SULFHYDRL OXIDATION REDUCES HIPPOCAMPAL SUSCEPTIBILITY TO HYPOXIA-INDUCED SPREADING DEPRESSION BY ACTIVATING BK-CHANNELS

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The cytosolic redox status modulates ion channels and receptors by oxidizing/reducing their sulfhydryl (SH) groups. We therefore analyzed to what degree SH-modulation affects hippocampal susceptibility to hypoxia. In rat hippocampal slices, severe hypoxia caused a massive depolarization of CA1 neurons and a negative shift of the extracellular DC potential, the characteristic sign of hypoxia-induced spreading depression (HSD). Oxidizing SH groups by DTNB (5,5’-dithiobis-2-nitrobenzoic acid, 2 mM) postponed HSD by 30%, while their reduction by DTT (1,4-dithio-DL-threitol, 2 mM) or alkylation by NEM (N-ethylmaleimide, 500 µM) hastened HSD onset. The DTNB-induced postponement of HSD was not affected by tolbutamide (200 µM), DL-AP5 (150 µM) or CNQX (25 µM). It was abolished, however, by Ni²⁺ (2 mM), withdrawal of extracellular Ca²⁺, charybdotoxin (25 nM), and iberiotoxin (50 nM). In CA1 neurons DTNB induced a moderate hyperpolarization, blocked spontaneous spike discharges and postponed the massive hypoxic depolarization. DTT induced burst firing, depolarized glial cells, and hastened the onset of the massive hypoxic depolarization. Schaffer-collateral/CA1 synapses were blocked by DTT but not by DTNB; axonal conduction remained intact. Mitochondria did not markedly respond to DTNB or DTT. While the targets of DTT are less clear, the postponement of HSD by DTNB indicates that sulfhydryl oxidation increases the tolerance of hippocampal tissue slices against hypoxia. As underlying mechanism we identified the activation of BK channels in a Ca²⁺-sensitive manner. Accordingly, ionic disregulation and the loss of membrane potential occur later or might even be prevented during short-term insults. Therefore, well directed oxidation of SH-groups could mediate neuroprotection.
INTRODUCTION

Reactive oxygen species (ROS) are mostly produced by the mitochondrial respiratory chain (Boveris and Chance 1973). Accordingly, changes in mitochondrial activity affect the cytosolic redox status, which is determined by the cellular H$_2$O$_2$ content as well as the two redox couples NAD$^+$/NADH and reduced/oxidized glutathione (Dröge 2001). It is now clear that ROS are not only involved in cellular damage resulting from metabolic disturbances (Chan 2001), status epilepticus (Kovaćs et al. 2002) and various neurodegenerative diseases such as Alzheimer disease, parkinsonism and amyotrophic lateral sclerosis (Halliwell 1992, Agar and Durham 2003), but also serve as subtle intracellular messengers under physiological conditions (Dröge 2001). The primary site of modulation of cellular proteins by redox signaling are the sulfhydryl (SH) groups of the amino-acid cysteine. Their reduction or oxidation critically influences the activity of the protein affected, since disulfide bonds and consequently protein structure might be modulated (Dröge 2001, Lipton et al. 2002).

In hippocampal neurons, various ion channels and receptors have been shown to be sensitive to redox modulation. Among these are NMDA-type glutamate receptors (Sucher and Lipton 1991), persistent Na$^+$ channels (Hammarström and Gage 2000), K$^+$ channels (Seutin et al. 1995) and also Schaffer collateral/CA1 neuron synapses are modulated by H$_2$O$_2$ (Pellmar 1987). Accordingly, there are various possibilities for redox modulation to control neuronal excitability. However, so far it is unknown to what degree redox signaling is involved in the response of neurons and glia to metabolic insults such as anoxia and ischemia. Could cellular redox signaling possibly be part of the oxygen sensing process in hippocampal neurons and trigger their immediate response to oxygen withdrawal? Could redox signaling even modulate the susceptibility of neural tissue against metabolic insults, could it possibly bear neuroprotective potential?
To address these questions we focused on the very end of the redox signaling cascade, the SH-groups: We analyzed to what degree the susceptibility of the hippocampal CA1 field towards anoxic insults, e.g. the generation of hypoxia-induced spreading depression (HSD), is affected by the modulation of SH groups. The specific targeting of SH groups was achieved by the SH oxidizing agent DTNB (5,5’-dithiobis 2-nitrobenzoic acid), the SH-reducing drug DTT (1,4-dithio-DL-threitol), and the SH-alkylating compound NEM (N-ethylmaleimide) (for review see: Lipton et al. 2002). The effects of these compounds were analyzed on the network level, the cellular level, and on the subcellular level.

Some of the results have been published as abstracts (Hepp et al. 2004, Hepp et al. 2005).
MATERIALS AND METHODS

Preparation

Hippocampal tissue slices were prepared from ether anesthetized Sprague-Dawley rats of 150-300 g body weight (4-8 weeks old, mostly males). Following decapitation the brain was rapidly removed from the skull and placed in chilled artificial cerebrospinal fluid (ACSF) for 1-2 minutes. The two hemispheres were separated, and 400 µm slices were cut using a tissue slicer (Campden Instruments, 752M Vibroslice). On a few occasions, the hippocampus was first isolated and then chopped (400 µm slices) using a custom-made tissue chopper. Slices were transferred to an interface recording chamber of the Oslo style and were left undisturbed for at least 90 min. The recording chamber was kept at a temperature of 35-36 °C. It was continuously aerated with 95% O₂ - 5% CO₂ (400 ml/min), and perfused with oxygenated ACSF (3-4 ml/min). Hypoxia was induced by switching the chamber’s gas supply to 95% N₂ - 5% CO₂. To protect the slices from drying out and to prevent oxygenation from the air during hypoxic episodes, the slice chamber was covered by a lid with a small (2 cm²) opening for the positioning of the electrodes.

