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Attenuating Ischemic Disruption of K^+ Homeostasis in the Cortex of Hypoxic-Ischemic Neonatal Rats: DOR Activation vs. Acupuncture Treatment

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Abstract Perinatal hypoxic-ischemic (HI) brain injury results in death or profound long-term neurologic disability in both children and adults. However, there is no effective pharmacological therapy due to a poor understanding of HI events, especially the initial triggers for hypoxic-ischemic injury such as disrupted ionic homeostasis and the lack of effective intervention strategy. In the present study, we showed that neonatal brains undergo a developmental increase in the disruption of K^+ homeostasis during simulated ischemia, oxygen-glucose deprivation (OGD) and neonatal HI cortex has a triple phasic response (earlier attenuation, later enhancement, and then recovery) of disrupted K^+ homeostasis to OGD. This response partially involves the activity of the δ-opioid receptor (DOR) since the earlier attenuation of ischemic disruption of K^+ homeostasis could be blocked by DOR antagonism, while the later enhancement was reversed by DOR activation. Similar to DOR activation, acupuncture, a strategy to promote DOR activity, could partially reverse the later enhanced ischemic disruption of K^+ homeostasis in the neonatal cortex. Since maintaining cellular K^+ homeostasis and inhibiting excessive K^+ fluxes in the early phase of hypoxic-ischemic insults may be of therapeutic benefit in the treatment of ischemic brain injury and related neurodegenerative conditions, and since many neurons and other cells can be rescued during the "window of opportunity" after HI insults, our first findings regarding the role of acupuncture and DOR in attenuating ischemic disruption of K^+ homeostasis in the neonatal HI brain suggest a potential intervention therapy in the treatment of neonatal brain injury, especially hypoxic-ischemic encephalopathy.

Keywords Neonatal hypoxia-ischemia · Cortex · δ-opioid receptor · K^+ homeostasis · Neuroprotection · Acupuncture

Abbreviations

$[K^+]e$	Extracellular K^+ concentrations
ACSF	Artificial cerebrospinal fluid
DOR	δ-opioid receptor
HI	Hypoxia-ischemia
HIE	Hypoxic-ischemic encephalopathy
MA	Manual acupuncture
NTI	Naltrindole hydrochloride
OGD	Oxygen-glucose deprivation
TTC	2,3,5-triphenyltetrazolium chloride

Introduction

The incidence of perinatal hypoxic-ischemic (HI) encephalopathy (HIE) has been estimated to occur in approximately 2 per 1000 live, term newborns. About 15 to 25 % affected newborns die in the postnatal period, and an additional 25 % develop long-term neurologic sequelae (e.g., cerebral palsy, mental retardation, epilepsy) [1, 2]. HIE is an evolving process rather than a single event, and the pathophysiology of HIE is closely related to energy failure during acute insults and the

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reperfusion period [1, 3]. The primary energy failure in acute insult leads to the rapid depletion of ATP and failure of Na^+/K^+ pumps, followed by the consequent disruption of ion homeostasis, which initiates the events associated with cytotoxic edema and free radical formation. This can lead to compromised cellular integrity and acute cell death (principally necrotic death) if the insults are severe and long enough. During the reperfusion and reoxygenation period, ATP levels recovered to baseline in 2 to 3 h, but then depleted within the next 48 h (a secondary energy failure), which further potentiates the events of excitotoxic-oxidation cascade and delayed cell death (predominantly apoptotic death) [2–4]. Though many neurons and other cells commit to dying during the period after HI insults, many survive over a period of days to weeks, and many of them can be rescued during this window of opportunity [1, 3]. However, despite the promising clinical trials of hypothermia [5, 6], there is still no effective therapy due to a poor understanding of HI events, especially the initial triggers for hypoxic-ischemic injury such as disrupted ionic homeostasis in the early phase of insult and the lack of effective intervention strategy.

Disruption of ionic homeostasis, including enhanced influx of Na^+ and Ca^{2+} and efflux of K^+, has been generally regarded as an initial and key alteration in anoxia/ischemia-induced neuronal injury [7, 8]. Among cellular ions, K^+ is the most abundant cation in the cytoplasm, and its sharp efflux has been shown to be closely associated with anoxia-induced depolarization, which is believed to be a crucial factor leading to neuronal death [7–9]. For example, sustained exposure to elevated extracellular K^+ (simulating ischemic extracellular K^+) causes significant neuronal death even under conditions of normoxia and abundant glucose supply [10], while blockade of K^+ efflux has been shown to attenuate hypoxia- and ischemia-induced neuronal death [11, 12]. These findings suggest that maintaining cellular K^+ homeostasis and inhibiting excessive K^+ fluxes in the early phase of insults may be of therapeutic benefit in the treatment of stroke and related neurodegenerative conditions [11, 12]. Presently, little is known about the developmental changes of K^+ homeostasis in the neonatal brain during ischemia, and there are also no pharmacological agents available for the maintenance of K^+ homeostasis in the neonatal brain during insults.

The δ-opioid receptor (DOR) is widely distributed throughout the central nervous system [13–17] and has been proven more sensitive than other opioid receptors to stressful stimuli, such as hypoxia and ischemia [18–20]. Studies from our laboratory and others have shown that activation of DOR is neuroprotective against hypoxic and excitotoxic stress, while inhibition of DOR induces major injury in cortical neurons not only during hypoxia but also in normoxic conditions [8, 21]. One of the underlying mechanisms of DOR neuroprotection is its capacity to stabilize ionic homeostasis during hypoxic-ischemic insults [8, 21]. A large amount of enkephalin has been found to be released in hypoxia/ischemia-exposed brains [22–25]. Enkephalin preferentially binds to DOR and activates it [26], thus possibly giving the brain adaptive protection against insult through the attenuation of hypoxic/ischemic disruption of ionic homeostasis. Therefore, it is important to know whether DOR is involved in ischemic disruption of K^+ homeostasis in neonatal hypoxia-ischemia brain.

Moreover, several lines of evidence have suggested that acupuncture may be a beneficial strategy against hypoxic-ischemic brain damage and cerebral palsy that is the known sequelae of HIE in neonatal rodents and in children [27–29], and can induce rapid and delayed brain ischemia tolerance in adult animals [30, 31]. On the other side, acupuncture can induce brain opioid release and subsequent DOR activation with an upregulation of DOR expression [21, 32–35]. Therefore, we asked if acupuncture treatment can attenuate ischemia-disrupted K^+ homeostasis in the neonatal HI rats.

The aims of this study were to (1) delineate the developmental changes of ischemia-induced disruption of K^+ homeostasis in the cortex of normal neonates and the possible different responses between neonates with/without suffering HI, (2) determine the possible involvement of DOR and its role in ischemic K^+ derangement in HI brain, and (3) to test if acupuncture treatment can attenuate ischemia-disrupted K^+ homeostasis in the neonatal HI rats. Our data showed that DOR is involved in cortical responses to ischemic disruption of K^+ homeostasis in neonatal HI brain, and acupuncture is beneficial against the ischemic disruption of ionic homeostasis, presumably via DOR activation in the brain. Our study suggests a therapeutic potential in treatment of hypoxia-ischemia brain injury in neonates.

Results

General Effects of Hypoxia-Ischemia on the Animals

No significant differences in body weight were found between the control ($n=3$) and their HI counterparts without any treatment (HI0) ($n=4$) for postnatal day 7 (P7) rats (16.2 ± 0.8 vs. 17.0 ± 1.4 g, $p=0.680$). After 1 day, the body weight of HI rats (HI1) ($n=8$) was significantly lower than the age-matched control ($n=3$) (16.0 ± 0.8 vs. 20.4 ± 0.7 g in control, $p=0.01$). Unlike control animals that steadily increased their body weight, HI1 rats showed a slight decrease in body weight. After 7 days of HI, rats (HI7) ($n=18$) began to partially recover from their lower body weight as compared to their control counterparts ($n=3$) (30.3 ± 1.4 vs. 37.9 ± 4.5 g in control, $p=0.067$). After 21 days of HI, the body weight did not show any significant differences between HI rats (HI21) ($n=3$) and the age-matched control ($n=3$) (83.0 ± 16.0 vs. 93.6 ± 2.6 g in control, $p=0.551$) (Fig. 1).

