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## Post-Hypoxic Changes in Rat Cortical Neuron GABA<sub>A</sub> Receptor Function Require L-type Voltage-Gated Calcium Channel Activation

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### Summary

Hypoxia modifies GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) function, and can cause seizures, encephalopathy or myoclonus. To characterize the effects of hypoxia on neuronal GABA<sub>A</sub>Rs, we subjected rat cortical neurons to 1% O<sub>2</sub> for 2, 4 or 8 h, followed by recovery times of 0 to 96 h, and used whole-cell and perforated-patch patch clamp recording to assess GABA<sub>A</sub>R currents and pharmacology. Hypoxic exposure for 4 h caused downregulation of maximal GABA current immediately following hypoxia and after 48 h recovery without changing the EC<sub>50</sub> for GABA. Two and eight hour hypoxic exposures had inconsistent effects on GABA<sub>A</sub>R currents. Maximal diazepam potentiation was increased immediately following 4 h hypoxia, while potentiation by zolpidem was increased after 48 h recovery. Pentobarbital enhancement and zinc inhibition of GABA currents were unchanged. Hypoxia also caused a depolarizing shift in the reversal potential of GABA-induced Cl<sup>-</sup> currents after 24 h recovery. The L-type voltage-gated calcium channel (L-VGCC) blocker, nitrendipine, during hypoxia or control treatment prevented the reduction in GABA<sub>A</sub>R currents, and increased control currents over baseline. Nitrendipine also prevented the increase in zolpidem potentiation 48 h after hypoxia, and blocked the depolarizing shift in Cl<sup>-</sup> reversal potential 24 h after hypoxia. The effects of hypoxia on maximal GABA<sub>A</sub>R currents, zolpidem pharmacology and Cl<sup>-</sup> reversal potential thus require depolarization-induced calcium entry via L-VGCCs, and constitutive L-VGCC activity appears to reduce maximal GABA<sub>A</sub>R currents in control neurons via a calcium-dependent process. Calcium-dependent modulation of GABA<sub>A</sub>R currents via L-VGCCs may be a fundamental regulatory mechanism for GABA receptor function.

### Keywords

Patch clamp; reversal potential; gramicidin; nitrendipine; benzodiazepine; zolpidem

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## 1. Introduction

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) mediate fast inhibitory synaptic neurotransmission in the central nervous system. They are members of the ligand-gated ion channel family, composed of five subunits from multiple subunit subtypes ( $\alpha$ 1- $\alpha$ 6,  $\beta$ 1- $\beta$ 3,  $\gamma$ 1- $\gamma$ 3,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$  and  $\rho$ 1- $\rho$ 3) (Macdonald and Olsen, 1994). The binding of  $\gamma$ -aminobutyric acid (GABA) regulates gating of the chloride ion channel, and a number of positive and negative allosteric regulators, including the benzodiazepines (BZ), barbiturates, neurosteroids, Zn<sup>2+</sup> and other divalent cations, interact with GABA<sub>A</sub>Rs at binding sites formed by the presence of specific subunit subtypes in the holoreceptor (Korpi et al., 2002). The BZ full agonist diazepam binds to GABA<sub>A</sub>Rs containing  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 or  $\alpha$ 5 subunits, combined with a  $\beta$  and  $\gamma$ 2 subunit (Korpi et al., 2002). High affinity zolpidem binding is conferred by the presence of an  $\alpha$ 1 subunit, combined with  $\beta$  and  $\gamma$  subunits. Presence of  $\alpha$ 2 or  $\alpha$ 3 subtype results in receptors with moderate zolpidem affinity (MacDonald and Kapur, 1999). Zn<sup>2+</sup> inhibits GABA-evoked currents with higher potency at receptors containing an  $\alpha$ 4,  $\alpha$ 5 or  $\alpha$ 6 and lacking an  $\alpha$ 1 and/or a  $\gamma$  subunit (Macdonald et al., 1996). The pharmacological and biophysical properties of GABA<sub>A</sub>Rs thus depend on subunit composition (Sieghart et al., 1999).

Both excitatory and inhibitory neurotransmission are affected during and after hypoxia, leading to excitotoxicity (Yue et al., 1997). Modification of GABA<sub>A</sub> receptor function has been implicated in a range of hypoxia-related pathologies, including encephalopathy (Low et al., 1985), seizures (Bergamasco et al., 1984) and myoclonus (Hallett, 2000). GABA-mediated currents were reduced in CA1 pyramidal neurons in hippocampal slices exposed to hypoxia both *in vivo* and *in vitro* (Xu and Pulsinelli, 1994; Congar et al., 1995). Decreased GABA<sub>A</sub>R current in cultured hippocampal neurons subjected to experimental ischemia was attributed to depletion of ATP and increased intracellular Ca<sup>2+</sup> (Harata et al., 1997). Within 1 h after cerebral ischemia, GABA-gated <sup>36</sup>Cl<sup>-</sup> flux dramatically decreased in gerbil neurons (Verheul et al., 1993). Intracellular Cl<sup>-</sup> in rat hippocampal slices was increased early after ischemia, resulting in reduced Cl<sup>-</sup> driving force and GABA<sub>A</sub> responses (Inglefield and Schwartz-Bloom, 1998).

Our previous studies demonstrated that maximal GABA currents in NTera2 (NT2-N) neuronal cells changed in a biphasic manner after 8 h exposure to 1% O<sub>2</sub>. Maximal currents were significantly increased immediately following 8 h hypoxia, but were dramatically reduced after 48 h recovery, and then returned to baseline after 96 h recovery. GABA<sub>A</sub> receptor  $\alpha$ 1,  $\alpha$ 5,  $\beta$ 2 and  $\gamma$ 2 subunit mRNAs were downregulated after hypoxia (Gao et al., 2004). Since NT2-N cells are derived from a human tumor cell line, they may not completely reflect the behavior of brain-derived neurons. Here we investigated the effects of different durations of hypoxia and normoxic recovery on GABA<sub>A</sub>R currents and pharmacology in rat cortical neurons in primary culture. Hypoxic exposure reduced GABA currents immediately following hypoxia and after 48 h recovery, altered benzodiazepine pharmacology and shifted the chloride equilibrium potential. These post-hypoxic changes could be prevented by the voltage-gated calcium channel (L-VGCC) blocker, nitrendipine, during hypoxic treatment.

## 2. Methods

### 2.1 Cortical Neuron Culture

Cortices were dissected from E18 fetal Sprague Dawley rats after euthanasia of the dam by a protocol approved by University of Toledo College of Medicine IACUC. Primary culture of cortical neurons was performed according to a procedure modified from standard techniques (Banker and Cowan, 1997; Ransom et al., 1977; Porter et al. 1997). Dissected cortices were transferred to 0.25% Trypsin-ethylene diaminetetraacetic acid (EDTA) for 10 min at 37 °C, washed three times with Spinner's modification of Eagle's minimum essential medium (SMEM), and then dispersed by repeated trituration. The cell suspension was diluted with

SMEM plus fetal bovine serum (5%) and horse serum (5%) to a final concentration of  $3\text{--}5 \times 10^5$  cells/ml and plated onto a poly-D-lysine coated 35 mm plastic culture dishes. To inhibit proliferation of non-neuronal cells at 3 days *in vitro* (DIV), half of the medium was replaced with SMEM with horse serum (SMEM/HS) containing 5-fluoro-2'-deoxyuridine (FUdR) and uridine. Half of the medium was exchanged for fresh SMEM/HS at least twice a week (Porter et al., 1997). Cells were cultured for 13–15 DIV before use in the experiments below.

## 2.2 Hypoxia and reoxygenation

Neurons were examined microscopically to confirm viability before starting the experiment. Deoxygenated culture medium (SMEM/HS) was prepared by bubbling for 10 min with 95% N<sub>2</sub>/5% CO<sub>2</sub> at 37°C. SMEM/HS was replaced with deoxygenated SMEM/HS and then placed in an O<sub>2</sub>- and CO<sub>2</sub>-controlled incubator (Innova CO-48, New Brunswick Scientific Co. Inc.) pre-equilibrated to 1% O<sub>2</sub>, 5% CO<sub>2</sub> at 37 °C for 2, 4 or 8 h. After hypoxic exposure, the medium was replaced with fresh aerated SMEM/HS and neurons were either studied immediately or returned to the normoxic incubator (95% air/5% CO<sub>2</sub>, 37°C) for 24 h or 48 h prior to recording. For the “0 h” recovery time point, neurons were recorded within 1–2 h of termination of hypoxia; for 24 and 48 h recovery times, recordings occurred within  $\pm 2$  h of the stated time. For L-VGCC antagonist experiments, SMEM/HS was replaced with deoxygenated SMEM/HS containing 3  $\mu$ M nitrendipine and kept in the hypoxic incubator (1% O<sub>2</sub>, 5% CO<sub>2</sub> at 37 °C) for 4 h. After hypoxic exposure, the medium was replaced with fresh aerated SMEM/HS without nitrendipine and cells were returned to the normoxic incubator. Control experiments were similarly handled but maintained in a normoxic environment and solutions at all times.

