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RESEARCH ARTICLE

# Attenuated effects of Neu2000 on hypoxia-induced synaptic activities in a rat hippocampus

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**Abstract** Neu2000 (NEU; 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid), a recently developed derivative of acetylsalicylic acid and sulfasalazine, potently protects against neuronal cell death following ischemic brain injury by antagonizing NMDA receptor-mediated neuronal toxicity and oxidative stress. However, it has yet to be determined whether NEU can attenuate hypoxia-induced impairment of neuronal electrical activity. In this study, we carried out extracellular recordings of hippocampal slices in order to investigate the effects of NEU on the electrical activity of neurons exposed to a hypoxic insult (oxygen and glucose deprivation). NEU prominently suppressed hypoxia-induced impairment of neuronal activity in a concentration-dependent manner. NEU, at a low dose (1 μM), competently depressed the hypoxia-induced convulsive activity in a manner similar to trolox. Furthermore, high concentrations of NEU (50 μM) markedly abolished all hypoxia-mediated impairment of neuronal activity and accelerated the slow recovery of neuronal activity more efficiently than ifenprodil and APV. These results suggest that NEU attenuates hypoxia-induced impairment of neuronal activity more potently than the antioxidant, trolox, and the NMDA receptor antagonists, ifenprodil and APV. We propose that NEU is a striking pharmacological candidate for neuroprotection against hypoxia because of its defensive action on hypoxia-mediated impairment of electrical neurotransmission as well as its neuroprotective action against neuronal cell death induced by exposure to pathological hypoxic conditions.

**Keywords** Derivative of acetylsalicylic acid · Extracellular recording · Neuronal toxicity · Oxidative stress · Hypoxia

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## Introduction

The pathogenesis of hypoxic-ischemic brain injury is associated with a series of events, including the deletion of cellular energy sources, release of excitatory amino acids such as glutamate, mitochondrial dysfunction, and excessive generation of oxygen free radicals (White et al. 2000). In the central nervous system, hypoxia-induced glutamate release impairs neuronal homeostasis, leading ultimately to excitotoxic damage and neuronal cell death (Nicholls and Attwell 1990; Lipton 1999). The abnormal accumulation of glutamate triggers an influx and intracellular rise in $Ca^{2+}$, which in turn results in $Ca^{2+}$ overload and activation of cytotoxic proteins that induce cell death (Simon et al. 1984; Rothman and Olney 1986; Choi and Rothman 1990). Another big consequence of severe hypoxia is the generation of injurious free oxygen and nitrogen species, which drives molecular tissue impairments that result in neuronal and glial cell death (White et al. 2000).

Brain hypoxic ischemia results in a loss of electroencephalographic activity and consciousness, followed by a high-risk period of irreparable neuronal damage; this reflects the rapid effect that hypoxia has on the brain. Hypoxia resulting from reduced blood perfusion results in impaired neuronal excitability and abnormal synaptic

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transmission in corresponding brain areas, such that their neuronal and synaptic electrical activities are silenced (Astrup et al. 1981). Indeed, it has been reported that severe cerebral hypoxia causes cessation of neurotransmission in the cortex (Mayevsky and Chance 1975; Luhmann and Heinemann 1992) and in hippocampal neurons (Lipton and Whittingham 1979; Leblond and Krnjević 1989). Although synaptic activity is inhibited during hypoxia, a paradoxical increase in glutamate release has been observed, and this excitatory neurotransmitter is known to play a role in the cascade of events leading to neuronal injury (Hershkowitz et al. 1993; Fleidervish et al. 2001).

Neu2000 (NEU; 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid), a derivative of the lead structure of sulfasalazine and aspirin, has demonstrated excellent neuroprotection against *N*-methyl-D-aspartate (NMDA) and free radical-induced cell death in the ischemic brain (Gwag et al. 2007). Since hypoxia can lead to aberrant excitatory neurotransmission and abnormal oxidative stress with deleterious consequences, it is imperative to find a therapeutic solution. Thus, the aim of this study was to determine the effect of NEU on hypoxia-mediated disturbances in neuronal transmission. To address this, we carried out electrophysiological extracellular recordings of acute rat hippocampal slices in order to characterize the pattern of neural activities induced by hypoxia and to examine if these patterns were altered by NEU.