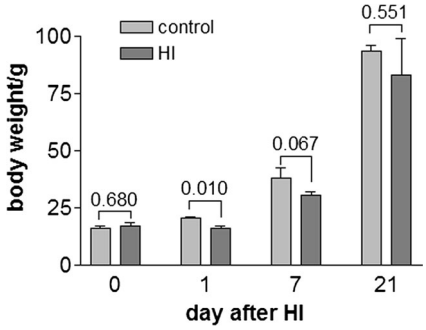


Fig. 1 Effect of hypoxia-ischemia (HI) on body weight. Note that on the day of HI treatment, there was no difference in body weight between control and HI counterparts. One day after HI, the body weight of HI rats (HI1) was significantly lower than the age-matched control. After 7 days of HI, rats (HI7) began to partially recover from their lower body weight as compared to their control counterparts. After 21 days of HI, body weight did not show any significant differences between HI rats (HI21) and the age-matched control

Meanwhile, we also observed that rats undergoing HI treatments showed weakness in grip capability in the unilateral limbs in days 0, 1, and 7, but not 21 days after HI. Unlike their control siblings, these animals were much more unbalanced and declined along the bottom of cages when their cages were slightly down-sloped. They swirled their body towards the left when their tails were pinched. The eyes in both sides of HI animals were asymmetric in size, with the left eye opening later and less than the right one in HI7. These rats gradually recovered and showed no difference 21 days after HI.

We also performed 2,3,5-triphenyltetrazolium chloride (TTC) staining, as described previously [36, 37], on the serial thick slices of the whole brains (2 mm of thickness) of the neonatal HI rat model under the same experimental conditions in our preliminary study. We found no apparent morphological damages of cerebral hemispheres of rats receiving hypoxia-ischemia 2 days after HI (data not shown). Therefore, this model under our experimental condition provides an ideal model for our functional study of K^+ homeostasis since we previously found that the slices with obvious morphological damage due to prolonged/severe hypoxia had very bad viability and were almost silent to oxygen-glucose deprivation (OGD) stimulation in extracellular K^+ activity (unpublished observation).

Developmental Changes in OGD-Induced Disruption of K^+ Homeostasis in the Cortex of Normal Animals

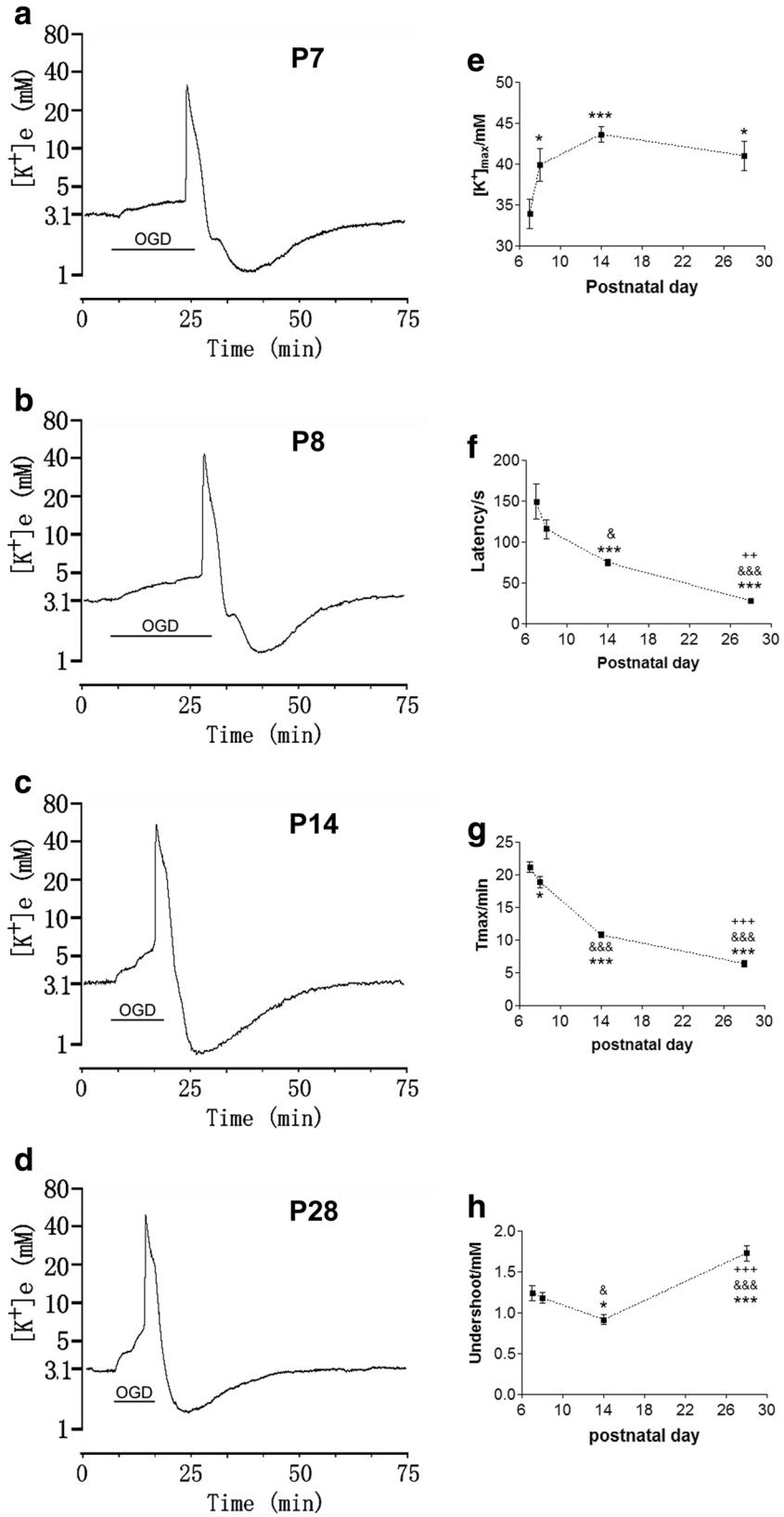
First, we observed the developmental changes of ischemic disruption of K^+ homeostasis in the cortical slices of rats with ages of P7, P8, P14, and P28 without undergoing hypoxia-ischemia treatment. As shown in Fig. 2, the basal levels of extracellular K+ concentrations ($[K^+]e$) in resting condition were around 3 mM. OGD evoked a very large increase in $[K^+]e$ in the cortical slices of these rats. OGD elicited an increase of 33.93 ± 1.78 mM in $[K^+]e$ in P7 cortical slices ($n = 11$), and this change was steadily increased to 39.87 ± 1.99, 43.64 ± 0.95, and 41.02 ± 1.80 mM in P8, P14, and P28 cortex, respectively ($n = 12$, 12, and 11, respectively) ($p = 0.038$, <0.001, and 0.011 in comparison to P7, respectively). The response latency to OGD was also steadily shortened with the development of rats. For P7 rats, OGD-evoked $[K^+]e$ changes began after 150 ± 22 s of perfusion of cortical slices with OGD artificial cerebrospinal fluid (ACSF), while for P8, P14, and P28 rats, it was reduced to 116 ± 12, 75 ± 4, and 28 ± 2 s, respectively. Tmax was steadily shortened too, with 21.2 ± 0.8 ($n = 11$), 18.9 ± 0.9 ($n = 12$), 10.8 ± 0.4 ($n = 12$), and 6.4 ± 0.5 min ($n = 11$) for P7, P8, P14, and P21 cortex, respectively. The undershoot of $[K^+]e$ after re-introducing O_2 and glucose was 1.24 ± 0.09, 1.18 ± 0.07, 0.92 ± 0.06, and 1.73 ± 0.09 mM for P7, P8, P14, and P21 cortex, respectively.

