Regulation of GABA$_A$ Receptors by Protein Kinase C and Hypoxia in Human NT2-N Neurons

Submitted by

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Regulation of $\text{GABA}_A$ Receptors by Protein Kinase C

and Hypoxia in Human NT2-N Neurons

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DEDICATION

I dedicate this dissertation to my wife, whose love has made me stronger, smarter and happier. Thank you for your strength through the hardest personal challenge of our lives within this enduring education. Your support and unwavering respect for me will always be the most motivating forces in my life.
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\textbf{INTRODUCTION}

\textit{\textgamma -Aminobutyric acid (GABA) is the most widely distributed inhibitory neurotransmitter in the mammalian brain.} The GABA binds to GABA\textsubscript{\textgamma} receptors (GABARs) to regulate gating (opening and closing) of the chloride ion channel (Macdonald and Olsen, 1994). The GABAR belongs to the superfamily of ligand-gated ion channels that includes the nicotinic acetylcholine receptor, the glycine receptor and the 5-hydroxytrytamine type 3 receptor. Like the other members of this receptor superfamily, GABARs are pentameric hetero-oligomers that can be assembled from seven subunit families with multiple subtypes: \textalpha(1-6), \textbeta(1-3), \textgamma(1-3), \textdelta, \textepsilon, \textpi, and \texttheta (Macdonald and Olsen, 1994; Sieghart and Sperk, 2002). All GABAR subunits consist of a large N-terminal extracellular domain, four transmembrane (TM) domains, a large intracellular loop between TM3 and TM4, and a small C-terminal extracellular domain (Macdonald and Olsen, 1994). The GABAR currents can be modulated by positive and negative allosteric agents including benzodiazepines (BZs), barbiturates, neurosteroids, bicuculline and zinc. Studies of recombinant GABARs have shown that different subunit or subtype compositions influence their pharmacological and biophysical properties including agonist efficacy, channel kinetics, and allosteric properties (Olsen and Macdonald, 2002; Rudolph et al., 2001; Sieghart et al., 1999). For example, BZ augmentation of GABAR currents requires a \textgamma subunit, and BZ specificity is determined by the \textalpha subtype (Korpi et al., 2002). Presence of \textalpha1 results in a receptor with high affinity
for zolpidem, while the $\alpha_2$ or $\alpha_3$ subtypes, combined with $\beta$ and $\gamma$ subunits, result in receptors with moderate zolpidem affinity. The GABARs containing $\alpha_5$ are sensitive to the BZ, diazepam, but are insensitive to zolpidem, while GABARs with $\alpha_4$ or $\alpha_6$ are insensitive to most BZs. Specific GABAR $\alpha$ subunits also appear to mediate the different clinical properties of the BZs, with $\alpha_1$ responsible for sedative, amnestic and anticonvulsant actions and $\alpha_2$, $\alpha_3$ and $\alpha_5$ responsible for anxiolytic and other properties (Rudolph et al., 2000). Changes in subunit composition may thus alter GABAR pharmacology (Macdonald and Olsen, 1994) and affect the inhibitory pathways involved in encephalopathy and seizures.

Neurotransmission by GABA is essential in controlling the excitability of the brain (Jones-Davis and Macdonald, 2003; Olsen and Avoli, 1997), anxiety status (Pratt, 1992), feeding and drinking behavior (Cooper, 1989), and cognition, memory and learning (Chapouthier and Venault, 2002; Rayevsky and Kharlamov, 1983). Modulation of GABAR expression, cellular distribution, and function, therefore, has profound consequences for neural excitability under both physiological and pathophysiological conditions. Substantial evidence links GABAR dysfunction to a variety of neurological disorders such as seizures and myoclonus. Particular interests of this dissertation are mechanisms that regulate GABAR function and expression, including GABAR transcriptional modification, translational regulation, and modification of recycling or a change in receptor phosphorylation status. These studies investigate the roles of hypoxia and protein kinase C (PKC) in the regulation of GABAR function in human NT2-N cells. Furthermore, we will examine whether
hypoxia regulates GABAR function and expression via the hypoxia inducible factor-1α (HIF-1α) pathway.

In these studies, we have used a neuronal cell model derived from a human tumor cell line. NTera2 cells (NT2), derived from a human teratocarcinoma, terminally differentiate into neuron-like NT2-N cells when treated with retinoic acid (RA) (Andrews, 1984). NT2-N cells extend dendritic and axonal processes and express neuron-specific markers (Pleasure et al., 1992) and functional neurotransmitter receptors (Beczkowska et al., 1997). The NT2-N cells express human GABARs with a consistent pharmacological profile and a pattern of GABAR subunit mRNA expression (Neelands et al., 1998) that develops over time in vitro (Neelands et al., 1999). Undifferentiated NT2 cells strongly expressed α5, β3, and π subtype mRNA and weakly express γ3, while NT2-N cells after 5 wk of retinoic acid treatment expressed high levels of α2, α3, α5, β3, γ2, γ3, ε and π subunit mRNAs. The changes in GABAR subunit expression and pharmacology were similar to many developmental changes in mammalian CNS neurons. The consistent expression of GABAR subunits at specific time-points allows our investigation of the regulation of GABAR function and expression by PKC and hypoxia.