## Materials and methods

### Preparation of hippocampal slice

All experiments regarding the ethical use of animals for experimentation conformed to the guidelines issued by Ewha Womans University. To obtain hippocampal slices, Sprague–Dawley rats (21–28 days old) were briefly anesthetized with isoflurane and decapitated. Brains were quickly removed from rat skulls and immersed in an oxygenated (95 % $O_2$/5 % $CO_2$), ice-cold sucrose solution containing the following (in mM): 201 sucrose, 3 KCl, 1.25 $NaH_2PO_4$, 3 $MgCl_2$, 1 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose. Transverse hippocampus slices, 400 μm-thick, were cut using a vibratome (1000S, TedPella). Before each recording, slices were incubated with warm (30 °C) oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): 126 NaCl, 3 KCl, 1.25 $NaH_2PO_4$, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 10 D-glucose. After a recovery period of 60 min, slices were left at room temperature. For the recording, individual slices were transferred to a submerged-type recording chamber that was fixed to the microscope stage (SZ-STU1, Olympus); the slices were kept at a temperature of 24–29 °C and superfused with oxygenated ACSF at a rate of 2–4 ml/min.

### Induction of hypoxia conditions

Episodes of hypoxia were produced by switching the gas flow mixture over the slice from 95 % $O_2$/5 % $CO_2$ to 95 % $N_2$/5 % $CO_2$ which bubbled for more than 60 min. Within 30 s of operating a three-way tap, hypoxic solutions, which were deprived of glucose by eliminating glucose from the ACSF, were added to the recording chamber. A stable control period of neuronal activity was recorded for 10–15 min, followed by a 5 min exposure period to the hypoxic condition, followed in turn by a washout period for 40–60 min.

### Spontaneous extracellular recordings

We recorded spontaneous spikes in the hippocampal CA3 area using a borosilicate glass capillary electrode (O.D. 1.5 mm/I.D. 0.84 mm, WPI) with ACSF connected to a microelectrode AC amplifier (model 1800; A-M Systems Inc.). Recording pipettes were pulled with a horizontal micropipette puller (P-9, Sutter Instrument Company) and polished with a microforge (MF-830, Narishige). Neuronal signals were recorded differentially using an AC-coupled four-channel amplifier at a gain of 1000, with a ground electrode located nearby in the bathing medium. Analog signal was digitized by an A/D converter (Digidata 1322A, Molecular Devices) and collected with pClamp10.0 software (Molecular Devices). The number of spontaneous spikes was counted by a threshold search method of the pClamp10.0 software. We took real spikes that had an amplitude >0.05 mV and the rest were ignored. Spike amplitude for control was 0.1–0.5 mV and the frequency was 3–20 Hz. All histograms were constructed to reflect the number of spikes per minute. Trolox, APV and ifenprodil were obtained from Tocris. NEU (a kind gift from Neurotech Corp.) was prepared as a stock solution of 100 mM dissolved in dimethylsulfoxide.

### Statistical analysis

We performed 48 independent experiments; 11 for control, 5 for NEU (1 μM), 8 for trolox (200 μM), 15 for NEU (50 μM) and 9 for ifenprodil (10 μM). Data were presented as mean ± standard error of the mean and analyzed using Prism5 software (GraphPad software Inc.). To normalize the number of spikes as a function of time, we divided the number of spikes in the presence of drugs by the basal average value which is the average number of spikes in the absence of drugs.

## Results

### Effect of hypoxia by oxygen–glucose deprivation on hippocampal neuronal excitability

In order to determine the effect of hypoxia on neuronal excitability, we induced hypoxic conditions by applying an oxygen- and glucose-deprived solution to acute hippocampal slices for 5 min; alterations in spontaneous neuronal activity under these conditions were recorded (Fig. 1). Stable spontaneous neuronal activity was collected for 10–20 min prior to hypoxia exposure. After onset of hypoxic conditions, a gradual decline in neuronal activity frequency was observed; at 3 min, neuronal activity in hypoxic condition was 60 % compared to control activity (at 10 min in time axis), and this was followed by a rapid explosive boost in activity frequency for 2–3 min. Explosive spontaneous activity increased by threefold (at 11 min) and was instantly diminished by 90 % compared with control activity (at 14 min). In the hypoxia-free condition, the reduced activity was very slow to recover; even after 20 min (at 40 min) the spontaneous activity was not completely restored: An average of 50 % neuronal activity developed in 5 out of 11 cases and only 10 % of the original neuronal activity was produced in 6 out of 11 cases. This suggests that brief exposure to hypoxia can induce severe irreparable damage to electrical neuronal function, which is in agreement with previous reports (Hershkowitz et al. 1993; Fleidervish et al. 2001).

