebral cortical cultures of the rat	Naloxone treatment increases glutamate release and the ⁴⁵ Ca ²⁺ uptake induced by depolarization; opioid agonists (DOR, MOR, KOR) inhibit the ⁴⁵ Ca ²⁺ uptake induced by K ⁺ depolarization. Stimulating neurons with DOR agonist DPDPE(1 μM) decreases [Ca ²⁺] _i by inhibition of L-type Ca ²⁺ channels with DOR activation, which can be blocked by DOR antagonist, ICI 174868 (10 μM).	Vlaskovska et al, 1997; Thorlin et al, 1998;
	<i>In vitro</i> neurons of turtle cortex	DOR inhibition with naltrindole (1–10 μM) greatly potentiates NMDA receptor activities and NMDA-mediated increase in [Ca ²⁺] _i in neurons.	Pamenter and Buck, 2008
	Neurons/neuroblastoma cell lines	DOR activation inhibits diverse calcium currents (e.g. L-, N-, P- and Q-type Ca ²⁺ channel currents) which link to Ca ²⁺ influx through different high-voltage-activated channels types.	Nah et al, 1997; Toselli et al, 1997, 1999; Adams and Trequatrini, 1998; Morikawa et al, 1998, 1999; Acosta and López, 1999;

Condition	Preparation	Observation	References
Anoxia/ ischemia	Acutely isolated dentate granule cells of the rat hippocampus	Na⁺ DOR agonist, SNC80 (1–1000 μ M) reduces the maximal Na ⁺ current amplitude in a dose-dependent manner, which is “opioid-receptor independent”.	Piros et al, 2000 Remy et al, 2004
	Mouse cortical slices	During 20 min of perfusion of DOR agonist, UFP 512 (1–10 μ M) before hypoxia/ ischemia, no appreciable changes in [Na ⁺] _o were observed.	Chao et al, unpublished data
	<i>Xenopus</i> oocytes with co-expressed DOR and Na ⁺ channels	DOR activation inhibits Na ⁺ channel function by decreasing the amplitude of Na ⁺ currents and increasing its threshold of activation	Kang et al, 2008, 2009
	Mouse cortical slices	K⁺ During 20 min of perfusion of DOR agonists (DADLE, UFP 512) and antagonist (Naltroindole) before hypoxia/ ischemia, no appreciable changes in [K ⁺] _o were observed, suggesting little effect of DOR on K ⁺ homeostasis under normoxic condition.	Chao et al, 2006, 2007a, 2007b, 2008, 2009
	Mouse cortical slices	Activation of DOR with DADLE or UFP 512 (1–10 mM) attenuated the anoxia/OGD-induced disruption of K ⁺ homeostasis via regulation of the function of several membrane channel proteins and involving a PKC-dependent and PKA-independent pathway in the downstream of G proteins; Transgenic over-expression of DOR significantly attenuates anoxia-induced disruption of K ⁺ homeostasis. Activation of DOR also directly attenuated Na ⁺ influx induced by anoxia via a PKC-dependent pathway.	Chao et al, 2006, 2007a, 2007b, 2008, 2009 unpublished data Kang et al, 2009

Table 3

Recent studies demonstrating DOR neuroprotection against hypoxic/ischemic insults

Stress	Preparation	Observation	References
Transient MCAO	In vivo striatum of the rat	Pretreatment with DADLE (i.p., 4mg/kg, 4 injections at 2h intervals) prior to MCAO completely blocked the striatal infarction and behavioral dysfunctions, and protected against necrotic and apoptotic cell death processes associated with ischemia-reperfusion, which transiently blocked by naltrexone (0.1 mg/kg, i.p.) in the early phase of DADLE effects.	Borlongan et al, 2004, 2009
OGD	Purkinje cells of rat cerebellar brain slices	Preconditioning with morphine ($\geq 0.3 \mu\text{M}$) significantly increased the survival rate of Purkinje cells, which was abolished by naloxone, a non-selective opioid receptor antagonist, naltrindole, a selective DOR antagonist.	Lim et al, 2004
OGD; MCAO; hypotension + bilateral common carotid artery occlusion	<i>In vivo</i> brain of the mouse and rat; Hippocampal slice culture of the rat	Preconditioning with DOR agonist TAN-67 (100 nM for 30 min at 24h before OGD) induced neuroprotection, which was inhibited by DOR antagonist, 7-benzylidenenaltrexone (0.5 μM); Administration of DOR agonists, DADLE (i.c.v., 0.2–20 mM; i.p., 16 mg/kg), DPDPE (i.c.v., 5–50 μg), Tan-67 (i.p. 3mg/kg), Deltorphine-D _{variant} (i.p., 2–4 mg/kg), or BW373U86 (i.c.v., 10–100 nmol) 15 min–24 hours before forebrain ischemia significantly reduced brain infarct volume, protected against hippocampal neuronal loss, and improved neurological outcome, which was abolished by naltrindole (i.c.v, 2 mM, 20–100 nmol; i.p., 10 mg/kg).	Zhao et al, 2006 Su et al, 2007; Iwata et al, 2007; Charron et al, 2008; Govindaswami et al, 2008; Tian et al, 2008b Kao et al, 2008
Transient MCAO	In vivo brain of the rat	Electroacupuncture (EA) significantly reduced ischemic infarction and neurological deficits, increased the expression of DOR, naltrindole (i.c.v., 20 nmol; i.p., 1mg/kg) completely abolished the EA protection.	Xiong et al, 2007; Tian et al, 2008a
HPC	Rat retinas	HPC markedly increased DOR expression and attenuated the loss of neuronal marker expression and the increase in pro-apoptotic caspase-3 activities induced by elevated intraocular pressure, which can be attenuated by DOR antagonist, naltrindole (1 μM).	Peng et al, 2009
Inhibition of mitochondrial respiratory chain	Cortical neuronal cultures of the rat	Activation of DOR with DADLE (0.1–10 μM) but not MOR with DAGO (0.1–10 μM) reduces NaN_3 -induced neuronal damage, and this protection is abolished by naltrindole (0.1 μM); prolonged DOR inhibition further increases NaN_3 -induced neuronal damage.	Zhu et al, 2009
Anoxia	<i>In vitro</i> cortical neurons of the turtle	Blockade of DOR with naltrindole (1–10 μM) potentiated normoxic NMDAR currents and not only prevented but also reversed the anoxic decrease in NMDAR activity, leading to excitotoxic events, increased Ca^{2+} influx and terminal depolarization in a significant percentage of anoxic neurons.	Pamenter and Buck, 2008

Stress	Preparation	Observation	References
Ischemia	<i>In vivo</i> rat spinal cord	Intrathecal treatment with a DOR agonist SNC80 (400 nmol in 10 μ l) 9–15 min before the induction of ischemia (9–22 min of aortic occlusion) attenuated hind-limb motor dysfunction and neuronal and white matter injury following spinal cord ischemia.	Horiuchi et al, 2004, 2008

HPC, Hypoxic Preconditioning;

MCAO, Middle Cerebral Artery Occlusion;

OGD, Oxygen-Glucose Deprivation.