## 2.3 Electrophysiology

**2.3.1. Whole-cell patch-clamp recording**—Whole-cell recordings were obtained using standard patch-clamp technique (Hamill et al., 1981) with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Cortical neurons were taken out from the incubator and the medium was replaced with external recording solution containing (in mM): 142 NaCl, 10 CaCl<sub>2</sub>, 6 MgCl<sub>2</sub>, 8.1 KCl, 10 glucose, 10 HEPES, 315–325 mOsm, pH 7.4. Patch-clamp electrodes of 2.5–5.0 M $\Omega$  were filled with internal solution containing (in mM): 153.3 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, 5 EGTA, 4 MgATP, 305–312 mOsm, PH 7.3. Signals were digitized on line at 1000Hz using a Digidata 1200A Data Acquisition System (Axon instruments, Foster City, CA) running Clampex 9.2 software and subsequently analyzed off-line using Clampfit 9.2 (pClamp 9.2, Axon Instruments). Patch-clamp electrodes were pulled from Fisher Microhematocrit capillary tubes (Fisher Scientific) using a P-97 Flaming-Brown micropipette puller (Sutter Instrument Co.). For whole cell recordings, membrane potential was held at  $-70$  mV. GABA and other drugs were sequentially applied in increasing concentrations using a pressurized drug application system to facilitate flow through 100  $\mu$ M applicator tips. Peak GABA<sub>A</sub>R currents elicited by increasing GABA concentrations were fitted to a sigmoidal function using a four parameter logistic equation (sigmoidal *C-R*) with a variable slope:  $I = (I_{\text{max}})/(1 + 10^{(\log(\text{EC}_{50} - [\text{drug}]) \times \text{Hill slope}))}$ , where *I* is the peak current at a given GABA concentration, and *I*<sub>max</sub> is the maximal GABA<sub>A</sub>R current. Curve fitting was performed using Prism 4.0 software (Graph Pad Software Inc., San Diego, CA). Maximal current and *C-R* curve fits were obtained from individual neurons, then averaged and compared by ANOVA with post hoc *t*-tests using Bonferroni's correction for multiple comparisons. Cell capacitance was measured shortly after obtaining whole-cell configuration using the “Membrane Test” function of Clampex 9.2. The percent enhancement or inhibition produced by co-application of a modulator was determined by dividing the peak amplitude in the presence of modulator by the average of control currents elicited by GABA alone before and after co-application and multiplying by 100. Neurons with significant rundown (>20% over 10 min) were excluded from analysis.

**2.3.2. Gramicidin perforated-patch recording**—Gramicidin perforated-patch recordings were performed as previously reported (Gao et al., 2005) using similar methods as whole-cell recordings, except the plasma membrane was not mechanically ruptured after gigaseal formation. Gramicidin was dissolved in DMSO (10 mg/ml) and then diluted in the internal solution to a final concentration of 10  $\mu\text{g/ml}$ . The electrode tip was filled with a small amount of gramicidin-free internal solution to avoid problems with seal formation. After seal formation, the progress of gramicidin  $\text{Cl}^-$  impermeable pore formation was evaluated by monitoring the gradual reduction in membrane resistance. GABA (10  $\mu\text{M}$ ) was applied to the cell after the membrane resistance had stabilized to 300–400  $\text{M}\Omega$  which usually took about 12–20 min. The reversal potential for GABA currents ( $E_{\text{Cl}}$ ) was determined by varying holding potential of neurons from  $-90$  mV to  $+50$  mV in 20 mV increments and measuring the resulting peak GABA-evoked currents. Linear regression was used to calculate a best-fit line for the voltage dependence of GABA currents, and the interpolated intercept of this line with the abscissa was taken as the  $E_{\text{Cl}}$  value. The slope of this line was used as the corresponding slope conductance. Perforated-patch recordings were terminated by increased suction on the micropipette or when the patch spontaneously ruptured into whole-cell configuration as evidenced by large depolarizing currents due to  $E_{\text{Cl}}$  of 0 mV.

### 3. Results

#### 3.1. Effects of duration of hypoxia on GABA<sub>A</sub>R currents

After 13–15 DIV, healthy cortical neurons developed long axons and dendrites. No overt changes in cortical neuron morphology were observed immediately after exposure to 1%  $\text{O}_2$  for 2h, 4h or 8h of hypoxia or after 24h or 48h recovery under normoxic conditions (95% air/5%  $\text{CO}_2$ , 37°C). This was consistent with our previous studies demonstrating that 8 h hypoxic exposure on NT2-N neuronal cells did not result in significant death or injury by trypan blue staining and lactate dehydrogenase (LDH) assay. There was no difference in initial membrane potential or membrane capacitance ( $C_m$ ) between control cells and cells recorded after hypoxic exposure with recovery durations up to 48 h (Table 1). Since membrane capacitance is proportional to cell membrane surface area, hypoxia did not appear to cause somatic swelling or dendrite retraction, features commonly associated with neuronal injury.

GABA-activated currents in cortical neurons were obtained by whole-cell patch-clamp recordings voltage clamped at  $-70$ mV. Brief (5 s) applications of increasing GABA concentrations (1  $\mu\text{M}$ –1 mM) evoked concentration dependent inward currents from both control and hypoxia-treated cortical neurons (Fig. 1). Peak currents values were then fitted to a sigmoidal logistic function (Fig 2A, 2C, 2E) which showed no significant change in  $EC_{50}$ . Maximal GABA-evoked currents after 2 h hypoxia were decreased after 0 h and 24 h recovery (Table 2,  $p < 0.05$  at 24 h, t test with Bonferroni correction after one way ANOVA) but trended toward an increase after 48 h recovery (Table 2,  $p > 0.05$ ). After 4 h hypoxia, maximal GABA current decreased after hypoxia; these reductions were significant the 0 h and 48 h recovery times at (Table 2,  $p < 0.05$ , t test with Bonferroni correction after one way ANOVA). Hypoxic exposure for 8 h caused non-significant trends toward reduction in GABA currents at each recovery time (Table 2,  $p > 0.05$ ). These changes in maximal current were not due to altered cell size, as there was no significant difference in membrane capacitance between time points (Table 1). Current density, defined as maximal GABA<sub>A</sub>R current normalized to membrane capacitance, was significantly reduced immediately after 4 h hypoxia and after 48 h recovery (Table 3, Fig 2C). Current density did not change at any recovery time after 2 h (Fig. 2B, Table 3,  $p > 0.05$ , t test with Bonferroni correction after ANOVA) or 8 h hypoxic exposures (Fig 2F, Table 3,  $p > 0.05$ ). Since both maximal currents and current density were significantly affected after 4 h hypoxia, with no change in the  $EC_{50}$  for GABA activation, this suggests either a reduction in the density of functional GABA<sub>A</sub>R channels per unit membrane, or a decrease in

the conductance or kinetics of individual channels. A change in the driving force for chloride is also possible (see below), but not relevant to these recordings, as equal chloride concentrations were used in the extracellular and intracellular pipette solutions, creating a reversal potential of 0 mV and minimizing problems related to large ion gradients. Since the most consistent changes in GABA<sub>A</sub>R current occurred after 4 h of exposure to 1% O<sub>2</sub>, we investigated whether this hypoxic exposure also affected GABA<sub>A</sub>R modulator pharmacology, which might suggest altered GABA<sub>A</sub>R subunit composition. In the following experiments, 4 h exposure to 1% O<sub>2</sub> was used as the standard hypoxia treatment.

### 3.2. Effects of 4 h hypoxia on GABA<sub>A</sub>R pharmacology

To investigate whether there was a change in GABA<sub>A</sub>R pharmacology after 4h hypoxia, individual cortical neurons were recorded at multiple recovery times after sham or 4 h hypoxic exposure, and the effects of subunit-specific modulators to enhance or inhibit GABA elicited currents was assessed by co-application of each modulator with GABA compared to currents elicited by GABA alone. The positive modulators diazepam, zolpidem and pentobarbital and the negative allosteric modulator, Zn<sup>2+</sup> were chosen on the basis of their association with specific subunits for their binding and efficacy.