### Effect of NEU as an antioxidant on hypoxia-induced abnormal activities in rat hippocampus

Next, we examined the effect of NEU on spontaneous neuronal activity in hippocampal slices exposed to pathological hypoxic conditions. In the presence of NEU (1 μM), hypoxia-induced convulsive abnormal activity was not detected and the spontaneous neuronal activity was left at 60 % under hypoxic condition (Fig. 2; at 10 min in time axis, 50 % activity; at 11 min, 40 %; at 14 min, 40 %). Following 10 min in the hypoxia-free condition, most neuronal activity was recovered in the presence of NEU (at 40 min, 96 %). This neuroprotective action of NEU (1 μM) against hypoxia-mediated impairment was identical to that of the trolox (200 μM), which acts as a representative peroxyl radical scavenger (at 10 min in time axis, 20 % activity; at 11 min, 10 %; at 14 min, 50 %). The reduced spike activity was recovered to 70 % by re-oxygenation in the presence of trolox (at 40 min), whereas the decreased activity was regained to 100 % in the presence of NEU. This suggests that low concentrations of NEU attenuate the pathological neuronal activity induced by hypoxia likely through the reduction of free-radicals.

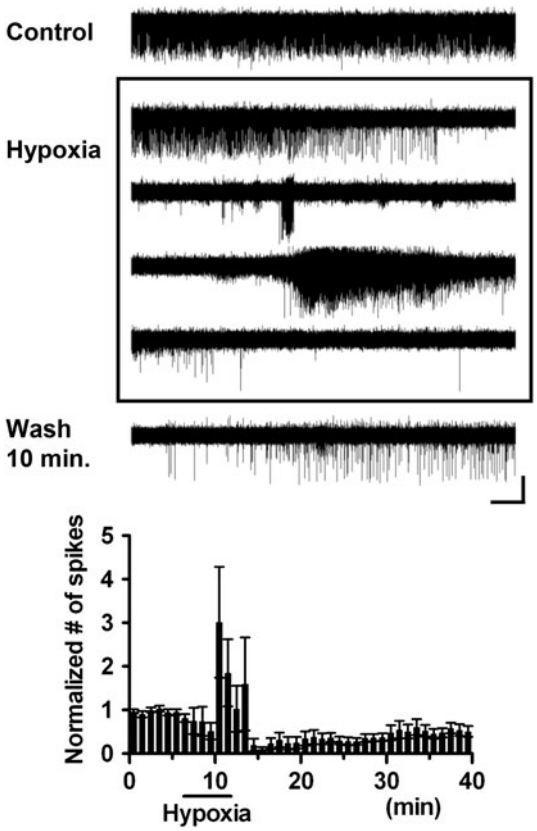


**Fig. 1** Effect of hypoxia on spontaneous activity in the CA3 area of the hippocampus. (*upper*) Spontaneous activity in a hippocampal slice before (control), during (for 5 min, Hypoxia), and after exposure to hypoxic conditions (wash). Note that under brief periods of hypoxia, the amount of spontaneous neuronal activity was gradually decreased, and the frequency of neuronal activity convulsively increased for 1–2 min. All explosive activity was then rapidly depressed. Neuronal activity slowly and incompletely recovered following re-oxygenation (re-admission of $O_2/CO_2$ mixture gas, Wash). *Scale bar* 0.2 min for *X axis*; 0.2 mV for *Y axis*. (*lower*) Normalized number of spikes in all conditions as a function of time before and after exposure to hypoxic conditions (*horizontal bar* Hypoxia, 5 min; $n = 11$). Data were presented as mean ± standard error of the mean