The first study in this dissertation, published in Neuropharmacology (Gao and Greenfield, 2005), examined the hypothesis that alteration of GABAR phosphorylation status by PKC changes GABAR sensitivity to BZs. GABAR function can be regulated by phosphorylation by Ser/Thr and Tyr protein kinases at
consensus phosphorylation sites in the major intracellular domains of several different GABAR subunits (Brandon et al., 2002; Moss et al., 1992b, 1995). Phosphorylation by PKC has frequently been reported to modulate GABAR function. Most results suggest that PKC activation decreases GABAR function (Filippova et al., 2000; Krishek et al., 1994; Leidenheimer et al., 1992) though in some systems PKC activation increases GABAR currents (Lin et al., 1996, 1994; Poisbeau et al., 1999). However, relatively few studies have focused on the effects of PKC on BZ- and barbiturate-mediated potentiation of GABAR currents, and the results have been inconsistent or even contradictory. Activation of PKC enhanced BZ and pentobarbital potentiation of \( \alpha_1\beta_1\gamma_2 \) GABAR currents expressed in *Xenopus laevis* oocytes (Leidenheimer et al., 1993). However, others have found no PKC-induced change in sensitivity to diazepam or pentobarbital using the same expression system (Ghansah and Weiss, 2001). In this study, we evaluated the effects of PKC activity on GABAR sensitivity to BZs by comparing GABA currents in NT2-N cells before and after exposure to different PKC modulators. Using whole-cell and gramicidin “perforated-patch” voltage-clamp recording techniques, we found that PKC activation had no direct effect on maximal GABAR currents or the GABA concentration-response relationship, but reduced diazepam potency to enhance GABAR currents without changing maximal enhancement. In cells exposed to diazepam for 7 d, maximal enhancement of GABAR currents by diazepam was decreased with no change in the concentration required to produce half-maximal currents (EC\(_{50}\)). In these diazepam-treated cells, PKC activation also shifted the EC\(_{50}\) rightward, suggesting that the effects of PKC to reduce diazepam potency are
distinct from the reduction in maximal efficacy induced by chronic diazepam exposure. Hence, PKC may reduce diazepam affinity at its GABAR binding site by decreasing the allosteric coupling between BZ and GABA sites on NT2-N GABARs.

The second manuscript, also published in *Neuropharmacology* (Gao et al., 2004), assessed the effect of hypoxia on GABAR function and expression in NT2-N cells. Hypoxia or ischemia can cause severe central nervous system (CNS) dysfunction including encephalopathy (Low et al., 1985), seizures (Bergamasco et al., 1984) and myoclonus (Hallett, 2000). Substantial evidence links dysfunction of GABARs to seizures and myoclonus. The GABARs play a key role in the control of cell and network activity, and therefore, modification of GABAR function by hypoxia will have important consequences to neural excitation and communication (Schwartz-Bloom and Sah, 2001). After hypoxia, significant changes in GABARs number and function have been observed in many experimental models. Binding to GABARs in gerbil hippocampus was reduced after hypoxia, which was thought to result from receptor internalization (Alicke and Schwartz-Bloom, 1995). Electrophysiological studies (Harata et al., 1997) also have demonstrated a down-regulation of hippocampal GABAR function, which were attributed to changes in the transmembrane Cl⁻ gradient (Inglefield and Schwartz-Bloom, 1998) resulting from decreased adenosine trisphosphate (ATP) or increased intracellular Ca²⁺ (Harata et al., 1997). However, the actual mechanisms underlying changes in GABAR function remain unknown. In this study, we have used whole-cell patch clamp recordings and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to
investigate the effects of hypoxia (8 h at $O_2 \leq 1\%$) on GABAR pharmacology and subunit mRNA expression in NT2-N neurons *in vitro*. Hypoxic exposure altered GABAR function with a biphasic time-course, accompanied by changes of GABAR subunit mRNA. The regulation of GABAR subunit gene expression by hypoxia may be a novel mechanism underlying changes in inhibitory neurotransmission after hypoxia.