**3.2.1 Diazepam**—The benzodiazepine full agonist, diazepam, potentiated GABA currents in a concentration-dependent manner (Fig. 5A). Diazepam concentration-response curves were derived from coapplication of increasing concentrations of diazepam with 3 μM GABA. After 4 h hypoxia, there was an initial significant increase in maximal diazepam potentiation compared to control cells ( $p < 0.01$ , see Table 4), with no significant difference in EC<sub>50</sub> ( $p > 0.05$  by one way ANOVA, see Table 5). This increase returned to control values after 24 h and 48 h recovery (Table 4).

**3.2.2 Zolpidem**—Zolpidem, a benzodiazepine site agonist with high potency at α1-containing GABA<sub>A</sub>Rs and minimal efficacy at α5-containing receptors, produced concentration-dependent enhancement of GABA<sub>A</sub>R current in cortical neurons (Fig 5B, Tables 4 and 5). Zolpidem concentration-response curves were derived from coapplication of increasing concentrations of zolpidem with 3 μM GABA. After 4 h hypoxia, maximal enhancement by zolpidem tended to be increased (Table 4) with no significant difference in EC<sub>50</sub> (Table 5,  $p > 0.05$  by one way ANOVA). The increase in maximal zolpidem potentiation was significant after 48 h normoxic recovery ( $p < 0.05$  by Bonferonni correction after one way ANOVA).

**3.2.3 Pentobarbital**—Pentobarbital potentiates GABA currents with no significant GABA<sub>A</sub>R subtype selectivity. Pentobarbital enhanced 3 μM GABA currents in a concentration-dependent manner (Fig 5C, Tables 4 and 5). There was no significant difference in maximal enhancement or EC<sub>50</sub> for enhancement between control neurons and neurons at different recovery times after hypoxic treatment.

**3.2.4 Zinc**—The divalent cation, Zn<sup>2+</sup>, negatively modulates GABA<sub>A</sub>R currents with higher potency at receptors containing an α4, α5 or α6 subtype and lacking the α1 subunit or γ subunits (Smart et al., 1991). Zn<sup>2+</sup> reduced whole-cell 10 μM GABA currents in a concentration-dependent manner (Fig 5D, Tables 4 and 5). Maximal inhibition and IC<sub>50</sub> for Zn<sup>2+</sup> inhibition of GABA currents were not significantly changed in post-hypoxic neurons at any of the recovery time points (Tables 4 and 5,  $p > 0.05$  by Bonferonni correction after one way ANOVA).

### 3.3 Effects of 4 h hypoxia on GABA current reversal potential

To examine whether hypoxia affected the Cl<sup>-</sup> reversal potential, we performed whole-cell gramicidin perforated-patch recording, which does not disturb intracellular Cl<sup>-</sup> concentration

( $[Cl^-]_i$ ). The antibiotic gramicidin forms membrane pores impermeable to  $Cl^-$ , but permeable to monovalent cations and small uncharged molecules and maintains  $[Cl^-]_i$  stable for times exceeding 60 min (Kyrozis and Reichling, 1995). I–V curves were plotted by peak currents obtained during application of 10  $\mu$ M GABA at multiple holding potentials (Fig. 4A). A sample experiment is shown in Fig. 4B, with an individual neuron studied 24 h after 4 h hypoxia, initially recorded in perforated-patch mode and again after rupture of the patch to go into whole-cell recording mode. With rupture of the perforated patch, the slope conductance increased due to reduction in access resistance and the  $Cl^-$  reversal potential shifted to  $\sim 0$  mV, due to the equivalent chloride concentrations between external and internal pipette solutions. With cells recorded after hypoxia in perforated-patch mode to preserve intracellular chloride gradients, there was a depolarizing (positive) shift in the  $Cl^-$  reversal potential after 4 h hypoxia relative to controls (Fig 4C) that was significant at the 24 h recovery point (Control:  $-59.5 \pm 3.2$  mV,  $n = 11$ ; H4,R24:  $-51.0 \pm 3.1$  mV,  $n = 9$ ,  $p < 0.05$  by Bonferonni correction after one way ANOVA). The chloride reversal potential returned toward control values after 48 h recovery (H4,R48:  $-54.3 \pm 2.5$  mV,  $n = 10$ ,  $p > 0.05$ ). Slope conductance at each recovery time point was not significantly different from control (Control :  $2.27 \pm 0.35$  pA/mV,  $n = 11$ ; 0 h:  $2.58 \pm 0.38$  pA/mV,  $n = 10$ ; 24 h:  $2.46 \pm 0.45$  pA/mV,  $n = 9$ ; 48 h:  $2.43 \pm 0.46$  pA/mV,  $n = 10$ ,  $p > 0.05$  by t test with Bonferonni correction after one way ANOVA, Fig. 6D). Hence, there was no evidence for a reduction in  $GABA_A$ R conductance or rectification. A depolarizing shift in  $Cl^-$  reversal potential could result in smaller outward GABA currents or even inward currents; however, it does not account for the change in GABA current amplitudes observed here under whole-cell recording conditions in which equivalent transmembrane  $Cl^-$  concentrations were used.

### 3.4 Prevention of hypoxic effects on GABA currents by nitrendipine

Hypoxia can regulate cell function through a variety of signaling pathways, including the elevation of intracellular calcium via several possible mechanisms. To determine whether the effects of hypoxia on cortical neuron GABA currents was due to calcium entry through L-type voltage-gated calcium channels (L-VGCCs), we co-incubated cortical neuron cultures in the L-VGCC blocker, nitrendipine (NT, 3  $\mu$ M), during 4 h control or hypoxic exposure, with subsequent washout and replacement with aerated SMEM/HS culture medium. Since several L-VGCC antagonists can inhibit  $GABA_A$ R currents, NT was chosen due to its lack of interaction with  $GABA_A$ R at the concentration used (Das et al., 2004). Whole-cell and perforated-patch recordings were performed at the same recovery time points.

**3.4.1 Maximal GABA current reduction blocked by nitrendipine—**Whole-cell currents elicited by GABA after incubation in NT (3  $\mu$ M, 4 h) were significantly larger than control currents from neurons not exposed to NT (Fig. 5A, B,  $p < 0.05$  by t test with Bonferonni correction after one way ANOVA). The  $EC_{50}$  value and Hill slope were unchanged from control. In cells recorded 0 h, 24 h or 48 h after 4 h hypoxia in the presence of NT (10  $\mu$ M), there was no significant change in maximal current,  $EC_{50}$  or Hill slope from control cells (exposed to NT), and maximal current values at each post-hypoxic time point were significantly larger than those recorded at these time points after 4 h hypoxia in the absence of NT (Table 2, Fig 5A, B). When normalized to cell capacitance, control + NT maximal current density was significantly increased from control without NT (Table 3, Fig. 5A, B) and at each post-hypoxic time point, suggesting an increased number of functional channels on the surface membrane. NT thus prevented the hypoxia-induced reduction in cell surface  $GABA_A$ Rs at the 0 h and 48 h recovery time points. There was no significant difference in membrane capacitance between neurons exposed or not exposed to NT (Table 1), hence exposure did not appear to alter cell size. These results suggest that L-VGCC activation may be required for hypoxia-induced reduction of  $GABA_A$ R currents.

### 3.4.2 Nitrendipine prevented the hypoxia-induced change in BZ pharmacology

—Since nitrendipine prevented the hypoxia-induced reduction in maximal GABA current, it was important to determine whether it could also affect the change in BZ pharmacology after hypoxia. Nitrendipine (3  $\mu$ M) during hypoxic exposure prevented the increase in zolpidem enhancement of GABA currents after 48h recovery (Fig. 6A). Interestingly, the potentiation of GABA current by zolpidem was significantly reduced at 48 h recovery after 4h hypoxia in the presence of NT (control+NT:  $269.4 \pm 18.7\%$ ; H4+NT,R48:  $194.0 \pm 16.3\%$ ;  $P < 0.05$ , student T-test; Fig 6B), in contrast to the increase observed at this time point after hypoxia in the absence of NT, suggesting that L-VGCC activation is involved in determining post-hypoxic BZ sensitivity.