OGD-Induced Disruption of K^+ Homeostasis in the Cortex of Neonatal HI Rats

Next, we asked whether OGD-induced a similar response in disrupted K^+ homeostasis in the cortical slices of rats undergoing HI treatment as their control. Therefore, we observed OGD-induced disruption of K^+ homeostasis in the cortical slices of HI rats at the time point just after HI treatment (HI0), and at 1 (HI1), 7 (HI7), and 21 days (HI21) after HI. As shown in Fig. 3, the cortical slices ($n = 9$) of HI0 rats had similar responses of extracellular K^+ activity to OGD as control ($n = 11$). OGD induced an increase in extracellular $[K^+]$max of 30.90 ± 3.47 mM (vs. 33.93 ± 1.78 mM in control, $p = 0.422$) with the response latency of 116.7 ± 15.0 s (vs. 149.6 ± 22 s in control, $p = 0.247$), Tmax in 19.4 ± 0.6 min of OGD perfusion (vs. 21.2 ± 0.8 min in control, $p = 0.112$), and an undershoot of 1.14 ± 0.10 mM in re-introduction of O_2 and glucose (vs. 1.24 ± 0.09 mM in control, $p = 0.47$).

However, 1 day after HI treatment (HI1), the cortical slices ($n = 12$) of HI1 rats showed a different response of extracellular K^+ activity to OGD in comparison to the control ($n = 12$). OGD-evoked $[K^+]e$ increase was much smaller in HI1 slices than in control (22.72 ± 3.08 vs. 39.87 ± 1.99 mM, $p = 0.0001$) with the response latency to OGD not being shortened as in control, but rather significantly elongated (152.3 ± 10.0 s vs. 115.8 ± 11.6 s in control, $p = 0.026$), and a trend of delayed Tmax (23.0 ± 2.0 vs. 18.9 ± 0.9 min in control, $p = 0.079$). HI1 and their age-matched controls showed a similar undershoot in the re-introduction of O_2 and glucose (1.43 ± 0.15 vs. 1.18 ± 0.07 mM in control, $p = 0.157$). These results suggest an adaption/intrinsic protective mechanism for ischemic disruption of K^+ homeostasis for the cortex 1 day after HI.

Seven days after HI treatment (HI7), OGD evoked a similar maximal $[K^+]e$ increase (43.06 ± 1.23 vs. 43.64



±0.95 mM, $p=0.723$) , the occurrence of the $[K^+]$max (10.2±0.3 vs. 10.8±0.4 min, $p=0.249$), and undershoot (0.93±0.03 vs. 0.92±0.06 mM, $p=0.881$) with O_2 and glucose re-introduced in cortical slices ($n=15$) as control ($n=12$). However, HI-treated cortical slices showed a much shorter response latency to OGD than control, with

◀ **Fig. 2** Developmental profiles of OGD-induced disruption of K^+ homeostasis in the cortex of normal animals. **a**–**d** Trace recordings showing OGD-induced changes in extracellular K^+ activity in the cortical slices of normal rats in postnatal day 7, 8, 14, and 28, respectively. **e**–**h** Statistical results of each recording parameter. *$p<0.05$, ***$p<0.001$ vs. P7; &$p<0.05$; &&&$p<0.001$ vs. P8; $^{++}p<0.01$, $^{+++}p<0.001$ vs P14. Note that OGD-induced disruption of K^+ homeostasis is developmentally augmented in the normal neonatal cortex of rats

the latency being shortened half of control (38.4 ± 4.1 vs. 75.3 ± 4.1 s, $p<0.0001$), suggesting HI-treated cortex is much more sensitive to a second stress stimulation 7 days after HI.

At 21 days after HI treatment (H21), OGD evoked the same response of extracellular K^+ activity in the cortex of both HI-treated rats ($n=11$) and control ($n=11$), including the same increase in $[K^+]e$ (40.32 ± 1.68 vs. 41.02 ± 1.80 mM, $p=0.781$), response latency to OGD (29.1 ± 3.7 vs. 27.9 ± 1.6 s, $p=0.755$), Tmax (5.9 ± 0.6 vs. 6.4 ± 0.5 min, $p=0.552$), and undershoot in re-introduction of O_2 and glucose (1.68 ± 0.13 vs. 1.73 ± 0.09 mM, $p=0.738$), suggesting hypoxic-ischemic changes of K^+ homeostasis to a second insult have recovered 3 weeks after HI.

Effects of DOR Antagonism on OGD-Evoked Changes of Extracellular K^+ Activity in the Cortex of Neonatal HI1 Rats

Our results suggested an adaptive protection mechanism for ischemic disruption of K^+ homeostasis for the cortex at 1 day after HI. Also, a large amount of enkephalin has been demonstrated to be released in hypoxia/ischemia-exposed brain [22–25], which preferentially binds to DOR and activates it [26] to stabilize hypoxic-ischemic disruption of ionic homeostasis in the cortex [38–43]. Therefore, we asked whether this adaptive protection against ischemic disruption of K^+ homeostasis for the cortex at 1 day after HI involves endogenous opioid release and DOR activation. To examine this hypothesis, we perfused HI cortical slices with DOR antagonist naltrindole (NTI) (5 μM) to examine the role of DOR. As shown in Fig. 4, with perfusion of naltrindole (5 μM), the attenuated peak increase of $[K^+]e$ induced by OGD in HI1 cortical slices ($n=12$) (22.72 ± 3.08 mM in HI vs. 39.87 ± 1.99 mM in control, $p<0.001$) was partially, but significantly reversed ($n=19$) (30.76 ± 2.11 mM in HI+ NTI vs. 22.72 ± 3.08 mM in HI alone, $p<0.05$; vs. 39.87 ± 1.99 mM in control, $p<0.01$) with a tendency of response latency being shortened (132 ± 11 vs. 152 ± 10 s in HI, $p>0.05$). This suggests that endogenously released opioid and subsequently activated DOR are involved in the adaptive protection against ischemic disruption of K^+ homeostasis for the cortex at 1 day after HI.