### Effect of NEU as an NMDA receptor antagonist on hypoxia-induced abnormal activities in rat hippocampus

Excitotoxic neuronal cell death induced by over-activation of the NMDA receptor plays a key role in the etiology of hypoxic brain injury (Rothman and Olney 1986), and a number of NMDA receptor antagonists have been shown to decrease hypoxic brain injury in various animal models. To determine the role of NEU as an NMDA receptor antagonist in hippocampal slices exposed to hypoxia, we examined the effects of NEU (50 μM) on hypoxia-induced spontaneous neuronal activity ($IC_{50}$ of NEU for NMDA = 35.4 μM) (Gwag et al. 2007; Noh et al. 2009). In the presence of NEU (50 μM), hypoxia-mediated neuronal

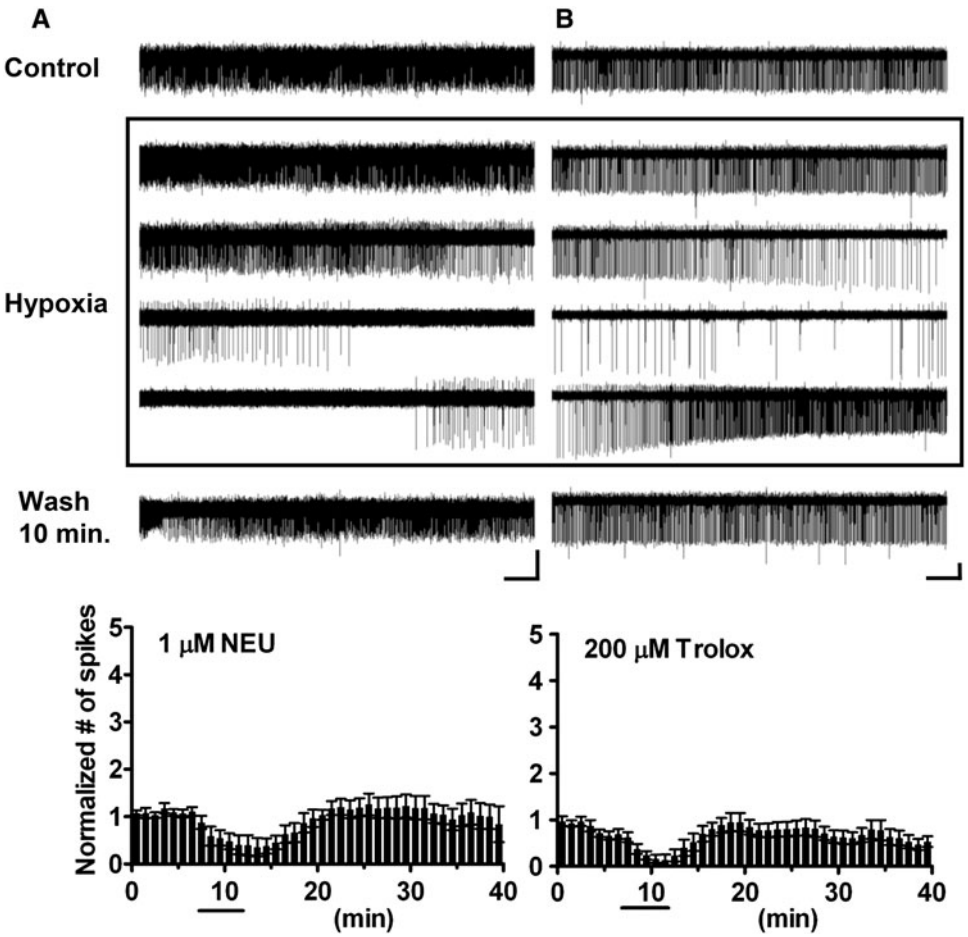


**Fig. 2** Effect of NEU as an antioxidant on hypoxia-induced spontaneous spike activity in the CA3 area of the hippocampus. **a** *Upper panel* shows spontaneous neuronal activity under hypoxic condition in the presence of NEU (1 μM). In the presence of NEU, approximately 5 min after exposure to hypoxia, the convulsive abnormal activities such as those observed in Fig. 1 were not detected. Moreover, the spontaneous activity was steadily decreased during the period of hypoxia (*horizontal bar* 5 min) and completely recovered in the hypoxic-free conditions (Wash) after 10 min (*lower*; $n = 5$). *Scale bar* 0.2 min for *X axis*; 0.2 mV for *Y axis*. **b** Spontaneous neuronal activity recorded (*upper*) and normalized number of spikes as a function of time before and after exposure to hypoxic conditions in the presence of 200 μM trolox (*lower*; $n = 8$). Data were presented as mean ± standard error of the mean