Cell cultures of hippocampal neurons were prepared from 2-4 days old Sprague-Dawley rats. After decapitation, the brain was removed and placed in ice-cold HBSS (Hanks’-balanced salt solution) containing 20% FCS (Biochrom). The dentate gyrus region was removed from the isolated hippocampi, and the remaining tissue was cut into smaller pieces and trypsinated (5 mg/ml) for 10 min at 37°C. Cells were then dissociated by gentle trituration and the suspension was centrifuged for 10 minutes (1500 rpm, 4°C). The pellet was re-suspended and plated on Matrigel (BD Biosciences)-coated glass cover slips, which were transferred to 4-well culture plates (Nunc). Cultures were incubated at 37°C in a humidified, 5% CO₂-containing atmosphere. After 24 h half of the 600 µl medium in each well was exchanged with growth-medium containing 4 µM cytosine arabinoside (Sigma-Aldrich). Cultures were kept for up to 2
weeks, refreshing medium and growth factors after 4 days. Within 2-3 days in culture, cells fully recovered well-pronounced dendritic processes and were suitable for experiments; most experiments were performed between 3 and 7 days in culture.

Solutions

Chemicals, unless otherwise mentioned, were obtained from Sigma-Aldrich. The artificial cerebrospinal fluid (ACSF) had the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.2 CaCl₂, 1.2 MgSO₄, and 10 Dextrose; aerated with 95% O₂ - 5% CO₂ to adjust pH to 7.4.

Minimum essential cell culture medium (MEM-medium, Invitrogen) was supplemented with 5 mg/ml glucose, 0.2 mg/ml NaHCO₃ and 0.1 mg/ml transferrin (Calbiochem). For initial plating it also contained 10% FCS (fetal calf serum), 2 mM L-glutamine, and 25 µg/ml insulin. The medium used after day 4 in culture contained 5% FCS, 0.5 mM L-glutamine, 20µl/ml B27 50x supplement (Invitrogen), and 100 µg/ml penicillin-streptomycin (Biochrom).

DTNB (5,5´-dithiobis 2-nitrobenzoic acid), DTT (1,4-dithio-DL-threitol), and TEA (tetraethylammonium chloride) were directly dissolved in ACSF. DL-AP5 (DL-2-amino-5-phosphonovaleric acid, Tocris) was prepared as 100 mM stock solution in 1M NaOH and was kept frozen. Rhodamine 123 (Molecular Probes) was dissolved in absolute EtOH (20 mg/ml). Charybdotoxin (Sigma-Aldrich and Alomone Labs) and iberiotoxin (Alomone Labs) were dissolved in 100 mM NaCl, 10 mM Hapes plus 0.1% BSA. Paxilline, CNQX and tolbutamide were dissolved in DMSO as 10, 50 and 100 mM stocks, respectively, and stored at 4°C. NEM (N-ethylmaleimide) was prepared freshly as 250 mM stock in DMSO. To allow for sufficient
diffusion of drugs into the interfaced slices, all drugs were applied for at least 20 min. Final DMSO and EtOH concentrations were \( \leq 0.2\% \).

**Microelectrodes**

Single barreled glass microelectrodes for extracellular recordings were pulled from thin-walled borosilicate glass (GC150TF-10, Harvard Apparatus) using a horizontal puller (P-97 Flaming/Brown Micropipette Puller, Sutter Instruments). They were filled with ACSF and their tips were broken to a final resistance of 5-10 M\( \Omega \). Sharp microelectrodes for current-clamp recordings were made from thick-walled borosilicate glass (GC 150F-10, Harvard Apparatus) and filled with 2 M K-Acetate + 5 mM KCl + 10 mM Hepes ((N-(2-hydroxyethyl) piperazine-N'- (2-ethanesulfonic acid); Sigma); pH 7.4. Their resistances were around 80 M\( \Omega \).

**Hypoxia-protocol and electrical recordings**

Severe hypoxia was induced by switching the recording chamber’s gas supply from carbogen (95% O\(_2\)-5% CO\(_2\)) to 95% N\(_2\)-5% CO\(_2\), while the carbogen aeration of the experimental solutions was continued. Such treatment resulted within a few minutes in the occurrence of HSD. The time point of reoxygenation critically determines the reversibility of the hypoxia-induced changes. Therefore, we resubmitted oxygen soon (15-20 s) after the onset of HSD, which was indicated by a sudden drop in extracellular DC potential. Within that time the extracellular DC potential shift had reached its nadir.

All signal amplitudes were measured between the pre-hypoxia baseline and the maximal change. Only rapid negative extracellular DC potential changes (\( \Delta V_o \)) of at least 10 mV amplitude were considered as HSD, and only those slices were accepted for the experiments, which produced an
HSD no earlier than 90s after oxygen withdrawal (no earlier than 60 s in Ca$^{2+}$ free solutions). HSD onset was defined as the occurrence of the sudden $\Delta V_o$.

Evoked responses were elicited by 0.1 ms unipolar stimuli delivered via microwire electrodes made from bare stainless steel wire (50 $\mu$m diameter, AM-Systems) and recorded as described in detail earlier (Müller and Somjen, 1998). Orthodromic responses were elicited by stimulation of Schaffer collaterals and recorded in st. radiatum of the CA1 region; antidromic responses were elicited by stimulating pyramidal cell axons in the alveus and recorded in st. pyramidale of the CA1 region. All extracellular recordings were performed with a locally constructed extracellular DC potential amplifier.

Current-clamp recordings from CA1 neurons were performed with an intracellular recording amplifier (SEC-05L, NPI Instruments, Tamm, Germany). Bridge balance and electrode-capacitance compensation were adjusted before insertion of the electrode and continuously controlled during the entire recording. CA1 pyramidal neurons were identified by their location in st. pyramidale, membrane potential, spontaneous activity, action-potential shape and input resistance (Morin et al. 1996). Glial cells (astrocytes) lining the pyramidal cell layer were identified by their negative membrane potential of -80 to -90 mV, low input resistance, the absence of spikes in response to depolarizing stimuli (D’Ambrosio et al. 1998). Successful cell impalement was achieved by slowly advancing the electrode into the slice and applying a brief high frequency current pulse ("buzz") to the electrode. Only CA1 neurons and glial cells with stable membrane potentials of at least -55 mV and -80 mV, respectively, were accepted. Neuronal input resistance was probed every 10 s by a hyperpolarizing current of 400-600 pA amplitude and 300 ms duration. Data were sampled at 2.5 kHz using a TL-1/Labmaster acquisition system and the Axotape V2 software (Axon Instruments). Input resistance was measured at the steady state
level of the voltage deflections and averaged over 10 successive current injections. Changes in input resistance were expressed as percent of pretreatment value.