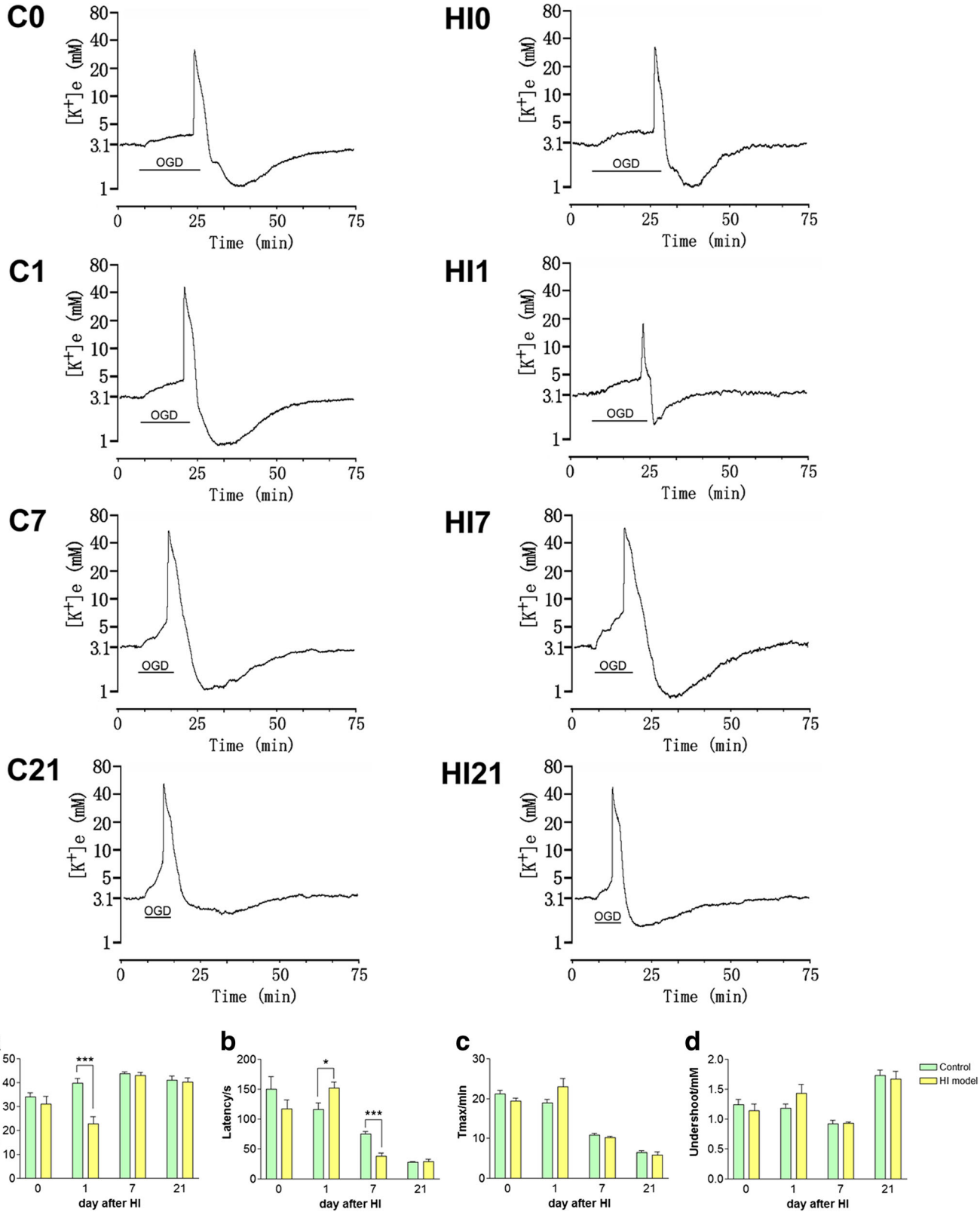
DOR-Induced Attenuation of Ischemic Disruption of K^+ Homeostasis in the Cortex of Neonatal HI7 Rats

Our results showed that HI-treated cortex is much more sensitive to OGD 7 days after HI as manifested by highly shortened response latency to OGD (Fig. 3). Here, we examined the effects of DOR activation on OGD-evoked changes in extracellular K^+ activity in the cortical slices of HI7 rats. As shown in Fig. 5, UFP 512, a potent DOR agonist [38, 44], at 5 μM, significantly prolonged the response latency to OGD in HI7 cortical slices ($n=15$) (58 ± 4 vs. 38 ± 4 s in HI alone, $p<0.01$). Moreover, OGD-induced increase in $[K^+]$max was also significantly decreased by UFP 512 (5 μM) (37.00 ± 1.37 vs. 43.06 ± 1.23 mM in HI alone, $p<0.01$). To further confirm the involvement of DOR in the attenuation of ischemic K^+ derangement in HI7 cortical slices, we also concomitantly perfused the slices with UFP 512 (5 μM) plus naltrindole (5 μM). Our results showed that naltrindole completely abolished the effects of UFP 512 on OGD-induced changes in $[K^+]e$ increase and response latency ($n=12$). With perfusion of naltrindole, the attenuation of OGD-induced increases in $[K^+]e$ by UFP 512 disappeared (42.40 ± 1.70 vs. 37.00 ± 1.37 mM in UFP 512 alone, $p<0.01$, and 43.06 ± 1.23 mM in HI, $p>0.05$), and the elongated response latency to OGD by UFP 512 were again shortened to the level of HI cortex without UFP 512 perfusion (39 ± 5 vs. 58 ± 4 s in UFP 512 alone, $p<0.01$, and 38 ± 4 s in HI, $p>0.05$). These results suggest that DOR activation attenuated ischemic disruption of K^+ homeostasis in the cortical slices of HI7 rats.

Acupuncture-Induced Attenuation of Ischemic Disruption of K^+ Homeostasis in the Cortex of Neonatal HI7 Rats

Several lines of evidence have demonstrated that acupuncture is a beneficial modality against hypoxic-ischemic brain damage and cerebral palsy that is the known sequelae of HIE in neonatal rodents and in children [27–29]. Because disruption of ionic homeostasis is the initial and key alteration in hypoxic/ischemic brain injury, and since maintenance of ionic homeostasis, especially the maintenance of cellular K^+ homeostasis and inhibition of excessive K^+ fluxes, may be of therapeutic benefit in the treatment of stroke and related neurodegenerative conditions [8, 11, 12, 21], we asked whether OGD-evoked disruption of K^+ homeostasis in the brain can be attenuated by acupuncture.

As shown in Fig. 6, after the rats were treated with manual acupuncture (MA) (see "Methods" section), the cortical slices showed some different responses to OGD. For example, despite the similar OGD-evoked increases in $[K^+]e$ between HI rats with/without MA treatment (41.86 ± 0.94 mM in HI + MA vs. 43.06 ± 1.23 mM in HI alone, $p=0.442$, $n=16$ and 15, respectively), MA treatment did elongate response latency to OGD in regard to $[K^+]e$ increase in



the cortical slices (63 ± 6 vs. 38 ± 4 s, $p = 0.002$), and the peak increase of $[K^+]e$ occurred with a small but significant delay after MA (11.5 ± 0.5 vs. 10.2 ± 0.3 min in HI alone, $p = 0.034$), suggesting that acupuncture partially attenuated ischemic disruption of K^+ homeostasis in the cortical slices of HI7 rats.

◀ **Fig. 3** OGD-induced disruption of K^+ homeostasis in the cortical slices of rats undergoing hypoxia-ischemia (HI) treatment. **HI0**, **HI1**, **HI7**, and **HI21** are the trace recordings showing OGD-induced disruption of K^+ homeostasis in the cortical slices of HI rats at the time point just after HI treatment (HI0), and at 1 (HI1), 7 (HI7), and 21 day(s) (HI21) after HI, respectively. **C0**, **C1**, **C7**, and **C21** are age-matched, normal untreated control rats. **a**–**d** Statistical results of each recording parameter. $*p < 0.05$, $***p < 0.001$. Note that the cortex of neonatal HI-treated rats showed a triple phasic response to OGD in regards to the disrupted K^+ homeostasis, namely certain attenuation in the early period (e.g., 1 day after HI), but increased sensitivity in the latter (e.g., 7 days after HI), and finally compensatory recovery (e.g., 21 days after HI)

Discussion

The major findings in the present study with the neonatal hypoxia-ischemia rat are as follows: (1) there was a developmentally increased disruption of K+ homeostasis in the cortex of normal neonates under ischemic condition; (2) neonatal hypoxia-ischemia cortex showed a triple phasic response to OGD in regards to the disrupted K^+ homeostasis, namely certain attenuation in the early period (e.g., 1 day after HI), increased sensitivity in the later period (e.g., 7 days after HI), and finally compensatory recovery (e.g., 21 days after HI); (3) the earlier attenuation, and later enhancement, of ischemic disruption of K^+ homeostasis could be reversed by respective blockade and activation of DOR; and (4) similar to DOR activation, acupuncture could partially reverse the later enhanced ischemic disruption of K^+ homeostasis in the neonatal cortex. This is the first work to demonstrate the roles of acupuncture and DOR in attenuating ischemic disruption of K^+ homeostasis in the neonatal HI brain.