activity impairment was strikingly abolished (Fig. 3; at 10 min in time axis, 70 % activity; at 11 min, 105 %; at 14 min, 85 %; 40 min, 95 %). Ifenprodil (10 μM), which is a NR2B selective and activity-dependent inhibitor of the NMDA receptors, and APV (50 μM), which is a general blocker of the NMDA receptors, both attenuated the hypoxia-mediated convulsive abnormal activity and accelerated the slow recovery of reduced activity in hypoxia-free conditions. However, these effects were not as prominent as those of NEU (50 μM) (ifenprodil; at 10 min in time axis, 50 % activity; at 11 min, 110 %; at 12 min, 200 %; at 14 min, 20 %; 40 min, 55 %; APV; at 10 min in time axis, 80 % activity; at 11 min, 170 %; at 12 min, 200 %; at 14 min, 20 %; 40 min, 60 %). Out of 15 cases, NEU (50 μM) completely blocked the hypoxia-mediated disturbance in neuronal activity in 6 cases, displayed ifenprodil-like actions in 7 cases, and trolox-like actions in 2 cases, thus suggesting that under hypoxic conditions, NEU (50 μM) protects against hypoxia-mediated impairment of neuronal activity more potently than antioxidant, trolox and the NMDA receptor antagonists, ifenprodil and APV.

## Discussion

The results of this study reveal that NEU markedly protects hypoxia-induced impairment of neuronal activity in hippocampal slices by acting more efficiently than other NMDA receptor antagonists, such as ifenprodil and APV and/or antioxidants, such as trolox. Under our hypoxic condition, we classified the pattern of neuronal firing activity into three components: (1) under hypoxia, gradually decreased firing activity; (2) under hypoxia, explosive boost in firing activity; and (3) in the hypoxia-free period, recovery of activity. Given that the "gradually decreased" component of hypoxia showed little alteration in activity compared to those experiments carried out in the presence of either trolox or ifenprodil and APV, this component might be mediated by another mechanism, such as $K^+$ conductance-mediated neuronal hyperpolarization (Erdemli et al. 1998). It has been established that acute hypoxia modulates the activity of a wide range of different ion channels, such as $Ca^{2+}$ channels (Franco-Obregón et al. 1995), $Na^+$ channels (O'Reilly et al. 1997), and $K^+$ channels (Liu et al. 1999), all of which have been described