Based on the above findings, the third manuscript, in preparation for submission, deals with the theory that hypoxia induced factor 1 (HIF-1) is sufficient to regulate GABAR expression and function. Several lines of evidence suggest that hypoxia regulates GABAR subunit gene expression (Gao et al., 2004; Li et al., 1993), which could be part of the mechanism of GABAR regulation in post-hypoxic state. Studies of transcriptional signaling by hypoxia have focused on several transcription factors, such as activator protein 1 (AP-1) (Premkumar et al., 2000), the early growth response factor 1 (EGR-1) (Yan et al., 2002, 1999), the nuclear factor κB (NF-κB) (Koong et al., 1994), the CCAAT-enhancer binding protein (C/EBPβ/NF-IL-6) and HIF-1 (Semenza, 2001), which were found to be involved in the modulation of gene expression by oxygen. Among them HIF-1 has been shown to have a key role. In the second manuscript, we demonstrated that hypoxia altered GABAR function and subunit expression in the NT2-N neuronal cell model (Gao et al., 2004). We have also found that hypoxia induced the accumulation of HIF-1. The HIF-1 is a heterodimeric transcription factor composed of two subunits, HIF-1α, which is degraded during normoxia by interaction with the tumor suppressor von
Hippel Lindau (VHL) protein, and HIF-1β, which is constitutively expressed (Semenza, 2001). In addition to hypoxia, other means to induce HIF-1α include exposure to cobalt chloride, the iron chelator, deferoxamine (DFX), as well as other factors, including insulin, thrombin and angiotensin II (AngII) (Page et al., 2002; Semenza, 2002). Activated HIF-1 has been shown to be a key regulator in cellular and physiological responses to the stress of hypoxia. In this study, using western immunoblotting, we demonstrated that HIF-1α protein level increased after both cobalt and DFX treatment. Using whole-cell recording and RT-PCR techniques, we found that maximal GABA currents increased 48 h after exposure to either cobalt or DFX, and gene expression of some GABAR subunits increased 24 h after cobalt or DFX treatment. These changes confirm that stimulation of HIF-1α protein accumulation can alter GABAR expression and function, but in the opposite direction from hypoxia, suggesting that additional factors induced by hypoxia may override hypoxic stimulation of HIF and result in downregulation of GABAR function and subunit expression.
GABAR Structure and Pharmacology

γ-Aminobutyric acid (GABA) is the most widely distributed inhibitory neurotransmitter in the mammalian brain. It has been estimated that about 30% of all synapses use GABA as a transmitter (Bloom and Iversen, 1971; Leung and Xue, 2003). The GABA binds to the GABAR to open its chloride ion channel, which mediates most fast inhibitory neurotransmission in the CNS. The GABAR is a member of a superfamily of ligand-gated ion channels that also includes the nicotinic acetylcholine receptor, the 5-hydroxytryptamine type 3 receptor and the glycine receptor. Like other members of this receptor superfamily, GABARs are pentameric hetero-oligomers that can be assembled from seven subunit families with multiple subtypes: α(1-6), β(1-3), γ(1-3), δ, ε, π, and θ, as well as β2 and γ2 splice variants (Table I) (Macdonald and Olsen, 1994; Sieghart and Sperk, 2002). Each GABAR subunit is a polypeptide of about 50 kDa. There is 30-40% sequence identity among the subunit families and about 70-80% identity in subtypes within each subunit family. All GABAR subunits consist of a large N-terminal extracellular domain, four TM domains, a large intracellular loop between TM3 and TM4, and a small C-terminal extracellular domain (Macdonald and Olsen, 1994). The major intracellular domains of GABAR subunits contain a number of consensus phosphorylation sites for both Ser/Thr and Tyr protein kinases (Brandon et al., 2002).
Table I. GABA\textsubscript{A} Receptors

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<thead>
<tr>
<th><strong>GABA\textsubscript{A} receptors</strong></th>
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<tbody>
<tr>
<td>Channel type</td>
<td>Cl\textsuperscript{–} channel, also permeable to HCO\textsubscript{3}\textsuperscript{–}</td>
</tr>
<tr>
<td>Conductance</td>
<td>27-30 pS</td>
</tr>
<tr>
<td>Mean channel open time</td>
<td>25-30 ms</td>
</tr>
<tr>
<td>Channel composition</td>
<td>Heterooligomeric</td>
</tr>
<tr>
<td>Subunit composition</td>
<td>(\alpha)1-6, (\beta)1-3, (\gamma)1-3, (\delta), (\varepsilon), (\theta)</td>
</tr>
<tr>
<td>Chromosomal location of coding genes</td>
<td>Chromosomes 1, 4, 5,15 and X</td>
</tr>
<tr>
<td>Selective agonist</td>
<td>Muscimol, GABA</td>
</tr>
<tr>
<td>Potency order of common agonists</td>
<td>Muscimol &gt; GABA &gt; TACA</td>
</tr>
<tr>
<td>Competitive antagonist</td>
<td>Bicuculline</td>
</tr>
<tr>
<td>Non-competitive antagonist</td>
<td>Picrotoxin, PTZ, TBPS</td>
</tr>
<tr>
<td>Channel blocker</td>
<td>Penicillin</td>
</tr>
<tr>
<td>Benzodiazepine receptor agonists</td>
<td>Diazepam, clonazepam</td>
</tr>
<tr>
<td>Benzodiazepine receptor inverse agonists</td>
<td>DMCM, (\beta)CCM</td>
</tr>
<tr>
<td>Benzodiazepine receptor antagonist</td>
<td>Flumazenil</td>
</tr>
<tr>
<td>Anchoring protein</td>
<td>GABARAP, gephyrin</td>
</tr>
</tbody>
</table>