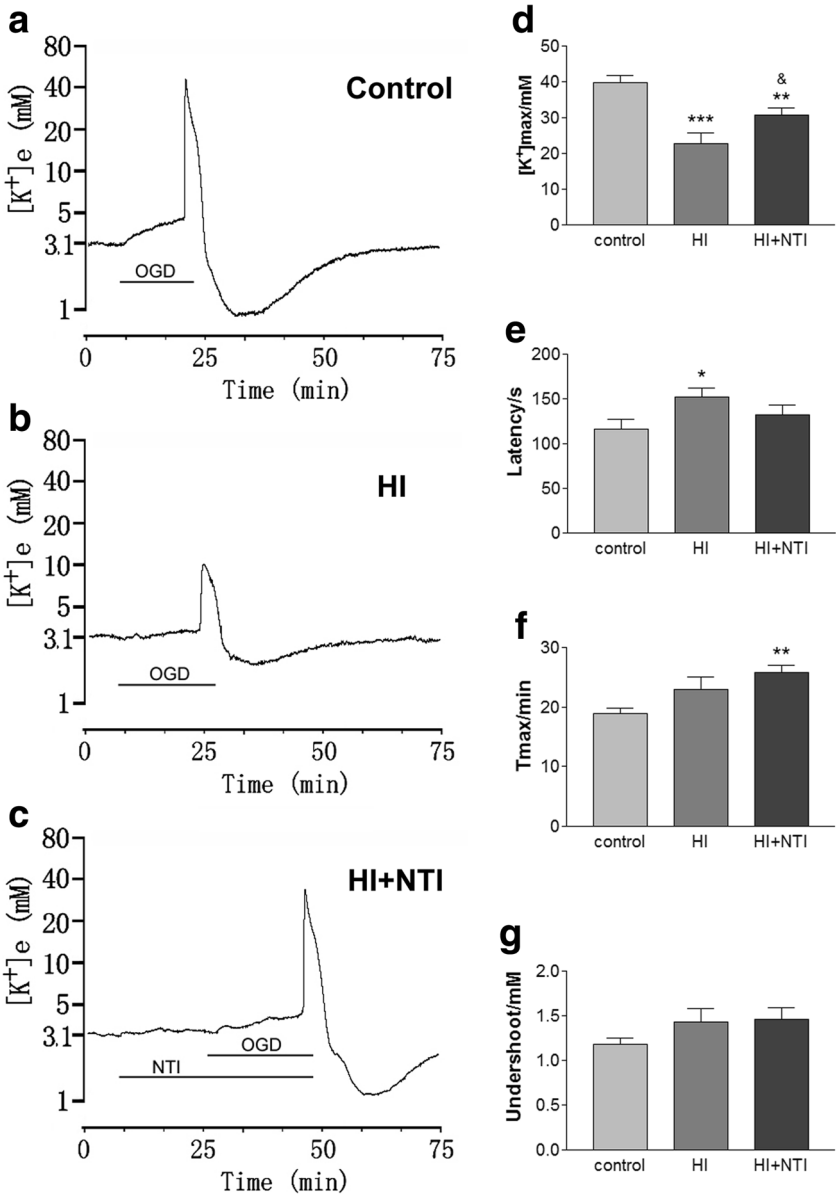
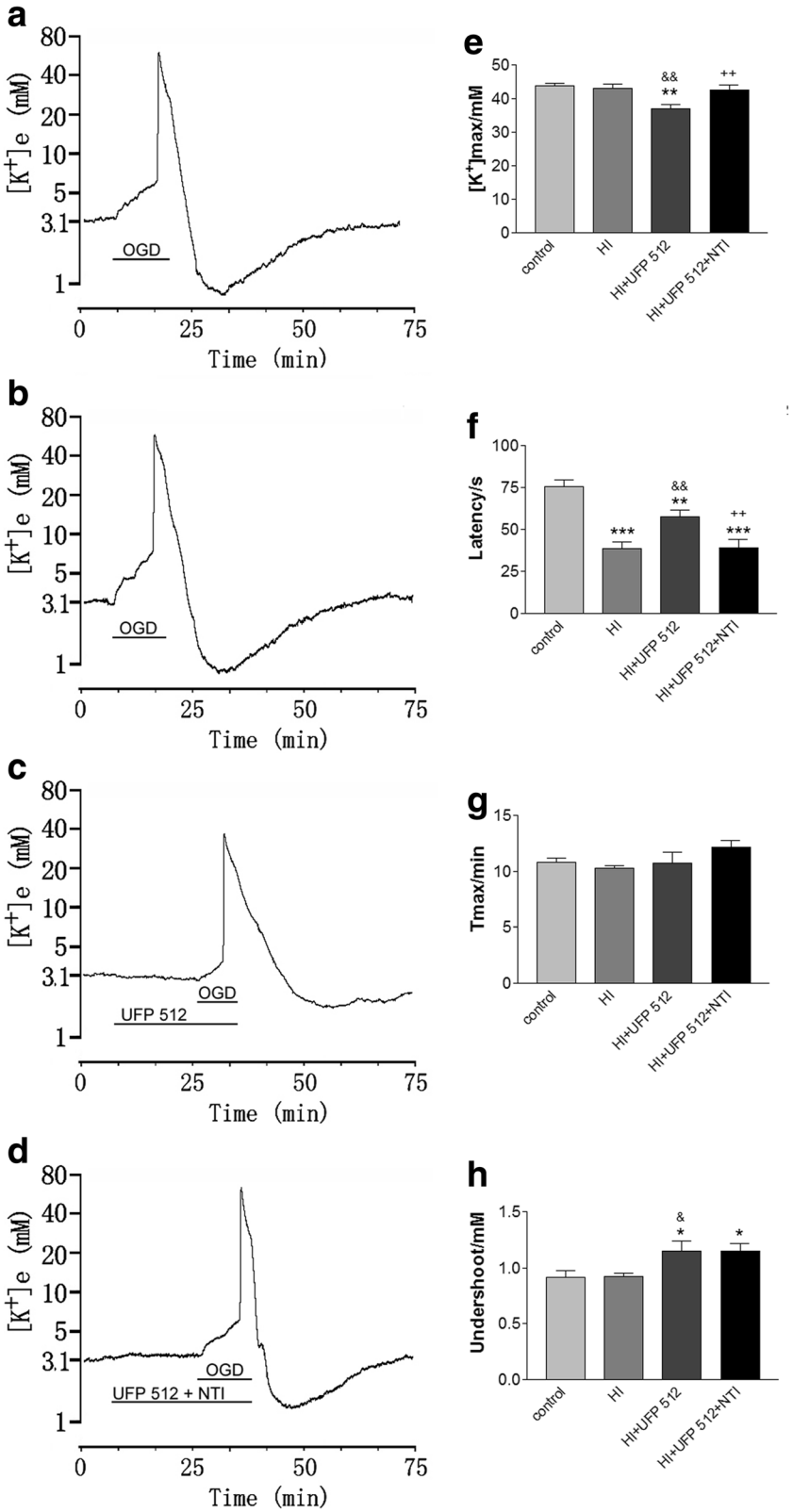


Fig. 4 Effect of DOR blockade on OGD-induced disruption of K^+ homeostasis in the cortical slices of rats 1 day after undergoing hypoxia-ischemia (HI) treatment. Tracing recording of **a** control, **b** HI, and **c** HI slice perfused with DOR antagonist naltrindole (5 μM) (HI + NTI). **d**–**g** Statistical results of each recording parameter. $**p < 0.01$, $***p < 0.001$ vs. control; $\&p < 0.05$ vs. HI. Note that the attenuated ischemic disruption of K^+ homeostasis of the cortex at 1 day after HI (that might be an adaptive mechanism to subsequent insults) involves endogenous opioid release and DOR activation since it could be partially blocked by DOR antagonist naltrindole



It is well established that the neonatal brain is much more resistant to hypoxia-ischemia insults than the adult brain. For example, we previously observed that 4 min of hypoxia increased $[K^+]e$ by 32 mM in adult brainstem, but only 2.6 mM in that of neonates (P5 rats) [45]. The slow rise in $[K^+]e$ before the onset of the rapid steep increase lasts for 25 min in the cerebral cortex of 4-day-old rats during nitrogen inhalation, but only for 2 min in adult rats [7]. In the present study, we