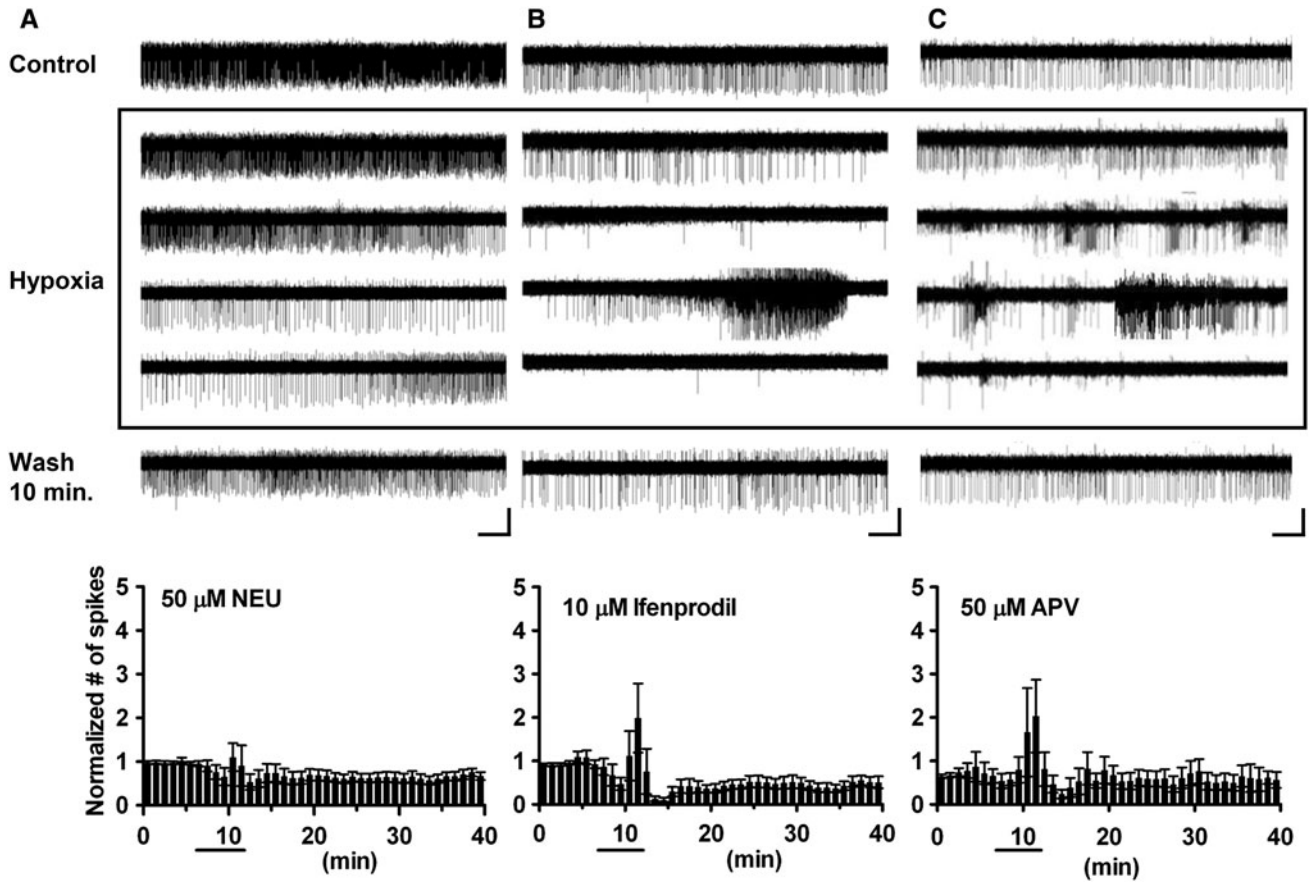


**Fig. 3** Effect of NEU as a NMDA receptor antagonist on hypoxia-induced spontaneous spike activity in the CA3 area of the hippocampus. **a** Spontaneous neuronal activity recorded (*upper*) and normalized number of spikes as a function of time before and after exposure to hypoxic conditions in the presence of 50 μM NEU (*lower*; $n = 15$). During and after the period of hypoxia (*horizontal bar* 5 min), convulsive activity and gradually decreased activity were not observed or only sparsely detected in the presence of NEU. Scale bar, 0.2 min for *X axis*; 0.2 mV for *Y axis*. **b** Spontaneous activity recorded (*upper*) and normalized number of spikes as a function of time before and after exposure to hypoxic conditions in the presence of 10 μM ifenprodil (*lower*; $n = 9$). Approximately 5 min following exposure to hypoxia, the convulsive abnormal activity increased by twofold in the presence of ifenprodil. After 10 min, the spontaneous activity was recovered to 60 % by re-oxygenation (Wash) in the presence of ifenprodil. **c**. Spontaneous activity recorded (*upper*) and normalized number of spikes as a function of time before and after exposure to hypoxic conditions in the presence of 50 μM APV (*lower*; $n = 5$). Approximately 5 min following exposure to hypoxia, the convulsive abnormal activity increased by twofold in the presence of APV. After 10 min, the spontaneous activity was recovered to 60 % by re-oxygenation (Wash) in the presence of APV. Data were presented as mean ± standard error of the mean

as oxygen sensitive. Because of our finding that NEU (50 μM) blocks this part of the neuronal activity (Fig. 3), future experiments showing the effect of NEU on different ion channels would clarify the precise mechanism by which NEU mediates protection against hypoxia-induced impairment of neuronal activity. The convulsive abnormal boost response of neuronal activity appeared to be induced by free radical-mediated glutamate release under hypoxic conditions since trolox, as well as NEU, completely abolished this part of the firing activity (Fig. 2); this finding is consistent with a recent report that free radicals mediate hypoxia-induced glutamate release in the cortex (Dong et al. 2012). In the hypoxia-free period, the decreased firing activity was quickly recovered in the presence of either trolox or NEU, suggesting that NEU efficiently blocks the impairment in neural activity that is mediated by a burst of excess oxygen radicals during reperfusion. The recovery of firing activity by ifenprodil or APV in hypoxic condition is less effective than that evoked by NEU (Fig. 3).