Optical recordings

Optical recordings of intracellular Ca\(^{2+}\) concentrations, mitochondrial membrane potential and NADH/FAD autofluorescence were performed with a standard computer-controlled fluorescence imaging system composed of a monochromatic light source (Polychrome II; Till Photonics, Gräfelfing, Germany) and a highly-sensitive CCD camera (Imago QE, PCO Imaging, Kelheim, Germany) attached to an upright microscope (Axiotech vario, Zeiss, Oberkochen, Germany). The computer controlled monochromatic lightsource selects the pre-defined wavelength(s) by a galvanometer-mounted grating which projects the desired range of the spectrally dispersed light onto a narrow slit. Therefore, the final excitation light shows a Gaussian wavelength distribution around the selected center-wavelength; bandwidth at half maximum is 15 nm. In the following just the center wavelengths of excitation light are mentioned. Rh123 was excited at 485 nm and fluorescence was recorded using a 505 nm beamsplitter and a 535/35 nm bandpass filter. Cellular NADH and FAD were excited alternately at 360 nm (NADH) and 460 nm (FAD), which is close to their respective absorption maxima, and the resulting autofluorescence was recorded simultaneously using a 505 nm beamsplitter and a 510/40 nm bandpass filter. This beamsplitter/emitter constellation used sacrificed a large part of NADH autofluorescence (which is highest around 400-450), yet it made possible the simultaneous detection of NADH and FAD autofluorescence. For imaging experiments tissue slices and cell cultures were placed in a submersion-style chamber at 33-35°C.
Statistics

The data were obtained from ~70 rats, and since most experiments did not last longer than 1-2 hours, up to five slices could be used from each brain. However, to ensure independence of observations, each experimental treatment was performed on at least 3 different rats. All numerical values are represented as mean ± standard deviation. Significance of the observed changes was tested using a two-tailed, unpaired Student’s t-test and a significance level of 5% (unpaired observations). Drug-induced changes were compared to untreated control slices that also underwent the corresponding number of HSD episodes. In the case of paired observations, a one-sample t-test was used to compare normalized drug effects against pre-treatment control conditions, defined as unity or as 100%. In the diagrams significant changes are marked by asterisks (* p < 0.05; ** p < 0.01). Statistical calculations were done with the Excel software (Office 2000).

RESULTS

Effects on hypoxia-induced SD

Severe hypoxia induces within a few minutes a nearly complete depolarization of hippocampal neurons and glial cells, which is associated by the occurrence of a negative shift in the extracellular DC potential, termed hypoxia-induced spreading depression (for review see: Somjen, 2001, 2004). In untreated slices HSD occurred within 156.6 ± 54.5 s, the associated negative shift of the extracellular DC potential (ΔV_o) averaged -16.1 ± 4.2 mV and measured at its half amplitude level it lasted 45.6 ± 10.4 s (n=115). As we have shown previously, HSD of short duration can be induced repeatedly in a given slice, and with a sufficient recovery time of
~30 min in between the hypoxic episodes, the characteristic parameters are not significantly affected for the first three HSD episodes (Müller and Somjen, 1998).

Modulation of sulfhydryl groups did, however, result in characteristic changes of HSD. As compared to the previously induced control HSD, application of the sulfhydryl oxidizing agent DTNB (2 mM, 20 min) postponed the onset of HSD by 29.2 ± 18.8 % and decreased its amplitude by 15.7 ± 8.8 % (n=9); HSD duration was not affected (Fig. 1A, C). By contrast, treatment with the SH-reducing agent DTT (2 mM, 20 min) shortened the time to HSD onset by 25.0 ± 17.3 % and decreased its amplitude by 26.1 ± 14.9 % (n=8), the duration of HSD was not affected (Fig. 1B, C). The sulfhydryl alkylating agent NEM (N-ethylmaleimide, 500 µM, 20 min) shortened the time to HSD onset by 48.4 ± 10.4 % and reduced its duration by 29.9 ± 5.5 %; HSD amplitude was not affected (n=7, Fig. 1C). Application of the rather unspecific oxidizing agent H$_2$O$_2$ (1 mM, 20 min) caused a moderate, less consistent postponement of HSD by 12 ± 19 % (n=7).

In general, treatments increasing neuronal excitability facilitate the generation of SD, while those decreasing excitability postpone its occurrence. So it seems that DTT might increase excitability, while DTNB reduces it. We therefore tested for a possible involvement of Ca$^{2+}$ channels, glutamate receptors, and K$^+$ channels in the DTNB- and DTT-induced modulation of HSD. In these experiments, the control HSD was already induced in the presence the given blocker and the second HSD was elicited in the continued presence of the blocker plus DTNB or DTT.

Withdrawal of extracellular Ca$^{2+}$ (n=7, Fig. 2A, C) or application of 2 mM Ni$^{2+}$ (n=5, Fig. 2B, C) prevented the DTNB-induced postponement of HSD, indicating the requirement of Ca$^{2+}$-influx from extracellular space for the delayed HSD onset. In the presence of the NMDA antagonist...
DL-AP5 (150 µM, n=6) or the AMPA/kainate antagonist CNQX (25 µM, n=8) the DTNB-induced postponement of HSD remained unchanged (Fig. 2C).

The postponement of HSD by DTNB could indicate an increased activity of K⁺ channels. We therefore tested for the DTNB-sensitivity of different K⁺ channel types. Low concentrations of TEA (500 µM, n=6), which are assumed to target BK channels (Beck et al. 1997), or treatment of slices with the K<sub>ATP</sub> channel blocker tolbutamide (200 µM, n=6) did not affect the DTNB-induced postponement of HSD (Fig. 3B). However, pretreating slices with 25 nM charybdotoxin (CTX, n=5 Fig.3A) or 50 nM iberiotoxin (IBX, tested in three trials only) consistently abolished the DTNB-induced postponement of HSD (Figs. 3A, B). It therefore seems that sulfhydryl modulation activates Ca<sup>2+</sup>-activated, high conductance K⁺ channels of the BK type.