◀ **Fig. 5** DOR attenuation of enhanced ischemic disruption of K^+ homeostasis in the cortical slices of rats 7 day after undergoing hypoxia-ischemia (HI) treatment. Trace recordings of **a** control, **b** HI, **c** HI slice perfused with DOR agonist UFP 512 (5 μM) (HI + UFP 512), and **d** HI slice perfused with UFP 512 (5 μM) plus naltrindole (5 μM) (HI + UFP 512 + NTI). **e**–**h** Statistical results of each recording parameter. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control; &$p < 0.05$, &&$p < 0.01$ vs. HI; ++$p < 0.01$ vs. HI + UFP 512. Note that DOR agonist UFP 512 attenuated enhanced ischemic disruption of K^+ homeostasis (e.g., decreased peak $[K^+]e$ and increased response latency to OGD) in the cortical slices of HI7 rats, which could be completely reversed by DOR antagonist, naltrindole

found that P28 rats began $[K^+]e$ increase after about 30 s, and reached a peak (41.02 ± 1.80 mM) in 6.4 ± 0.5 min of OGD in the cortical slices. In contrast, P7 rats started to have slight increases in $[K^+]e$ after about 3 min of the same stress, and reached a peak level (33.93 ± 1.78 mM) after 21 min of OGD (Fig. 2). These data further supports the viewpoint that the immature brain is more tolerant to hypoxic-ischemic stress than the adult. The tolerance of the neonatal brain to OGD may stem on diversified mechanisms, including the age differences in metabolic pathways (e.g., higher glycolytic capacity and reduced metabolic rate), expression of membrane functional proteins (e.g., energy-costly Na^+ channels), and ion pumping (e.g., small demands in the neonatal brain to preserve ATP levels) during OGD [7, 45–48]. Moreover, neurons in immature brains are smaller and less branched with fewer synapses, which give these neurons less functional surface area than adult neurons for ion

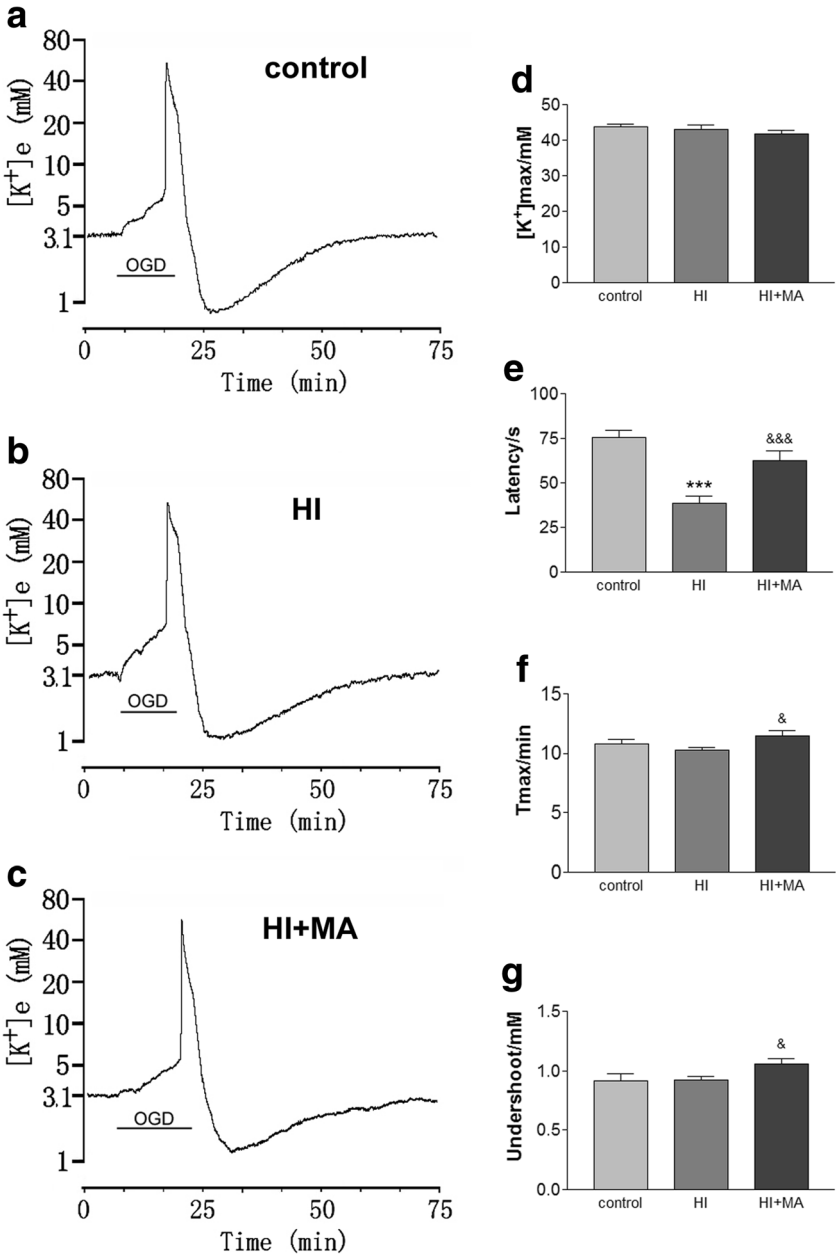


Fig. 6 Effect of acupuncture on enhanced ischemic disruption of K^+ homeostasis in the cortical slices of rats 7 day after undergoing hypoxia-ischemia (HI) treatment. Trace recordings of **a** control, **b** HI, and **c** HI-treated with manual acupuncture (HI + MA). **d**–**g** Statistical results of each recording parameter. ***$p < 0.001$ vs. control; &$p < 0.05$, &&$p < 0.01$ vs. HI. Note that manual acupuncture partially attenuated enhanced ischemic disruption of K^+ homeostasis (e.g., increased response latency and delayed occurrence of peak $[K^+]e$ to OGD) in the cortical slices of HI7 rats

dissipation. Another contributing factor is that the rate of energy metabolism in the immature brain is only 5–20 % of the adult depending on age, but the glycolytic capacity to regenerate ATP in the immature brain is twice as high as in adult. Therefore, immature brains have an extraordinary capability to combat ischemic ionic dyshomeostasis compared to adult brains [7]. Moreover, we found that the intensity of ischemic disruption of K^+ homeostasis in the neonatal cortex is age-dependent, as observed when examining different time points from P7 to P28 in our study. Namely, there is an age-dependent increase in $[K^+]e$ and shortness of both response latency and the occurrence of peak $[K^+]e$ increase (Fig. 2). This phenomenon may be related to the increased rate of energy metabolism with age [7] and the rapid developmental changes in the immature brain, such as increased expression of some key functional proteins in neurons. Increases in ion channel expression such as Na^+ channels and ionotropic glutamate receptor channels (include NMDA receptor, AMPA/KA receptors) have been found in neonatal brains in the postnatal period. For example, Nav1.1 and Nav1.2 are primarily expressed in the central nervous system. Nav1.1 expression is first detectable at postnatal day 7, increases during the third postnatal week, and peaks at the end of the first postnatal month, after which levels decrease by about 50 % in the adult. Nav1.2 expression also increases during the third postnatal week and continues to increase thereafter, until the maximal level is reached in adulthood [33]. NMDA glutamate receptors first appear at new synapses followed by AMPA/KA receptors, and the densities of both receptors increase rapidly in several brain regions including the cortex and hippocampus in the second postnatal week [49–51]. In addition, NMDA receptors undergo developmentally regulated alterations in the ratio of NR subunits and phosphorylation of the component subunits [48, 52]. The large and rapid increases in these channels and receptors in the brain increase neuronal excitability and the capacity to generate action potentials, which may increase K^+ leakage in hypoxic/ischemic condition [8]. In addition, these increases in channel and receptor expression increase Na^+ loading during neuronal activity, which also increases the burden of Na^+/K^+ ATPase. Consequently, exhaustion of ATP is accelerated during insults, and contributes to hypoxic-ischemic disruption of ionic homeostasis and consequent perinatal brain injury [1, 2, 52, 53]. This possibility is further supported by the fact that blockade of Na^+ channels and ionotropic glutamate receptors significantly dampens hypoxia-induced sharp increases in $[K^+]e$ [40, 41, 54]. In addition, K^+ efflux from K^+ channels is also an important source of the increase in $[K^+]e$ during hypoxia/ischemia [8]. Many studies have indicated that the expression of a variety of structurally and functionally diverse K^+ channels and/or their dynamic properties also highly increase in the central nervous system from the second postnatal week [55–59]. These changes may also contribute to the age-dependent enhancement of the disruption of K^+ homeostasis in neonatal cortex during OGD.