Under hypoxic condition, free radical and NMDA receptor activation seem to act interdependently of each other in neuronal damage. Free radical is a downstream target of NMDA receptor and vice versa. An excessive increase in extracellular accumulation of glutamate following hypoxia has been demonstrated to be a critical event involved in the induction of neuronal damage and death (Rothman and Olney 1986). It has also been shown that $Ca^{2+}$ influx, through activation of the NMDA glutamate receptor, is associated with ischemia/reperfusion-induced cell damage (Simon et al. 1984) and generation of

free radicals (Lafon-Cazal et al. 1993). In addition, free radicals are reportedly involved in hypoxia-induced glutamate release (Dong et al. 2012). Moreover, recovery of synaptic transmission in the hippocampus of glutathione peroxidase transgenic mice after hypoxia is related to improved detoxification of peroxides that might be produced in response to NMDA receptor activation (Furling et al. 2000). In this study, under hypoxia, an explosive boost in firing activity was completely blocked by the antioxidant trolox, but not by NMDA receptor antagonism, indicating that free radical activation precedes NMDA receptor activation. In the recovery period, both antioxidant approaches and NMDA receptor antagonism effectively blocked the impairment of activity. It suggests that antioxidative behavior can protect the impairment of activity not only during the initial hypoxic period but also during reperfusion after hypoxic exposure, whereas NMDA receptor antagonism mainly plays a role in protecting reperfusion-induced activity impairment. A dose of 50 μM NEU illustrated a little impairment of activity (Fig. 3). In the recovery period, NEU more effectively and quickly recovered the excitability compared to the antioxidant, trolox, and the NMDA receptor antagonists, ifenprodil and APV.

Accumulating reports suggest that since perturbations in the balance between synaptic and extrasynaptic NMDA activity contributes to neuronal dysfunction (Hardingham and Bading 2010), the extrasynaptic NMDA receptor, which is mainly composed of NR2B subunits, could be therapeutically targeted without compromising normal synaptic function (Thomas et al. 2006). However, our results suggest that NR2B-selective NMDA receptor antagonism is insufficient to protect against abnormal firing activity, which is in agreement with a recent report (Wroge et al. 2012). Instead, we suggest that the neuroprotective strategy against hypoxia should aim to both enhance antioxidant activities and disrupt NMDA-dependent death signaling. It has previously been demonstrated that NEU can block biphasic mitochondrial free radicals production and attenuate delayed neuronal death in the hippocampus (Park et al. 2011). Furthermore, NEU can markedly prevent the neuronal death mediated by excitotoxicity and oxidative stress after hypoxic-ischemic brain injury (Gwag et al. 2007), in the treatment of amyotrophic lateral sclerosis (Shin et al. 2007) and acute spinal cord injury (Springer et al. 2010). We propose that NEU is a striking pharmacological candidate for neuroprotection against hypoxia due to its defensive action on hypoxia-mediated impairment of electrical neurotransmission as well as its neuroprotective action against neuronal cell death induced by exposure to pathological hypoxic conditions.

In the hippocampal slice, various features of the early events that occur during hypoxia are well-established. The cessation of neuronal firing is a prominent early feature because the greater diffusion distance slows down exchanges between the interior of the slice and its surroundings. Three reasons have been uncovered to explain the early cessation of electrical activity: (1) neurons are hyperpolarized by a sharp rise in membrane permeability to potassium ions and a substantial drop in membrane resistance (Leblond and Krnjević 1989; Fujimura et al. 1997; Erdemli et al. 1998), (2) membrane currents that generate sodium and calcium action potentials are suppressed, and (3) excitatory synaptic transmission is selectively blocked (Fowler 1989; Leblond and Krnjević 1989; Fujimura et al. 1997). Hypoxia-induced signals trigger a rise in cytoplasmic free calcium, fall in adenosine triphosphate, and extracellular accumulation of adenosine. Our results are the first to demonstrate that NEU can improve the capacity of hippocampal cells to recover synaptic transmission after a short period of hypoxia in vitro. However, it remains unclear whether NEU effects neurotransmission differently under physiological conditions compared to pathological conditions and whether NEU can modulate normal neurotransmission in a neural circuit. Moreover, it should be elucidated that which one of three molecular mechanisms underlying the attenuation of abnormal electrical neuronal activities is affected by NEU.

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