### 3.4.3 Nitrendipine prevented the hypoxia-induced change in chloride reversal potential

—We next explored whether NT exposure during hypoxia would affect the hypoxia-induced depolarizing shift in the chloride reversal potential. Gramicidin perforated-patch recordings were performed as before on cortical neurons under control conditions and at the 24 and 48 h recovery time points after 4 h hypoxia in the presence of NT (3  $\mu$ M). The chloride reversal potential in control neurons exposed to NT ( $-50.41 \pm 3.59$  mV,  $n = 7$ ) was less negative than control neurons not exposed to NT ( $-59.5 \pm 3.1$  mV,  $n = 11$ ) but this difference was not significant ( $p > 0.05$ , students t-test). The chloride reversal potential at 24 h and 48 h recovery after 4 h hypoxia in the presence of NT did not change compared with that at normoxia (H4 +NT,R24:  $-52.93 \pm 4.36$  mV; H4+NT,R48:  $-51.77 \pm 4.17$  mV,  $p > 0.05$ , t test with Bonferroni correction after one way ANOVA). Thus, NT also prevented the depolarizing shift in chloride reversal potential after hypoxia, suggesting that L-VGCC-dependent processes are involved in the post-hypoxic regulation of chloride homeostasis in cortical neurons.

## 4. Discussion

As in our previous studies using NT2-N neuronal cells (Gao et al., 2004), GABA<sub>A</sub>R currents in cortical neurons were significantly affected by transient exposure to hypoxia. However, GABA<sub>A</sub>R currents in rat primary cortical neurons in culture had variable responses to hypoxia. After 2 h exposure to 1% O<sub>2</sub>, maximal GABA-evoked currents tended to be smaller immediately after hypoxia, were significantly smaller 24 h afterwards, and tended to be larger than control after 48 h recovery. After 4 h hypoxic exposure, currents were significantly smaller at 0 and 48 h after hypoxia and non-significantly reduced 24 h after hypoxia. After 8 h hypoxia, currents were non-significantly reduced at all 3 post-hypoxic time points. These results differed from our prior studies in NT2-N neuronal cells, in which cells had a biphasic response after 8 h exposure to 1% O<sub>2</sub>. NT2-N cell GABA currents were increased immediately after hypoxia, returned toward control after 24 h recovery, were decreased (60% of control) after 48 h recovery, and returned to baseline at 96 h recovery (Gao et al., 2004). The key differences are that cortical neuron GABA currents did not increase at any post-hypoxic time-point after any of the 3 hypoxic durations. Moreover, the most significant reductions in maximal GABA current in cortical neurons were observed after 4 h rather than 8 h hypoxia.

Such contrasting effects of hypoxia on GABA-evoked currents might be due to differences in GABA<sub>A</sub>R subunit composition between NT2-N cells and cortical neurons, and to the variability of neuronal phenotypes in cortical cultures. NT2-N neuronal cells are derived from a human teratocarcinoma, and consistently express a subset of GABA<sub>A</sub>R subunit mRNAs at different stages of *in vitro* development (Neelands et al., 1999). These cells can be used as a model for GABA<sub>A</sub>R regulation due to the relative homogeneity of their responses to GABA and GABA<sub>A</sub>R modulators (Neelands et al., 1998). Although NT2-N cells are quite 'neuronal' in many respects, the present results suggest that the details of GABA<sub>A</sub>R regulation, function and subunit expression may differ in actual CNS neurons. Indeed, the present findings in

cortical neurons in culture might also differ from neurons within the intact brain and in different brain regions.

Since the most consistent changes in maximal GABA-evoked current occurred after 4 h hypoxic exposure, this treatment was selected for further examination of post-hypoxic changes in GABA<sub>A</sub>R function, focusing on subunit-dependent pharmacology. Hypoxia (4 h at 1 % O<sub>2</sub>) increased diazepam efficacy for enhancing GABA-evoked currents immediately after hypoxic exposure and non-significantly elevated enhancement at later post-hypoxic time points. In contrast, GABA<sub>A</sub>R current enhancement by the  $\alpha$ 1 subunit-selective imidazopyridine, zolpidem, was non-significantly increased at the 0 and 24 h time points but significantly increased after 48 h recovery. No changes in barbiturate or Zn<sup>2+</sup> sensitivity were detected at any post-hypoxic time point.

In NT2-N neuronal cells, similar changes in BZ pharmacology were noted, associated with altered subunit mRNA expression (Gao et al., 2004). Maximal potentiation of GABA currents by diazepam was increased 48 h after (8 h) hypoxia, while zolpidem enhancement was decreased, and responses to pentobarbital and zinc were unchanged. Semiquantitative reverse transcriptase (RT)-PCR showed decreased  $\alpha$ 1,  $\alpha$ 5,  $\beta$ 2 and  $\gamma$ 2 subunit mRNA after hypoxia, correlating with decreased maximal currents. Such changes may be clinically relevant, as mRNA expression of the  $\alpha$ 1 and  $\beta$ 2 subunits decreased within 30 minutes after reperfusion in hippocampal areas CA1, CA3 and dentate gyrus in an *in vivo* stroke model (Li et al., 1993). Moreover, in a rat stroke model, cortical reductions in the  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 5 and  $\gamma$ 2 subunits were observed 1, 7 and 30 days after infarct (Redecker et al., 2002).

In cortical neurons, the early change in diazepam sensitivity and the late change in zolpidem sensitivity, both specific to BZ binding site, might have resulted from incorporation of different  $\alpha$  subunits into the holoreceptor. The increase in diazepam efficacy could be related to incorporation of BZ-sensitive subunits ( $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 or  $\alpha$ 5) in place of BZ-insensitive subunits ( $\alpha$ 4 or  $\alpha$ 6), loss of “ $\gamma$ -less” receptors, or the preferential loss of BZ-insensitive  $\alpha$  subunits during downregulation of GABA<sub>A</sub>R current. The isolated increase in diazepam enhancement immediately after hypoxia, with no change in zolpidem enhancement, could be produced by an increased proportion of  $\alpha$ 5-containing receptors that respond to diazepam but not zolpidem. The later increase in zolpidem efficacy could have been produced by an increased proportion of GABA<sub>A</sub>Rs containing  $\alpha$ 1,  $\alpha$ 2 or  $\alpha$ 3, since zolpidem only binds to receptors containing these subunits. Further, the lack of increase in zolpidem potency argues against an increase in  $\alpha$ 1-containing receptors, since zolpidem has higher affinity at those sites. Hence, if subunit changes are involved, the early post-hypoxic period would require an increased proportion of  $\alpha$ 5-containing GABA<sub>A</sub>Rs, while the late changes would need an increase in  $\alpha$ 2 or  $\alpha$ 3. Such changes are likely to be quite different from those associated with BZ tolerance after chronic exposure, which results in decreased BZ sensitivity associated with a variety of subunit changes in different model systems (e.g. (Bateson, 2002; Chen et al., 1999; Primus et al., 1996)). Future examination of specific  $\alpha$  subunit expression patterns on the neuronal surface by immunocytochemistry could help determine whether such subunit changes occur.

The mechanism underlying the observed changes in GABA<sub>A</sub>R current and pharmacology might also involve regulation by protein kinases and/or phosphatases, particularly the Ca<sup>2+</sup>/calmodulin-dependent protein phosphatase 2B, calcineurin (CaN), as previously demonstrated in a perinatal model of hypoxia-induced seizures (Sanchez et al., 2005). In hypoxia-treated neonatal rats, a decrease in GABAergic inhibition in CA1 pyramidal neurons resulted from AMPA-receptor mediated activation of CaN, which dephosphorylated the GABA<sub>A</sub>R  $\beta$ 2 or  $\beta$ 3 subunit (Sanchez et al., 2005). CaN regulation of GABA<sub>A</sub>R function is important in mediating long-term depression (LTD), a decrease in GABA-mediated IPSP amplitude induced by an NMDA receptor-mediated mechanism similar to that underlying long term



potentiation of AMPA receptor synapses (Lu et al., 2000) and involves a direct interaction between CaN and the  $\gamma 2$  subunit (Wang et al., 2003). In our cortical culture model, calcium entry was unlikely to be mediated by AMPA or NMDA receptor mechanisms as synaptic connectivity in culture is typically sparse and ambient glutamate and glycine levels are low. Rather, hypoxia might induce membrane depolarization (possibly due to lowered ATP levels and decreased  $\text{Na}^+/\text{K}^+$  ATPase activity), which could lead to  $\text{Ca}^{2+}$  influx through VGCCs, particularly L-VGCCs which are slow to desensitize. An increase in intracellular  $\text{Ca}^{2+}$  could activate CaN, possibly resulting in the decrease in GABA current. We therefore sought to determine whether antagonism of L-VGCCs would block some or all of the effects of hypoxia on  $\text{GABA}_{\text{A}}\text{R}$  function.