In our neonatal hypoxia-ischemia rat model, OGD-induced disruption of K^+ homeostasis in HI rat cortex presents a triple phasic response during the period investigated (1, 7, and 21 days after HI). The three phases are (1) attenuation in the early period (1 day after HI), (2) increased sensitivity in the later period (7 days after HI), and (3) final recovery (e.g., 21 days after HI). The exact reasons for this phenomenon are unknown, but might be related to the output of the balance between adaptive protection and delayed damage in the brain after HI insult. Hypoxia-ischemia treatment may have either preconditioning effects or very harmful consequences depending on multiple inherent and environment factors such as the duration (short vs. long) and severity (mild vs. severe) of insult, the age of animals (young vs. old), the regions of the brain, and even the type of cells (neurons vs. glial cells). For example, a similar period of hypoxia increases $[K^+]e$ largely in the brainstems of adult rats, but only very slightly in those of neonates, and has no effects on turtle brain [45]. Under our experimental conditions, the hypoxia-ischemia treatment is not fatally severe because we did not detect any apparent morphological damages and edema in cerebral hemispheres after hypoxia-ischemia through TTC staining and other observations. The body weight of HI-treated rats only shows a slight decrease after 1 day of HI, and partially recovers after 7 days of HI, and no significant differences were seen between HI rats (HI21) and the age-matched control rats at 21 days after HI.

The attenuation of ischemic disruption of K^+ homeostasis in the phase 1 might reflect a "preconditioning-like" response of the cortex to the hypoxia-ischemia stress since hypoxic or ischemic stress may precondition the brain in a certain period of time after the onset of the stress with an upregulation of DOR activity, including the release of enkephalin, a DOR-preferential agonist, in the exposed brains [22–25]. Indeed, we found that such earlier attenuation of ischemic disruption of K^+ homeostasis could be blocked by DOR antagonism. On the other side, the enhanced sensitivity to OGD in the cortex of rats 7 days after HI could be attributed to a delayed neuroinflammatory response. Inflammatory gene expression in neonatal HI brain is evident at 8 h and increases further at 24 to 72 h post HI ([60], our unpublished data). Elevated cytokines such as IL-1β, IL-6, IL-8, and TNF-α initiate the inflammatory response and create windows of susceptibility to hypoxic-ischemic injury [1, 61], corresponding to a shorter response latency to OGD for the cortex of rats 7 days after HI in our study. However, this enhanced sensitivity may be limited in neonates because of the extraordinary compensatory ability in neonatal brains and the preconditioning effect of HI if the condition of hypoxia-ischemia is mild as it was in our

study. Therefore, it is not surprising that neither OGD-induced peak increases in $[K^+]e$ nor the occurrence of maximal $[K^+]e$ change showed significant differences between rats after 7 days of HI and their age-matched control, and the same can be said for OGD-induced disruption of K^+ homeostasis in rats after 21 days of HI and their age-matched control.

Interestingly, we found the attenuation of ischemic disruption of K^+ homeostasis in the early period (1 day after HI) could be partially reversed by blockade of DOR, while the increased sensitivity to OGD in the later period (7 days after HI) could also be reversed by activation of DOR, which can be abolished by DOR antagonist naltrindole. These findings suggest the involvement of DOR and its stabilizing role in ischemic K^+ derangement in HI brain. It has been found that endogenous enkephalin levels are greatly increased in hypoxia/ischemia-exposed brain [21–25]. This increase may be one of the strategies used to counteract hypoxic/ischemic stress by increasing the activity of DOR. Indeed, hypoxic preconditioning has been proven to be neuroprotective against more severe hypoxic/ischemic and excitotoxic injury in cultured cerebral cortical neurons as well as intact brains through the increase in endogenous enkephalin levels and DOR expression [19, 20, 62]. Interestingly, previous works in our laboratory did demonstrate that DOR activation is neuroprotective against hypoxia/ischemia-induced disruption of ionic homeostasis, including K^+ homeostasis [38–43], and our present study further proved this result in the cortical slices of rats 7 days after HI. Therefore, we believe that the attenuated disruption of K^+ homeostasis evoked by OGD in 1 day after HI is at least partially resulted from the activation of DOR by endogenously released enkephalin in HI-treated rat brain, since this attenuation indeed can be partially reversed by DOR antagonist, naltrindole.

Acupuncture is one of the most popular therapeutic techniques in traditional Chinese medicine, and is also widely practiced in Japan, Korea, and other Asian countries and immigrant communities of oriental origin in the West for treating various diseases including brain disorders such as stroke [63], epilepsy [32, 33], Parkinson's disease [64], Alzheimer's disease, and other neurological disorders [65]. Recent evidence also showed that acupuncture is beneficial to hypoxic-ischemic brain damage and cerebral palsy in neonatal rodents and in children [27–29]. However, the underlying mechanisms of the neuroprotection of acupuncture against hypoxic-ischemic brain damage and cerebral palsy in neonatal brains are not well understood since the immature brain is much more distinguished from the mature brain in response to hypoxic/ischemic insults despite some common features they shared in hypoxic/ischemic brain injury [7, 45, 48]. It was completely unknown if the beneficial effects of acupuncture on neonatal HI brain injury are related to its action on the key and initial response of K^+ efflux [8]. In this work, we present the first data showing that despite similar OGD-evoked increases in $[K^+]e$ between HI rats with/without acupuncture treatment, after several episodes of acupuncture treatment, the response latency to OGD in the cortical slices is in fact elongated, and the peak increase of $[K^+]e$ occurs after a small but significantly delay. This finding suggests acupuncture partially attenuated ischemic disruption of K^+ homeostasis in the neonatal HI brain. The mechanisms underlying acupuncture attenuation of ischemic disruption of K^+ homeostasis in the neonatal HI brain are not known. However, several clues render us to believe endogenous enkephalin release and DOR activation may partially account for it. Firstly, our DOR activation/blockade experiment in OGD-induced K^+ derangement in HI7 rat brain showed a similar result with that of acupuncture. Secondly, acupuncture can enhance the biosynthesis of central enkephalin and upregulate DOR expression [32, 33]. Thirdly, in cerebral ischemic rats, acupuncture indeed improves brain damage and induces tolerance against focal cerebral ischemia by stimulating the release of enkephalin and DOR activation [66, 67]. Finally, and most importantly, we have demonstrated in our previous study that DOR activation can stabilize hypoxia/ischemia-induced disruption of K^+ homeostasis in the mouse cortex [38–41]. Therefore, we speculate that acupuncture attenuates ischemic disruption of K^+ homeostasis in the neonatal HI brain via DOR activation. Further investigations are required to prove this hypothesis.

Conclusions

We made the first demonstration showing that neonatal brains undergo a developmentally increased disruption of K^+ homeostasis during ischemia, and neonatal hypoxia-ischemia cortex shows a triple phasic response of disrupted K^+ homeostasis to simulated ischemic stress with DOR being differentially involved in the attenuation of the ischemic insult. More importantly, we found that acupuncture is beneficial against ischemic disruption of ionic homeostasis, presumably via DOR activation in the brain. Since maintaining cellular K^+ homeostasis and inhibiting excessive K^+ fluxes in the early phase of insults may be of therapeutic benefit in the treatment of hypoxic-ischemic encephalopathy, and since many neurons and other cells can be rescued during the "window of opportunity" after HI insults, our study suggests a therapeutic potential in treatment of hypoxia-ischemia brain injury in the neonates and acute stroke in the adult.