Analogous pharmacological experiments were also performed to identify the target of DTT. Combination of Ca<sup>2+</sup> free solutions with DTT (2 mM) triggered within 19.7±4.7 min (n=4) spontaneous recurrent SD episodes which irreversibly damaged the slices under investigation. Subsequent oxygen withdrawal failed to trigger full size HSDs in these slices (Fig 4A). One slice which did not produce spontaneous SDs under these conditions showed a further reduction in HSD onset by 50% in DTT. Ni<sup>2+</sup> could not be tested, since it formed a massive brown precipitate with DTT. In the presence of the glutamate antagonists CNQX (25 µM, n=5) or DL-AP5 (150 µM, n=5) the DTT-induced hastening of HSD onset was unchanged, nor did tolbutamide (200 µM, n=5) prevent this effect of DTT (Fig. 4B). Since the peptidergic BK channel blockers charybdotoxin and iberiotoxin do possess three disulfide-bonds each, which are potential targets for reduction by DTT, we rather chose the non-peptidergic BK channel inhibitor paxilline (Sanchez and McManus, 1996). In our trials paxilline (10 µM) did not prevent the hastening of HSD onset by DTT (n=5, Fig. 4B), and it also failed to prevent the postponement of HSD by
Changes in cellular excitability and synaptic function

To explore whether DTNB and DTT affect other neuronal functions that are likely to be influenced by BK channels, we performed sharp electrode recordings from single CA1 neurons and glial cells in interfaced slices. In addition, we probed for changes in synaptic function by recording extracellular field potentials.

Since pyramidal cells and interneurons respond to hypoxia identically (Müller and Somjen, 2000), the data of both types of cells were pooled (11 pyramidal cells, 9 interneurons). Average membrane potential and input resistance of the recorded neurons were -65.9 ± 5.0 mV and 25.5 ± 7.5 MΩ (n=20). In untreated control slices, oxygen withdrawal triggered within 1-2 minutes an initial hyperpolarization of CA1 neurons, which then turned into a slow depolarization and finally triggered (after 177 ± 69 s, n=7) an explosive depolarization close to 0 mV (for details see: Müller and Somjen 2000 a, b) (Fig. 5A).

Application of DTNB (2 mM, 20-25 min) caused a moderate hyperpolarization, by 3.8 ± 2.8 mV, and decreased the input resistance, by 17.9 ± 13.2 % (n=6). As a result spontaneous spike discharges were reduced or even ceased (Fig. 5B). In the presence of DTNB oxygen withdrawal triggered the anoxic depolarization within 237 ± 64 s (n=7), i.e. the CA1 neurons tolerated hypoxia longer than untreated control cells (Fig. 5B). The initial hyperpolarization was slightly reduced or was even absent, probably due to the more negative resting membrane potential in the presence of DTNB (Fig. 5B).
Incubation with DTT (2 mM, 20-25 min) caused a moderate depolarization in 4 cells and a moderate hyperpolarization in the 2 others; the input resistance did not change significantly (Fig. 5C). However, in the presence of DTT spontaneous spike discharges became more frequent and within ~15 min well pronounced burst discharges developed (Fig. 5D). During these burst discharges single spikes became progressively broadened (Fig. 5D). In the presence of DTT, oxygen withdrawal triggered the massive hypoxic depolarization within 97 ± 45s. The initial hypoxic hyperpolarization was usually blocked and the neurons directly started to depolarize slowly when oxygen was withdrawn (Fig. 5C).

By incidence in hippocampal cell cultures we observed the retraction of glial processes in response to DTT (Fig. 6A), while neuronal processes remained intact. This retraction started within 7-10 minutes of DTT application and was completed within another 30-40 minutes. DTNB application did not cause such changes in cell shape. To elucidate, whether DTT disturbs glial function, which would severely interfere with neuronal and synaptic function, we performed sharp electrode recordings from CA1 glial cells in hippocampal slices. The average glial membrane potential was -89.7 ± 5.6 mV (n=6), their input resistance was too low to be probed reliably. In response to DTT glial cells showed a slow but continuous depolarization which averaged 19.2 ± 7.5 mV after 15-20 min of DTT treatment (Fig. 6, n= 6). The synchronized burst discharges elicited by DTT in pyramidal neurons, caused – apparently due to an increased extracellular K⁺ concentration – slow transient depolarizations of glial cells by up to 20 mV (Fig. 6). Continuing DTT treatment beyond 30 min usually caused spontaneous SD episodes, during which glial cells underwent the characteristic massive depolarization (see also Müller and Somjen 2000a).
Orthodromically evoked field potentials (fEPSPs) and antidromically evoked population spikes were recorded in st. radiatum and st. pyramidale of the CA1 region, respectively. Compared to the evoked responses previously recorded in the same slice, DTNB (2 mM, 20 min) tended to somewhat reduce the amplitude of evoked excitatory field potentials (Fig. 7A). Paired pulse facilitation was not significantly affected (Fig. 7A). Against expectation, DTT (2 mM, 20 min) depressed orthodromically evoked fEPSPs by 45-50 % (n=6), but it also induced multiple population spikes in response to single orthodromic stimuli (Fig. 7B). Paired pulse facilitation was also somewhat reduced by DTT. This depression of EPSPs by DTT also occurred in the continued presence of 3 mM glutamine (Fig 7C, n=6). Axonal conduction was not affected by either DTNB or DTT, as judged by the unchanged amplitudes of antidromic population spikes (Fig. 8). Yet again, DTT induced multiple population spikes in response to a single stimulus (Fig. 8B).

Is mitochondrial function modulated?

Since direct effects of DTNB and DTT on mitochondria would be expected to modulate neuronal susceptibility to hypoxia, we elucidated whether these compounds affect mitochondrial membrane potential and/or metabolism. In detail, we probed for changes in rhodamine 123 (Rh123) fluorescence, a marker for mitochondrial membrane potential (ΔΨ), and for changes in autofluorescence, representing the cellular redox couples NADH/NAD⁺ and FADH₂/FAD. These recordings were performed in a submersion style chamber (33-35°C).