L-VGCC are highly expressed in the rat cerebral cortex and hippocampus with high density on neuronal cell bodies (Ricci et al., 2002). Blockade of L-VGCC during hypoxia prevented the reduction of GABA current at all time points after 4h hypoxia (Fig. 5), suggesting that L-VGCC are activated during hypoxia and that the reductions in  $\text{GABA}_{\text{A}}\text{R}$  current may be  $\text{Ca}^{2+}$ -mediated. In addition, the maximal GABA current in control cells significantly increased after 4 h exposure to nitrendipine, indicating that  $\text{Ca}^{2+}$ -dependent downregulation of GABA currents likely occurs even under non-hypoxic conditions and may participate in setting the “basal tone” of  $\text{GABA}_{\text{A}}\text{R}$  inhibition by determining the number of functional cell surface receptors. Indeed, blockade of L-type voltage-gated  $\text{Ca}^{2+}$  channels by nifedipine has been shown to inhibit down-regulation of GABA<sub>A</sub>R number induced by 48 h exposure to 1 mM GABA (Lyons et al., 2001). VGCC regulation of  $\text{GABA}_{\text{A}}\text{R}$  surface expression may be a mechanism common to a variety of stimuli, as the decrease in IPSC  $\text{GABA}_{\text{A}}\text{R}$  conductance after chronic BZ treatment, likely induced by  $\text{GABA}_{\text{A}}\text{R}$ -mediated depolarization and resulting VGCC activation, was reversed by systemic nimodipine injection (Xiang et al., 2007b).

In addition to preventing the hypoxia-induced decrease in  $\text{GABA}_{\text{A}}\text{R}$  current, nitrendipine not only prevented the upregulation of zolpidem enhancement of GABA current after 48 h recovery, but actually reduced zolpidem potentiation at that recovery time point. Whether this was due to prevention of a change in  $\text{GABA}_{\text{A}}\text{R}$  subunit composition (e.g. a hypoxia/ $\text{Ca}^{2+}$ -induced decrease in  $\alpha 5$ -containing receptors) or a change in calcium-dependent phosphorylation is unclear. In earlier studies in NT2-N neuronal cells, we found that activation of protein kinase C (PKC) caused a rightward shift in the diazepam concentration-response curve without affecting maximal enhancement (Gao et al., 2005), hence PKC is unlikely to be directly involved. Other phosphorylation or dephosphorylation mechanisms are possible (Kittler et al., 2003), but few studies have investigated the effects of such post-translational regulation on BZ allosteric properties.

Finally, we used gramicidin perforated-patch recording to determine whether hypoxia altered the reversal potential for chloride in cortical neurons. We found a modest depolarizing shift of the chloride reversal potential which was significant 48 h after hypoxic exposure. Co-incubation with nitrendipine (3  $\mu\text{M}$ ) during hypoxia caused a non-significant depolarizing trend in the chloride reversal potential in control neurons and prevented the depolarizing shift after hypoxia. Hypoxia/ischemia has been shown to increase intracellular chloride concentration in adult rat CA1 hippocampal neurons (Comerford et al., 2003; Inglefield and Schwartz-Bloom, 1998; Katchman et al., 1994; Mittmann et al., 1998) though the mechanism remains controversial. Changes in  $\text{Cl}^-$  reversal potential in CA1 neurons were also seen after chronic BZ treatment, accompanied by  $\text{GABA}_{\text{A}}\text{R}$ -mediated depolarization (Zeng et al., 1997). In our previous experiments, the resting membrane potential in cortical neurons at 13–15 days *in vitro* was around  $-65$  mV (data not shown), hence the shift in chloride reversal potential from  $-59.5 \pm 3.2$  mV in control cells to  $-51.0 \pm 3.1$  mV recorded 24 h after hypoxia implies that  $\text{GABA}_{\text{A}}\text{R}$  currents become significantly depolarizing after hypoxia and could increase neuronal excitability. Indeed, GABA currents could facilitate L-VGCC-mediated calcium

currents as suggested by the findings of Tiez et al. after chronic BZ treatment (Xiang et al., 2007a). Downregulation of GABA<sub>A</sub>R function by a calcium-dependent process might thus be protective against excessive depolarization associated with seizures and myoclonus, though the mechanisms underlying these changes require further investigation.

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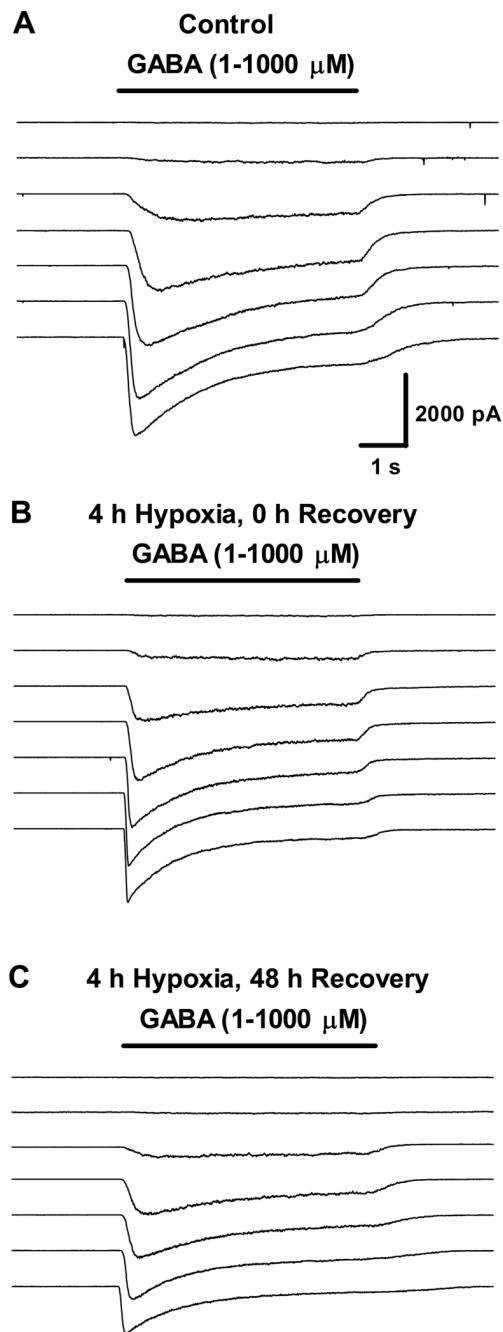
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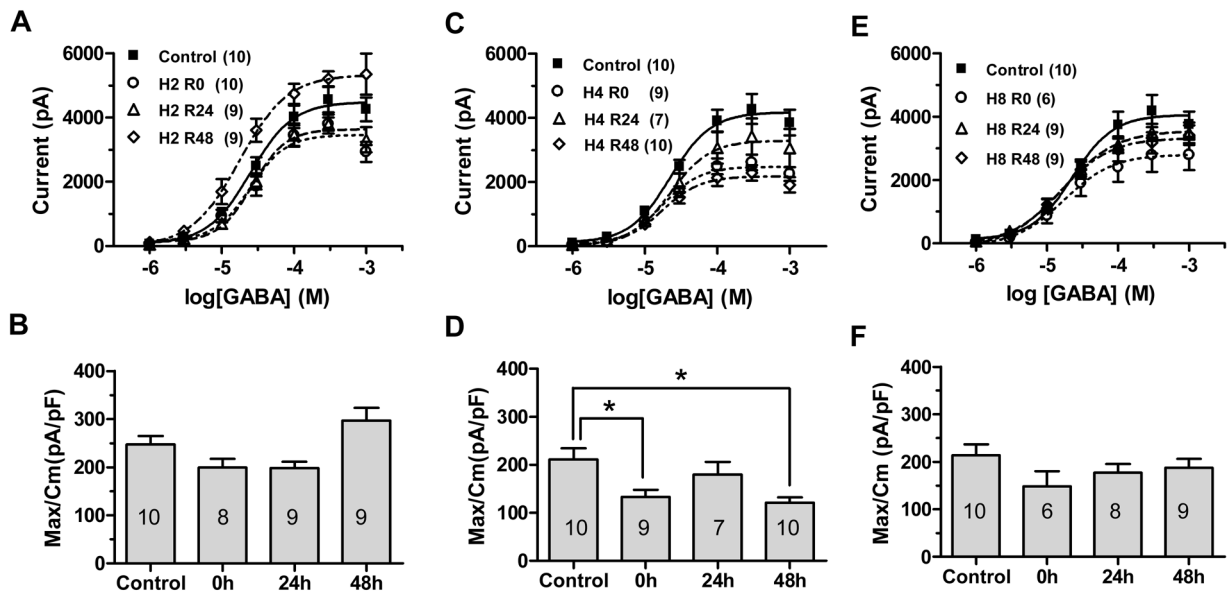
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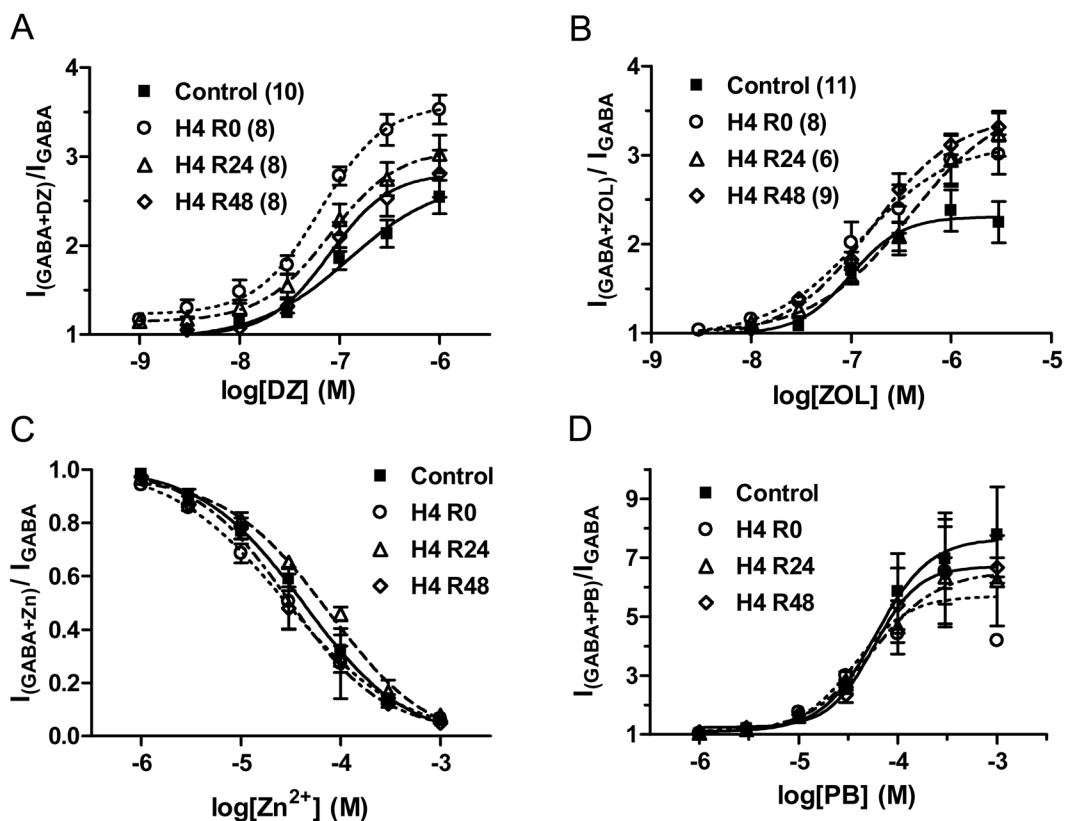