Methods

Induction of Neonatal Hypoxia-Ischemia

This study was approved by the University of Texas Medical School at Houston Animal Care and Use Committee (Animal

Welfare Assurance Number: HSC-AWC-10-144) and was in accordance with the National Institutes of Health Guide for the Care and Use of Animals in Research.

Timed pregnant Sprague-Dawley rats were obtained from Charles River Laboratory and were allowed food and water ad libitum under standard laboratory conditions (a 12-h light/12-h dark cycle and 22 °C in ambient temperature). The neonatal HI model was performed in postnatal day 7 (P7) with pups of both genders under inhalational anesthesia with isoflurane (initial 3 %, maintenance 1.5 %). Briefly, the body temperature of P7 pups was maintained at 37 °C with a heating pad. A midline skin incision (0.5 cm) was made on the neck, and the left common carotid artery was exposed, isolated from nerves and veins, and ligated with 4–0 surgical silk. After surgery, the rat pups were allowed to recover for 30–60 min. Pups were then placed in a plexiglass chamber (30″W × 20″D × 20″H) (Biospherix, Redfield, NY, USA) and the temperature was maintained with a 37 °C water bath to maintain a constant thermal environment. Oxygen levels in the chamber were strictly kept at 8.0 ± 0.5 % by constantly flushing the chamber with nitrogen that was automatically controlled by a ProOx P110 Oxygen Controller with E702 Oxygen Sensor (Biospherix, Redfield, NY, USA). The ProOx O_2 controller has an extremely wide range from 0.1 % O_2 all the way up to 99.9 % O_2 with the variation of 0.1 %, which provides a very important tool for our research in terms of accurate control of O_2 level. The pups were exposed to hypoxia for 1.5 h. Following hypoxic exposure, pups were either immediately used (HI0) or returned to their dams for later experiments after 1, 7, or 21 day(s) of HI (HI1, HI7, and HI21). Sham-operated animals without left common carotid artery ligation and hypoxic exposure were set as the control.

Acupuncture Treatment

A professional acupuncturist/physician (QW) who is skilled in acupuncture treatment in pediatric neurology carried out manual acupuncture (MA) treatment in neonatal HI rats. MA was applied at Baihui (GV20) and Shuaigu (GB8) once daily for 30 min beginning 2 days before HI treatment till the day HI surgery was performed. After that, MA was given every other day for 30 min until day 7 after HI. Acupuncture needles (specification = 0.18 × 13 mm, Suzhou Acupuncture and Moxibustion Appliance Co., Ltd, Suzhou, China) were punctured horizontally 10 mm and were twirled and rotated for 30 s every 5 min during each MA episode.

Slice Preparation

Slices of the cortex were prepared as described in our previous studies [38, 39]. Transverse cortical slices (400 μm) were cut from the brains of timed neonatal rats on a vibrotome containing carbogen (95 % O_2, 5 % CO_2) saturated ice-cold standard artificial cerebrospinal fluid (ACSF). Slices were then transferred to an incubation holder placed in a beaker containing 150 ml ACSF vigorously aerated with carbogen at ~35 °C. Standard ACSF consisted of (in mM) NaCl 125, KCl 3.1, $NaHCO_3$ 26, $CaCl_2$ 2.4, $MgSO_4$ 1.3, NaH_2PO_4 1.25, and D-glucose 10 at pH 7.4. After an equilibration period of at least 90 min in carbogen saturated ACSF at ~35 °C, slices were used for recording. The recordings were made in the outer layer (corresponding to layers II and III) of the cortex.

Induction of Oxygen-Glucose Deprivation in Cortical Slices

A slice was transferred to the recording chamber (Model RC-26, Warner Instrument Co, Hamden, CT) which was perfused with carbogen saturated ACSF (35.5 ± 0.5 °C) with a flow rate of ~5 ml/min. Slices were completely submerged 0.5–1 mm below the ACSF surface in the tissue chamber and kept under normoxic conditions for at least 10 min at ~35.5 °C before experimental measurements were taken.

OGD was induced by switching from the control superfusate (95 % O_2, 5 % CO_2) to D-glucose-deficient ACSF (D-glucose was substituted by equal molar mannitol) continuously bubbled with 95 % N_2 and 5 % CO_2. Each slice was subjected to a single period of OGD that continued for about 2 min after the onset of anoxic depolarization (as assessed by a rapid increase in extracellular $[K^+]$ several minutes after the onset of OGD).

Measurements of Extracellular $[K^+]$

Extracellular K^+ concentrations ($[K^+]e$) were measured using K^+ sensitive microelectrodes. K^+-sensitive microelectrodes were prepared as described previously [38, 39]. The reference electrode was an Ag/AgCl bridge electrode embedded in 2 % agar in 3 M KCl. Calibrations were carried out by detecting the responses generated in KCl solutions (1, 3.1, 5, 10, 20, 40, 80, 100, 160 mM) in triplicate. For each concentration, the average voltage changes in three separate tests were used as the final voltage change. Over this range, electrode response was nearly ideal, showing a logarithmic relationship to $[K^+]$.

Electrical signals were recorded by a direct current amplifier (Model IE-210, LPF 200, Warner Instrument Co., Hamden, CT) and digitized by an Axon mini-digitizer acquisition system (Model miniDigi 1A, Axon Instruments, Union City, CA) at a sampling rate of 100 Hz. The following parameters were derived to assess K^+ homeostasis: (1) the latency of OGD-induced $[K^+]e$ increase (Latency),

which was defined as a time period from the beginning of OGD to the time point when OGD induced a K^+ electrode voltage change greater than 1 mV; (2) Maximal $[K^+]e$ ($[K^+]max$), which was the peak change in extracellular potassium concentration induced by OGD; (3) The duration of OGD to the occurrence of peak K^+ increase, which was defined as the time period from the beginning of OGD to the occurrence of $[K^+]max$ (Tmax); and (4) the undershooting of $[K^+]e$ (Undershoot), which referred to the minimal value of $[K^+]e$ during re-introduction of oxygen and glucose.

After recording a stable baseline for at least 5 min, the slices were subject to experimental treatments. The electrophysiological recordings were continuously performed for at least 75 min with/without drug administration in the exact same conditions.

Drug Administration

For the brain slice study, drugs were applied to cortical slices by switching from control superfusate to one containing drugs, which was controlled by a six-channel valve-controlled solution perfusion system (Model VC-6, Warner Instrument Co, Hamden, CT). All drugs were perfused for 20 min before the induction of OGD, and continued till the end of OGD induction.

Chemicals and Reagents

Naltrindole hydrochloride (NTI), a highly selective δ-opioid receptor antagonist, mannitol, and the chemicals for preparing K^+ sensitive microelectrodes [N-(trimethysilyl)dimethylamine, valinomycin, potassium terrakis(p-chlorophenyl)borate, 2,3-dimethylnitrobenzene] were purchased from Sigma Chemicals Co. (St. Louis, MO). UFP 512 (H-Dmt-Tic-NH-CH(CH_2-COOH)-Bid), a specific and potent DOR agonist [38, 44], was synthesized by our research team. UFP 512 and NTI were prepared in high concentrations in ACSF as stock solutions, and diluted with ACSF to final concentration before experiments.

Statistics

All data are expressed as mean ± SEM. To assess the significance, two-tailed, unpaired Student's *t* test was used for comparison of two experimental groups, and one way analysis of variance (ANOVA) followed by Newman Keuls test was used for multiple pairwise tests. Changes were identified as significant if the probability value was <0.05.

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Compliance with Ethical Standard

Competing Interests The authors declare that they have no competing interests.

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