In Rh123 labeled slices (2-5 µg/ml Rh123 loading for 25 min) as well as in cultured CA1 neurons, the protonophore FCCP (1-2 µM) induced a clear increase in Rh123 fluorescence, 65 ± 21 % in st. radiatum of acute slices (n=7) and 63 ± 33 % in the cytosol (soma) of cultured CA1 neurons (n=8), thereby indicating pronounced mitochondrial depolarization (Figs. 9A, B).
contrast, DTNB and DTT (2 mM each, applied for 7-9 min) did not markedly affect mitochondrial membrane potential – neither in cultured CA1 neurons, nor in acute hippocampal slices (Figs. 9 A, B). Also, recording autofluorescence from acute slices did not reveal any pronounced effects of DTNB or DTT (2 mM each) on the cellular levels of NADH and FAD. Autofluorescence excited at 360 nm represents reduced NADH, while autofluorescence excited at 460 nm corresponds to oxidized flavins (FAD). As expected, in response to mitochondrial inhibition by anoxia, 1 mM CN− caused opposite changes in these simultaneously recorded measures (Duchen and Biscoe, 1992), shifting NADH to its reduced and FAD to its oxidized form (Figs. 9C, D). Accordingly, NADH autofluorescence increased by 9.2 ± 1.9 %, while FAD autofluorescence decreased by 7.4 ± 1.8 % (n=6 each). DTT induced only barely noticeable changes in autofluorescence levels. DTNB, which absorbs light in the range of 300-450 nm, caused an artificial drop in NADH fluorescence due to competitive absorption of excitation light (Fig 9D). The time course of these changes as well as the absence of a corresponding, opposite signal in FAD autofluorescence do confirm the artifactual nature of the DTNB-induced changes in NADH autofluorescence.

**DISCUSSION**

We demonstrated that sulfhydryl modulation affects the susceptibility of hippocampal tissue slices to the generation of the highly synchronized anoxic response, i.e. HSD. Oxidation of sulfhydryl groups by DTNB or H₂O₂ postponed the onset of HSD, while their reduction by DTT as well as their alkylation by NEM hastened HSD onset. The Ca²⁺ dependence of the DTNB-induced postponement as well as its sensitivity to charybdotoxin and iberiotoxin prove that an
enhanced activity of BK-type channels underlies the observed modulation of HSD. The mechanisms involved in the hastening of HSD onset by DTT are less clear.

How is SD postponement achieved?

The generation of HSD reflects the concerted activation of various types of ion channels and glutamate receptors enabling massive, self regenerative \( \text{Ca}^{2+} \) and \( \text{Na}^{+} \) influx into neurons that is paralleled by \( \text{K}^{+} \) release into extracellular space (Aitken et al. 1991, Müller and Somjen 1998, 2000b, Somjen 2001, 2004). Computer simulations, based on experimental data (Kager et al. 2002), as well as current source density analyses (Wadman et al. 1992) provided evidence that SD is ignited in the dendrites. The ignition point is reached, when the sum of all inward currents exceeds the total outward current, i.e. when the net dendritic current turns inward (Kager et al. 2002). Inhibition of voltage-gated \( \text{Na}^{+} \) channels, \( \text{Ca}^{2+} \) channels, or glutamate receptors does not prevent SD, but just postpones its onset. No matter what maneuvers are taken, in single neurons the anoxic depolarization, once triggered, evolves until completion in an all-or-none fashion (Müller and Somjen 2000b, Kager et al. 2002). If the hypoxic depolarization is less synchronized within the neuronal population this might result in a reduced extracellular DC potential shift, but again without dampening the massive depolarization in individual pyramidal cells and interneurons. But how does oxidation of SH groups and the resulting postponement of HSD onset fit into this scenario?

The immediate, early response of hippocampal CA1 neurons to hypoxia is an initial hyperpolarization, which is generated by activation of \( \text{K}^{+} \) channels and subsequent \( \text{K}^{+} \) release into interstitial space (Hansen et al. 1982, Donnelly et al. 1992, Müller and Somjen 2000a). Whether solely BK-type channels or \( \text{K}_{\text{ATP}} \) channels are activated or whether both types of channels are involved, with their contribution being weighed depending on the severity of an
insult and the experimental conditions, is still under discussion (Fujimura et al. 1997; Erdemli et al. 1998; Zawar and Neumcke 2000). Under our experimental conditions (slices of adult rats placed in an interface chamber at 35-36°C), the initial hyperpolarization seems to be mediated mostly by BK-type channels: DTNB was found to hyperpolarize CA1 neurons by activation of BK channels and subsequent oxygen withdrawal did not cause a further hyperpolarization of these cells. That the postponement of HSD by DTNB was abolished by charybdotoxin, iberiotoxin, Ni²⁺, and Ca²⁺ withdrawal further supports the activation of BK channels. By contrast, tolbutamide was without effect, ruling out an involvement of K_ATP channels – at least during the early phase of hypoxia. Also the time to onset of HSD in untreated control slices (167±63 s, n=19) and in slices treated with 200 µM tolbutamide (143±42 s, n=12) did not differ.

We therefore conclude that the DTNB-mediated oxidation of SH groups activates BK channels, thereby postponing the onset of HSD. Whether DTNB directly acts on BK channels - modulating their gating properties or rendering them more Ca²⁺ sensitive - cannot be decided yet on the basis of our data. Indications for an increased Ca²⁺-sensitivity of hippocampal BK channels were found during the postischemic period (Gong et al. 2002). Alternatively, DTNB could as well modulate voltage-gated Ca²⁺ channels and enhance Ca²⁺ influx during the initial phase of hypoxia, thereby activating BK channels indirectly. Such an activation of Ca²⁺ channels, leading to subsequent activation of a charybdotoxin-sensitive K_Ca current, was reported in dissociated hippocampal neurons by Nowicky and Duchen (1998) in response to cyanide poisoning of mitochondria or their uncoupling by FCCP. The fact is that in our experiments Ca²⁺ influx from extracellular space was required for the activation of BK channels and that it was carried via voltage-gated Ca²⁺ channels, not via glutamate receptors, because DL-AP5 and CNQX failed to antagonize the DTNB-induced postponement of HSD.
The different time course of BK channel activation in the presence of DTNB or the enhanced BK-mediated outward current resulted in a postponement of SD. This could be due to more efficient glial K\(^+\) buffering, which was demonstrated in computer simulations to postpone or even prevent SD (Somjen 2004, p. 309). BK channels were already open before the onset of hypoxia, thereby causing an earlier hyperpolarization and allowing the neurons to withstand the generation of SD for a longer time period. Interestingly, BK channels were also reported to control the generation of Ca\(^{2+}\) spikes in pyramidal cell dendrites (Golding et al. 1999). Accordingly, an increased BK channel activity could successfully shift the ratio of inward versus outward current more to favor of the outward current, thereby preventing dendritic depolarization and thus HSD ignition within the dendritic tree just a little bit longer. As BK channels are assumed to be expressed in astrocytes as well (Barres et al. 1990), they could facilitate K\(^+\) uptake and K\(^+\) buffering within the glial syncytium. However, since ignition of SD occurs in neurons while glial cells just follow passively, the extracellular increase in K\(^+\) (Müller and Somjen 2000a, Somjen 2001), we rather believe that the postponement of HSD due to modulation of BK channels is more likely to take place in neurons rather than glia.