**Figure 1.** GABA<sub>A</sub>R current traces after 4 h hypoxia. Whole-cell GABA<sub>A</sub>R currents evoked by increasing concentrations of GABA (1  $\mu$ M–1 mM) in a control neuron (A); immediately following 4 h hypoxia (B); 48 h recovery after 4 h hypoxia (C). Current amplitude and time scale as shown are the same for all traces.



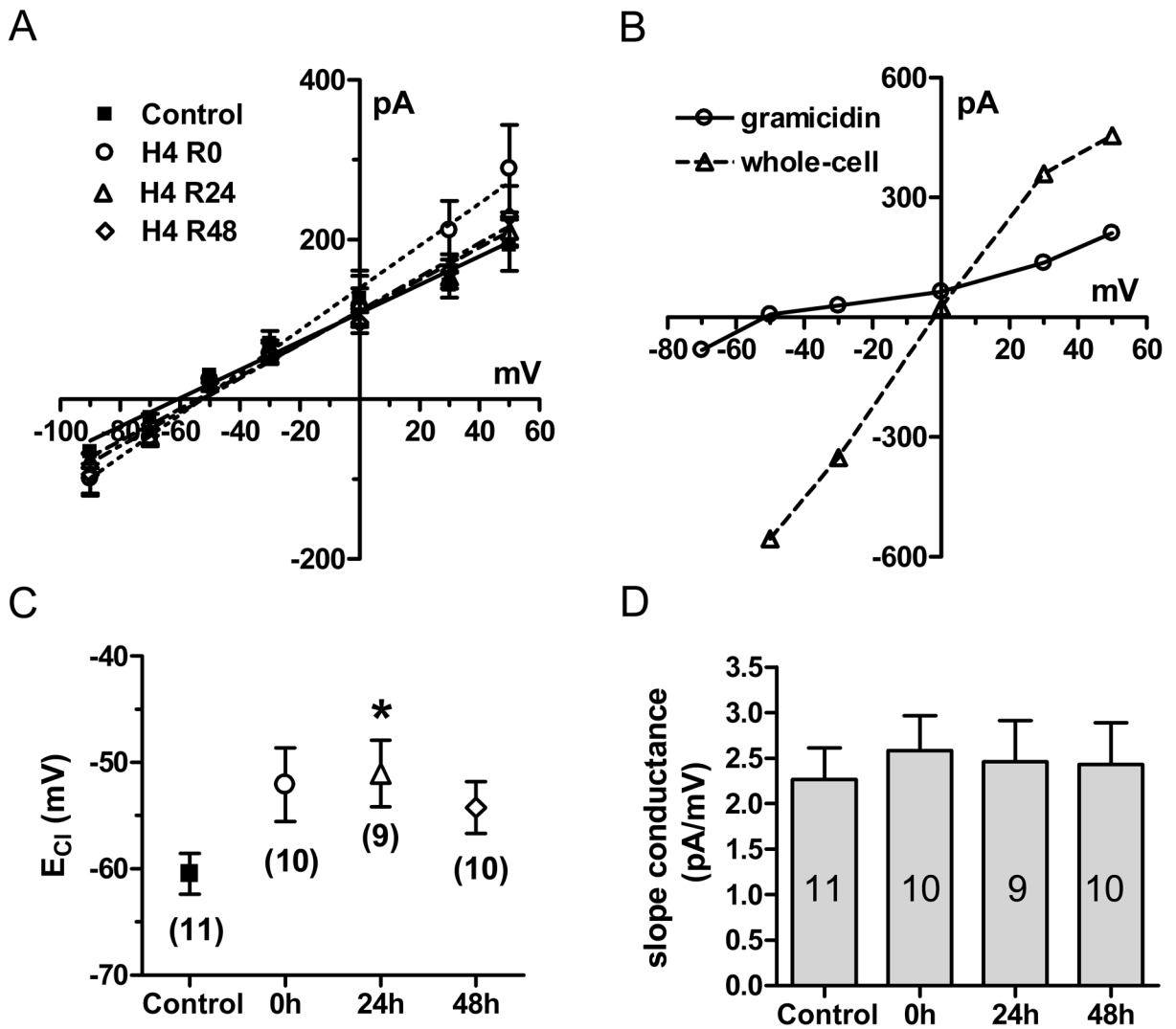
**Figure 2.**

GABA<sub>A</sub>R current after 2 h, 4 h or 8 h hypoxia. Peak currents elicited by increasing GABA concentrations in control cells (filled squares) and immediately (0 h, open circles), 24 h (open triangles) and 48 h (open diamonds) after 2 h hypoxia (A), 4 h hypoxia (C), or 8 h hypoxia (E). Each symbol represents means of peak currents evoked at that GABA concentration. Maximal GABA currents normalized to cell capacitance for control cells and at each recovery time points after 2 h hypoxia (B), 4 h hypoxia (D) or 8 h hypoxia (F). Bars represent mean  $\pm$  SEM; the n for each condition is shown in the appropriate bar. There was no significant difference in current density between control and 2h or 8h hypoxia-treated cells at any recovery time after hypoxia ( $p > 0.05$ , one way ANOVA). Current density was significantly reduced at 0 h and 48 h recovery after 4 h hypoxia (D). Asterisks show columns significantly different from each other as marked (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control by one way ANOVA).