**Redox Modulation of hippocampal circuitry and excitability**

DTNB tended to decrease synaptic efficacy only slightly, while axonal conduction was not affected (Figs. 5A, 6A). The slight dampening of synaptic function is probably the result of the moderate hyperpolarization of single CA1 neurons in response to DTNB, i.e. a reduced postsynaptic excitability due to the increased resting K\(^+\) conductance or a dampening effect of DTNB on the NMDA component of the EPSP (Tauck 1992). Paired pulse facilitation, which arises from residual Ca\(^{2+}\) remaining in the presynaptic terminal after the first stimulus (Zucker 1989), was not reduced by DTNB, suggesting that presynaptic Ca\(^{2+}\) cycling was not affected.
By contrast, DTT markedly reduced synaptic efficacy, but did not depress antidromic responses. In addition, it increased postsynaptic excitability (Figs. 7B, 8B). The moderate depolarization observed in single hippocampal neurons and the induction of burst discharges by DTT (Fig. 5D) correspond to earlier reports obtained in guinea pigs (Tolliver and Pellmar, 1988) and they indicate an enhanced excitability of CA1 neurons. Obviously these changes in CA1 neurons are also responsible for the firing of multiple population spikes in response to single antidromic stimuli (Fig. 7B), as well as for the observed depolarization of glial cells (Fig. 6B) which suggests an increase in extracellular $K^+$. Thus, the observed depression of synaptic function rather seems to be due to presynaptic effects of DTT in the Schaffer collateral terminal. If also induced presynaptically, the DTT-mediated depolarization or the increased extracellular $K^+$ could inactivate presynaptic $Na^+$ and $Ca^{2+}$ channels, thereby reducing $Ca^{2+}$ influx and transmitter release from the synaptic terminals (Eccles et al. 1963, MacDermott et al. 1999). As glutamine failed to prevent synaptic depression by DTT, glial poisoning by DTT can be excluded as a possible explanation for synaptic failure.

Besides the proven modulation of BK channels, additional channels and receptors are known to be sensitive to sulfhydryl modulation. DTT increases, while DTNB decreases NMDA currents (Sanchez et al. 2000, Tauck 1992), thereby enhancing NMDA-receptor mediated toxicity in hippocampal slice cultures (Pringle et al. 2000). In contrast, DTNB suppresses spontaneous ictal activity due to inhibition of NMDA receptors (Sanchez et al. 2000). Yet for our data, redox modulation of glutamate receptors can be ruled out, since the effects of DTNB and DTT on HSD persisted in the presence of NMDA and non-NMDA antagonists (Figs. 2C, 4B).

Persistent $Na^+$ channels are potentiated by hypoxia (Hammarström and Gage 1998) and an increase in extracellular $K^+$ (Somjen and Müller 2000), and they play a key role in the triggering
of normoxic SD and HSD (Somjen, 2001, Kager et al. 2002). Interestingly $I_{Na\beta}$ is also sensitive to SH modulation, being increased by NO donors in a DTT sensitive manner (Hammarström and Gage 1999). Nevertheless, persistent Na$^+$ channel modulation cannot explain our findings either, since it was BK-channel inhibition that abolished the DTNB-mediated postponement of SD. Also, if Na$^+$ channels were modulated by DTNB or DTT, one would expect effects on axonal conduction as well. Antidromic responses were, however, found not to be modulated by either DTT or DTNB (Fig. 8).

Changes in cellular redox state during hypoxia

The oxidation of SH groups was found to postpone HSD. Severe hypoxia itself causes a decrease of mitochondrial metabolism by inhibiting complex IV of the respiratory chain, which should decrease the formation of ROS (Boveris and Chance 1973, Votyakova and Reynolds 2001). In addition, the redox couple NAD$^+$/NADH is shifted more to the reducing form, thus shifting the cellular redox status towards reducing conditions (Riepe et al. 1996, Mills and Jöbsis 1972, and Fig. 9). In contrast, reoxygenation, rotenone, antimycin, Ca$^{2+}$-induced mitochondrial depolarization, increased ATP-synthesis and mitochondrial uncoupling by e.g. FCCP increase ROS production (Bindokas et al. 1996, Boveris and Chance 1973).

Could reducing conditions possibly favor the generation of HSD? NMDA-receptor mediated currents are increased under reducing conditions (Sanchez et al. 2000, Tauck 1992, Tolliver and Pellmar 1988), i.e. neuronal excitability increases. Also, BK channels are blocked by reducing conditions, but activated by oxidizers such as DTNB and H$_2$O$_2$. In addition to our findings, Nowicky and Duchen (1998) reported activation of Ca$^{2+}$-sensitive K$^+$ channels in dissociated CA1 neurons in response to mitochondrial depolarization by either FCCP or chemical anoxia induced by cyanide. So one might ask, whether sulfhydryl/redox modulation could be the missing
The link of how mitochondrial failure affects K\(^+\) channel activity, how mitochondrial dysfunction finally affects membrane conductances. The fact is that neurons respond immediately to metabolic compromise and they respond – with protective mechanisms – way before cellular ATP is depleted (Hansen et al. 1982, Lipton and Whittingham 1982, Müller et al. 2002). The detailed molecular events involved in the sensing of hypoxia by central neurons are only partly understood, but it seems that SH-modulation may play a pivotal role in these processes.

**Neuroprotective potential of SH-oxidation**

The postponement of HSD was found to depend on the activation of BK channels. In general, these channels decrease neuronal excitability, and others reported them to reduce cell death from ischemic insults in CA1 pyramidal cell cultures and to regulate presynaptic glutamate release (Runden-Pran et al. 2002). We found that - even though BK channels are activated anyway during the early phase of hypoxia - their increased activity already before oxygen withdrawal succeeded in stabilizing the membrane potential and preventing the ignition of HSD for some more time – on average 30 % longer than in untreated slices. As a result, the dramatic disturbance of ionic homeostasis and especially the massive Ca\(^{2+}\) load being associated with the hypoxic depolarization (Hansen 1985, Martin et al. 1994, Müller and Ballanyi 2003) were delayed. The decreased amplitude of HSD might indicate desynchronization of the anoxic depolarization in CA1 neurons. Both, postponement and desynchronization may prevent HSD during short term insults and possibly also reduce its spreading speed and -range. Accordingly, parts of the neuronal population could be affected later or might even be spared from the loss of membrane potential and the threatening Ca\(^{2+}\) overload. Stroke is of course rarely anticipated, but peri-infarct SD waves reaching out in the penumbra and aggravating neuronal damage of surrounding tissue that was initially not affected (Busch et al. 1996, Kempski et al. 2000) could be dampened or
depressed by well directed SH-modulation. Therefore, oxidizing SH-groups might mediate neuroprotection during metabolic insults.
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**FIGURE LEGENDS**

**FIGURE 1:** Sulfhydryl modulation shifts the susceptibility to HSD.

**A**) The sulfydryl oxidizing agent DTNB (2 mM, 20 min) clearly postponed the onset of HSD. The amplitude of the plotted extracellular voltage deflection (ΔV_o) decreased slightly, while its duration (measured at the 50% level) was unaffected. Severe hypoxia was induced by switching the recordings chambers gas supply from carbogen to 95% N₂ – 5% CO₂.

**B**) The sulfhydryl reducing agent DTT (2 mM, 20 min) shortened the time HSD onset, but did not affect its duration.

**C**) Statistical summary of sulfhydryl-modulator induced changes. Similar to DTT, NEM accelerated the onset of HSD and in addition it decreased its duration, while application of H₂O₂ (1 mM, 20 min) mimicked the effects of DTNB, also postponing HSD. The plotted parameters are normalized to the respective control HSD previously induced in each slice. For statistical comparison, the parameters of a second HSD induced in an untreated slice are plotted as well. Error bars represent standard deviations, asterisks mark significant changes as compared to this second HSD induced in an untreated slice, i.e. the data set 2nd hypoxia plotted on the very right (* P < 0.05; ** P < 0.01; n:= number of trials).

**FIGURE 2:** DTNB-mediated postponement of HSD requires Ca²⁺ influx, but does not depend on ionotropic glutamate receptors.

**A, B**) In Ca²⁺-free solutions and the presence of Ni²⁺ (2 mM) the DTNB-induced postponement of HSD was abolished, indicating that Ca²⁺ influx from extracellular space is required.

**C**) Statistical comparison showing that only withdrawal of extracellular Ca²⁺ or application of Ni²⁺ prevent the DTNB-induced postponement of HSD. Since the NMDA antagonist DL-AP5 (150 µM) and the non-NMDA inhibitor CNQX (25 µM) are without effect, Ca²⁺ influx obviously occurs via voltage-gated Ca²⁺ channels rather than glutamate receptors. Asterisks mark
significant changes of the respective HSD parameters as compared to the HSD induced in the presence of DTNB alone (shown in the first three bars, data are the same as shown in Fig 1C).

**FIGURE 3:** BK-type $K^+$ channels mediate the postponement of HSD.

A) In the presence of the inhibitor of $Ca^{2+}$-activated $K^+$ channels, charybdotoxin (20 nM), the DTNB-induced postponement of SD was abolished.

B) Statistical summary showing that neither tolbutamide nor low concentrations of TEA affected the DTNB-induced postponement of SD. However, charybdotoxin as well as iberiotoxin abolished the effects of DTNB. This clearly proves that activation of large conductance $Ca^{2+}$-activated $K^+$ channels of the BK type is responsible for the delayed onset of HSD in the presence of DTNB. Asterisks show significant changes as compared to the HSD induced in the presence of DTNB alone (first three bars and Fig. 1C).

**FIGURE 4:** Sulfhydryl reduction does neither target glutamate receptors, nor $K_{ATP}$ channels.

A) Recurrent spontaneous SD episodes induced by the combination of $Ca^{2+}$-free solutions and DTT. The slices were irreversibly damaged and HSD could not be induced anymore.

B) Statistical summary of the effects of glutamate antagonists and tolbutamide on the DTT-induced hastening of SD onset. Changes were compared to the data set plotted for DTT alone, which is identical to the data shown in Fig 1C. Since CNQX (25 µM) and DL-AP5 (150 µM) are without effect, non-NMDA or NMDA glutamate receptors do not seem to be involved in the hastening of SD onset by DTT. Tolbutamide (200 µM) was without effect as well, indicating that $K_{ATP}$ channels are not affected by DTT.

C) The non-peptidergic BK channel antagonist paxilline (10 µM) failed to prevent the hastening of HSD onset by DTT, yet it also failed to prevent the postponement of HSD by DTNB. Thus it is questionable, whether paxilline is effective under our experimental conditions at all.
FIGURE 5: Current-clamp recordings showing the effects of DTT and DTNB on single CA1 neurons during normoxia and subsequent hypoxia.

A) The hypoxic response of untreated control cells consists of an initial hyperpolarization which is followed by the regenerative, nearly total depolarization. Upon reoxygenation the cells start to recover, but during that phase intracellular recordings usually became unstable. Numbering refers to the characteristic points of the hypoxic response. 1:= resting membrane potential, 2:= initial hyperpolarization, 3:= ignition threshold, 4:= peak of fast depolarization, 5:= absolute peak. Their values are summarized in panel E for the various treatments.

B) DTNB treatment (2 mM, 20 min) caused a moderate hyperpolarization and abolished spontaneous spike discharges, in parallel the input resistance decreased slightly. The anoxic depolarization was postponed by DTNB, but otherwise occurred unchanged, in an all-or-none fashion.

C) DTT application (2 mM, 20 min) resulted in a moderate depolarization and an increasing rate of spontaneous spike discharges. The time to onset of the anoxic depolarization was shorter than under control conditions and the initial hyperpolarization was less pronounced or even absent.

D) In addition to more frequent spike discharges, DTT also induced pronounced burst firing. The displayed trace segments were recorded from the same neuron before and after 20 min DTT treatment. The trace displayed on the very right is a stretched segment of a burst discharge recorded in the presence of DTT (see arrow mark).

E) Summary of the characteristic potentials of the anoxic depolarization. For definition of potentials see numbering 1-5 in panel A. Marked differences did not occur in the presence of DTNB or DTT. Once the anoxic depolarization was triggered, it occurred in an all-or-none fashion. Compared to untreated control cells (n=7), its onset tended, however, to be postponed by DTNB (n=7) and hastened by DTT (n=5).
FIGURE 6: Targeting of glial cells by DTT.