**Figure 3.**

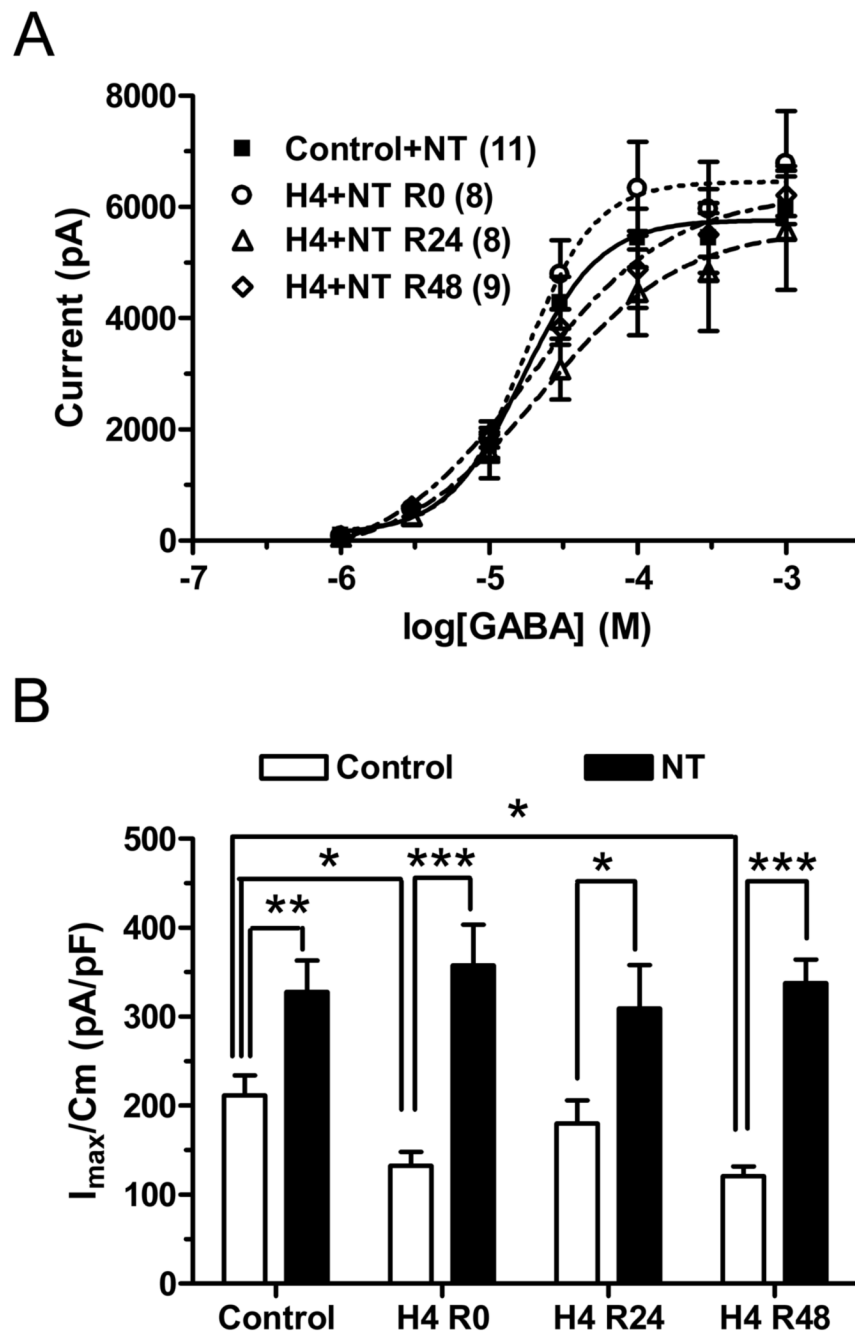
Modulation of GABA currents after 4 h hypoxia. (A) Enhancement of 3  $\mu$ M GABA currents by increasing concentrations of diazepam (DZ) in control cells (filled squares, n=10), 0 h (open circles, n=8), 24 h (open triangles, n=8) and 48 h (open diamonds, n=8) after 4 h hypoxia, normalized to control current amplitude. A significant increase in maximal enhancement was observed at 0 h after hypoxia ( $p < 0.01$ ) compared to control, with no change in  $EC_{50}$ . (B) Enhancement of 3  $\mu$ M GABA currents by zolpidem (ZOL) in control cells (filled squares, n=10), 0 h (open circles, n=8), 24 h (open triangles, n=6) and 48 h (open diamonds, n=8) after 4 h hypoxia. The  $EC_{50}$  for enhancement was unchanged, but maximal enhancement was significantly increased at the 48 h recovery time point after hypoxia ( $p < 0.05$ ). (C) Enhancement of 3  $\mu$ M GABA currents by pentobarbital (PB) in control cells (filled squares, n=6), 0 h (open circles, n=2), 24 h (open triangles, n=6) and 48 h (open diamonds, n=6) after 4 h hypoxia. Maximal enhancement and  $EC_{50}$  were not significantly different ( $p > 0.05$ ). (D) Inhibition of 10  $\mu$ M GABA currents by increasing concentrations of  $Zn^{2+}$  in control cells (filled squares, n=6), 0 h (open circles, n=5), 24 h (open triangles, n=6) and 48 h (open diamonds, n=6) after 4 h hypoxia. No difference in  $Zn^{2+}$  inhibition were observed ( $p > 0.05$ ). (See Table 4, 5)



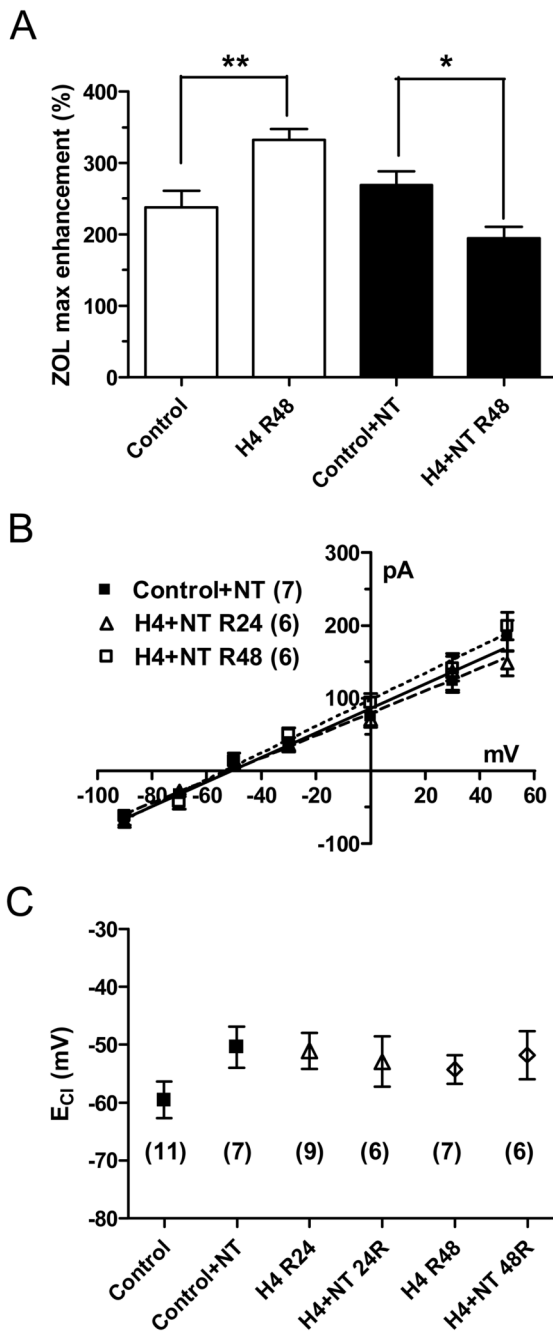
**Figure 4.**

Hypoxia (4 h) shifted the reversal potential of GABA-induced  $Cl^-$  currents ( $E_{Cl}$ ). (A) GABA current-voltage relationship in control conditions (filled squares), immediately following 4 h hypoxia (0 h, open circles), 24 h (open triangles) and 48 h (open diamonds) recovery after 4 h hypoxia. (B) Sample I-V relationship of GABA currents in gramicidin perforated-patch and whole-cell patch mode in a single cell recorded 24 h after 4 h hypoxia. (C) The changes in  $E_{Cl}$  were significantly different between control and 24 h recovery time point. Data represents mean  $\pm$  SEM; the n for each condition is shown in the appropriate symbol; Asterisks show columns significantly different from each other as marked (\* $p < 0.05$  compared to control by one way ANOVA with post-hoc t test). (D) There was no significant difference in slope conductance after 4 h hypoxic exposure compared with control ( $p > 0.05$ , one way ANOVA).





**Figure 5.** Nitrendipine prevented the decrease in GABA currents after 4h hypoxia. (A) Peak currents elicited by increasing GABA concentrations in control cells (filled squares) and immediately (0 h, open circles), 24 h (open triangles) and 48 h (open diamonds) after 4 h hypoxia in the presence of nitrendipine (NT, 3  $\mu$ M). Each symbol represents means of peak currents evoked at that GABA concentration. (B) Maximal GABA currents normalized to cell capacitance for control cells and at each recovery time point after 4 h hypoxia, with or without NT. There was no significant difference in current density between control and 4 h hypoxia-treated neurons in the presence of NT during hypoxia ( $p > 0.05$ , one way ANOVA).

**Figure 6.**

The effect of nitrendipine on zolpidem pharmacology and GABA reversal potential. (A) Enhancement of 3  $\mu$ M GABA currents by zolpidem (ZOL) significantly increased at 48 h recovery after 4 h hypoxia ( $p < 0.01$ , one way ANOVA). NT (3  $\mu$ M) during hypoxia not only prevented the increase in enhancement of GABA currents by ZOL, but significantly reduced ZOL enhancement at 48h recovery ( $p < 0.05$ , student t test). (B) GABA I–V relationship in control conditions (filled squares), and at 24 h (open triangles) and 48 h (open diamonds) recovery after 4 h hypoxia in the presence of NT. (C) Summary of changes in  $E_{Cl}$  in control conditions or hypoxia conditions with or without NT. In the presence of NT during hypoxia,

there was no difference in  $E_{Cl}$  between control and hypoxia-treated neurons. Data represents mean  $\pm$  SEM; the n for each condition is shown at the appropriate symbol.

**Table 1****Rat Cortical Neuron Membrane Capacitance after Hypoxia**Membrane capacitance (pF) at control and post-hypoxic recovery times after stated durations at 1% O<sub>2</sub>.

<b>Hypoxia Duration</b>	<b>Control Mean ± SEM.(n)</b>	<b>0 h Recovery Mean ± SEM.(n)</b>	<b>24 h Recovery Mean ± SEM.(n)</b>	<b>48 h Recovery Mean ± SEM.(n)</b>
2 h	19.5 ± 0.46 (9)	20.0 ± 0.93 (8)	19.3 ± 0.77 (9)	18.2 ± 1.3 (9)
4 h	20.3 ± 0.71 (10)	20.1 ± 0.75 (9)	19.1 ± 1.07 (7)	19.3 ± 0.71 (10)
8 h	19.9 ± 0.88 (10)	20.2 ± 1.06 (10)	20.2 ± 0.58 (8)	17.9 ± 0.84 (9)
4 h + NT	18.8 ± 0.67 (11)	19.1 ± 0.89 (8)	17.3 ± 0.75 (8)	18.5 ± 0.67 (9)

**Table 2****Maximal GABA<sub>A</sub>R Currents after Hypoxia and Recovery**

Maximal GABA-evoked peak currents at specified recovery times after 3 different hypoxic durations. NT= nitrendipine (3  $\mu$ M).

<b>Hypoxia Duration</b>	<b>Control (pA) Mean <math>\pm</math> SEM (n)</b>	<b>0 h Recovery (pA) Mean <math>\pm</math> SEM (n)</b>	<b>24 h Recovery (pA) Mean <math>\pm</math> SEM (n)</b>	<b>48 h Recovery (pA) Mean <math>\pm</math> SEM (n)</b>
2 h	4848 $\pm$ 376 (9)	3924 $\pm$ 281 (8)	3791 $\pm$ 208 (9) <sup>*</sup>	5303 $\pm$ 497 (9)
4 h	4289 $\pm$ 459 (10)	2813 $\pm$ 246 (9) <sup>*</sup>	3445 $\pm$ 547 (7)	2362 $\pm$ 239 (10) <sup>**</sup>
8 h	4290 $\pm$ 505 (10) <sup>*</sup>	2872 $\pm$ 502 (6)	3551 $\pm$ 310 (8)	3334 $\pm$ 338 (9)
4 h + NT	6341 $\pm$ 484 (11) <sup>*</sup>	6748 $\pm$ 820 (8) <sup>***</sup>	5409 $\pm$ 945 (8)	6212 $\pm$ 524 (9) <sup>***</sup>

\* p<0.05,

\*\* p<0.01,

\*\*\* p<0.001 compared to control, t test with Bonferroni correction after one-way ANOVA. For 4 h + NT, comparisons are made to 4 h control and post-hypoxic time points; post-hypoxic values did not differ from control in the 4h + NT group.

**Table 3****Maximal GABA<sub>A</sub>R Currents Normalized to Membrane Capacitance after Hypoxia and Recovery**

Maximal GABA-evoked currents normalized to membrane capacitance at specified recovery times after 3 different hypoxic durations.

<b>Hypoxia Duration</b>	<b>Control (pA/pF) Mean ± SEM (n)</b>	<b>0 h Recovery (pA/pF) Mean ± SEM (n)</b>	<b>24 h Recovery (pA/pF) Mean ± SEM (n)</b>	<b>48 h Recovery (pA/pF) Mean ± SEM (n)</b>
2 h	247.2 ± 17.3 (9)	199.5 ± 17.6 (8)	198.4 ± 12.7 (9)	296.9 ± 26.2 (9)
4 h	211.5 ± 22.5 (10)	132.7 ± 15.6 (9) <sup>*</sup>	180.0 ± 26.1 (7)	121.2 ± 10.7 (10) <sup>**</sup>
8 h	214.0 ± 22.7 (10)	148.4 ± 31.6 (6)	177.3 ± 18.0 (8)	187.4 ± 18.8 (9)
4 h + NT	341.1 ± 27.4 (11) <sup>**</sup>	357.5 ± 36.0 (8) <sup>***</sup>	309.5 ± 48.7 (8) <sup>*</sup>	337.8 ± 26.7 (9) <sup>***</sup>

<sup>\*</sup> p<0.05,

<sup>\*\*</sup> p<0.01 relative to control, t test with Bonferroni correction. after one way ANOVA.

**Table 4**  
**Maximal Enhancement or Inhibition of GABA Currents after 4 h Hypoxia Relative to Control**

	<b>Control (% of control)</b>	<b>0 h Recovery (% of control)</b>	<b>24 h Recovery (% of control)</b>	<b>48 h Recovery (% of control)</b>
	<b>Mean ± SEM (n)</b>	<b>Mean ± SEM (n)</b>	<b>Mean ± SEM (n)</b>	<b>Mean ± SEM (n)</b>
Diazepam	256 ± 16.6 (10)	358 ± 13.9 (8)**	303 ± 21.2 (8)	274 ± 20.1 (8)
Zolpidem	245 ± 21.7 (10)	317 ± 24.5 (8)	310 ± 25.3 (6)	334 ± 14.4 (8)*
Pentobarbital	777 ± 163 (6)	654 ± 177 (2)	635 ± 170 (5)	668 ± 33.1 (4)
Zinc	5.72 ± 1.39 (6)	6.58 ± 2.06 (5)	7.70 ± 1.77 (6)	4.90 ± 0.88 (6)

p<0.05,

\*\*  
p<0.01,

\*\*\*  
p<0.001 by Bonferroni correction after one way ANOVA

**Table 5**  
**EC<sub>50</sub> (or IC<sub>50</sub>) of Allosteric Agent Effects on GABA Currents After 4 h Hypoxia**

	<b>Control</b> EC <sub>50</sub> (μM) (95% CI, n)	<b>0 h Recovery EC<sub>50</sub></b> EC <sub>50</sub> (μM) (95% CI, n)	<b>24 h Recovery EC<sub>50</sub></b> EC <sub>50</sub> (μM) (95% CI, n)	<b>48 h Recovery EC<sub>50</sub></b> EC <sub>50</sub> (μM) (95% CI, n)
Diazepam	0.12 (0.05–0.30, 10)	0.06 (0.04–0.09, 8)	0.08 (0.04–0.14, 8)	0.08 (0.05–0.13, 8)
Zolpidem	0.10 (0.06–0.17, 10)	0.12 (0.05–0.30, 8)	0.38 (0.13–1.14, 6)	0.16 (0.11–0.24, 8)
Pentobarbital	59.7 (27.5–130, 8)	37.3 (10.3–134, 2)	54.7 (18.3–163, 6)	57.1 (35.9–90.9, 7)
Zinc	42.6 (29.5–61.6, 6)	27.3 (13.9–53.7, 5)	75.3 (48.3–117, 6)	30.0 (20.7–43.5, 6)