A) By coincidence, in hippocampal cell cultures, we observed the retraction of glial processes in response to DTT. It started a few minutes upon DTT application (2 mM) and was completed within 30-40 min. For better visibility the displayed glial cell was loaded with Fluo-3 AM (3 µM, 20 min).

B) Sharp electrode recordings from glial cells in slices revealed a progressive loss of glial membrane potential that started a few minutes upon addition of DTT (2 mM). Within 35 min the recorded glial cell terminally depolarized to -20 mV, obviously the result of a spontaneous spreading depression episode, which occurred in some of the DTT-treated slices.

FIGURE 7: Sulfhydryl reduction markedly depresses synaptic efficacy.

A, B) The plotted input-output curves as well as the sample recordings of EPSPs show that DTNB tended to somewhat reduce EPSP amplitudes (n=7), while DTT significantly depressed the amplitudes of orthodromically-evoked responses (n=6), indicating block of synaptic function upon reduction of SH groups. Paired pulse ratios of orthodromic responses, tested by twin pulses separated by 75 ms, were not affected significantly by either treatment. EPSPs were recorded in stratum radiatum of the CA1 region. The stimuli (0.1 ms, unipolar) are marked by the arrows; stimulation artifacts are truncated.

C) The depression of synaptic function by DTT could not be prevented by glutamine (3 mM). Plotted EPSPs amplitudes are normalized to the pre-treatment control amplitudes (100 µA, 0.1 ms stimulus) and they are compared for the effects of DTT in control and glutamine-treated slices (n=6 each). Sample traces show EPSPs elicited in glutamine-containing ACSF before and after addition of DTT (17 min, 2 mM).
**FIGURE 8:** Antidromic population spikes, i.e. axonal conduction, is not depressed by sulfhydryl modulation.

**A)** Significant changes in the antidromically evoked population spikes were not observed during DTNB treatment (n=6).

**B)** While the amplitude of antidromic responses was unaffected (n=5), stimulation in the presence of DTT evoked multiple population spikes, indicating enhanced excitability of CA1 pyramidal neurons upon reduction of SH groups.

**FIGURE 9:** DTT and DTNB do not exert marked effects on mitochondrial membrane potential or mitochondrial metabolism.

**A)** Recordings of Rh123 fluorescence revealed no marked effects of DTNB and DTT application on mitochondrial membrane potential (ΔΨ). Experiments were performed on single cultured CA1 neurons (upper trace) as well as acute slices in the CA1 region (lower trace), with regions of interest being placed in st. radiatum, st. oriens, and st. pyramidale.

**B)** Recordings of autofluorescence revealed no effects of DTNB and DTT on the cellular levels of NADH (excited at 360 nm) or FAD (excited at 460 nm). By contrast, inhibition of mitochondria by 1 mM cyanide caused the expected opposite changes, shifting NADH/NAD ratio to reducing conditions (increase in NADH autofluorescence) and the FAD/FADH ratio to oxidizing conditions (decrease in FAD autofluorescence). In the case of DTNB interpretation of the NADH levels is difficult, since DTNB itself shows pronounced competitive absorption in the wavelength range required for NADH excitation (360 nm), thereby causing an instantaneous and marked reduction in the intensity of excitation light, and thus NADH emission. However, no such changes are being observed in FAD autofluorescence, confirming the artifactual nature of the drop in NADH traces.
Figure 1

(A) ACSF vs. 2 mM DTNB under hypoxia conditions. The graphs show the amplitude and time to onset.

(B) ACSF vs. 2 mM DTT under hypoxia conditions. The graphs show the amplitude and time to onset.

(C) Bar graph showing normalized parameters for different conditions: DTNB (n=9), DTT (n=8), NEM (n=7), H$_2$O$_2$ (n=9), and 2nd hypoxia (n=9). The graph includes error bars and statistical significances indicated by asterisks.
Figure 2
Figure 3

A

20 nM CTX

Hypoxia

20 nM CTX + 2 mM DTNB

Hypoxia

B

Normalized parameters

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude</th>
<th>Time to onset</th>
<th>Duration</th>
</tr>
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<tbody>
<tr>
<td>DTNB only (n=9)</td>
<td></td>
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<tr>
<td>DTNB + Tolbut (n=6)</td>
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<tr>
<td>DTNB + TEA (n=6)</td>
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<tr>
<td>DTNB + CTX (n=5)</td>
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<tr>
<td>DTNB + IBX (n=3)</td>
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</table>
**Figure 4**

A. 2 mM DTT

Ca\(^{2+}\)-free solution

B.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude</th>
<th>Time to onset</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
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<tr>
<td>DTT + CNQX (n=5)</td>
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<td>DTT + DL-AP5 (n=5)</td>
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<td>DTT + Tolbut (n=5)</td>
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<tr>
<td>DTT + Paxilline (n=5)</td>
<td></td>
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</tbody>
</table>

C.

- **Paxilline**
  - Hypoxia
  - Paxilline + DTT
  - Hypoxia

- **Paxilline + DTNB**
  - Hypoxia
  - Paxilline + DTNB
  - Hypoxia

**Legend**

- Amplitude
- Time to onset
- Duration
Figure 5

A

V_m (mV)

Hypoxia 2 min

B

V_m (mV)

2 mM DTNB

C

V_m (mV)

2 mM DTT

D

V_m (mV)

ACSF

2 mM DTT (20 min)

0.5 s

10 ms

E

V_m (mV)

Time to onset (s)

ACSF DTNB DTT
Figure 6

A

Control

10 min DTT

20 min DTT

30 min DTT

5 μm

B

$V_m$ (mV)

DTT

$-100$

$-80$

$-60$

$-40$

$-20$

0

2 min
Figure 7
Figure 8

A

Antidromic responses

B

Antidromic responses

stimulus (µA)

relative amplitude

stimulus (µA)

relative amplitude

Control-1  DTNB-1

Control-2  DTNB-2

Control-1  DTT-1

Control-2  DTT-2

4 mV

10 ms

Ctl

DTNB

Ctl

DTT
Figure 9

A. Cultured CA1 neuron

B. Bar graph showing F/F₀ ratios for DTNB, DTT, and FCCP treatments.

C. Graphs showing F/F₀ ratios for NADH and FAD treatments with asterisks indicating significance.

D. Graphs showing F/F₀ ratios for [NADH] and [FAD] treatments with bars indicating different regions.

Figure 9

** *** **

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