This table is adapted from (Macdonald and Olsen, 1994).
Depending on their subunit composition, GABARs exhibit distinct pharmacological and electrophysiological properties (Sieghart, 1995). Most native GABARs in the brain are composed of two α-, two β- and one γ-subunits (Sieghart et al., 1999). The δ, ε and π subunits seem to be able to replace the γ subunit in GABARs, whereas the θ subunit might be able to replace a β subunit (Sieghart et al., 1999). The GABAR subunits are differentially expressed both temporally and spatially throughout the brain. For example, the α2, α3 and β3 subunits are the major α and β subunits in the fetal brain, respectively, whereas the α1 and β2 subunits are mainly expressed after birth (Laurie et al., 1992; Poulter et al., 1992). Several studies have characterized the regional distribution of GABAR subunits in the brain (Sperk et al., 1997; Wisden et al., 1992). Results from recent studies suggest that the α1, β2, and γ2 subunits are the most abundant GABAR subunits in the brain, while α2 and α3 are present in 35% and >14% of all brain GABARs, respectively (Sieghart and Sperk, 2002). The α4 and α5 subunits are present in only 6% and 7% of all GABARs, respectively, but these subunits are especially enriched in hippocampus, about 13% for α4 and 31% for α5. The α6 subunit is present only in cerebellar granule cells and in the cochlea. In the cerebellum, about 58% of GABARs contain α6 subunits. Most of these results are from rodent experiments, and the expression and regional distribution of GABAR subunits in rodents may not necessarily correspond to that in the human brain. For example, the α3 subunit is virtually absent in rodent hippocampus, but was strongly expressed in the CA1 area of human hippocampus and was present to varying degrees in dentate granule cells of human
brain (Loup et al., 2000). In addition, almost no $\alpha_1$-subunit staining was observed in human CA3 pyramidal cells, whereas moderate $\alpha_1$-subunit immunoreactivity has been reported in rodent CA3 dendritic fields (Loup et al., 2000). Because of these species differences, we should be cautious in interpreting findings from animal models of human diseases.

The GABARs can be pharmacologically defined by their responses to agonists, competitive and noncompetitive antagonists, and various allosteric modulators. The ligand binding sites on GABARs are formed by protein regions conserved among the subunits. The amino acid residues involved in ligand-binding pockets are homologous among super-family members. In searching for the GABA binding sites, help comes from the results of experiments on nicotinic acetylcholine receptors (nAChR) from the electric ray, Torpedo, and the pentameric water-soluble acetylcholine binding protein (AChBP), which is homologous to the extracellular domain of the nAChR. Experimental evidence suggests that the GABA-binding site lies within an asymmetric pocket formed at the interface between $\alpha$ and $\beta$ subunits. Specific amino acid residues, including F65, R67 and S69 in the $\alpha_1$ subunit (Boileau et al., 1999) and T202, S204, Y205, R207, S209 in the GABAR $\beta_2$ subunit (Wagner and Czajkowski, 2001), have been demonstrated to participate in GABA binding sites by mutagenesis and substituted cysteine accessibility modification (SCAM).

The binding of GABA to the GABAR regulates gating of the chloride ion channel. Studies have showed that each TM domain is $\alpha$-helical and that the TM2
domain from each subunit forms the water-filled pore. Inspection of the sequence of
the TM2 segments of the various GABAR subunits reveals the presence of several
highly conserved hydrophilic residues. This hydrophilic region of TM2 is unlikely to
be involved in ion selectivity. Jensen et al. (2002) used site-directed mutagenesis to
demonstrate that the TM1–TM2 loop of the β subunit plays a dominant role in
determining the ion selectivity of the GABAR, suggesting that the selectivity filter is
structurally asymmetric. Binding of agonist triggers a complex structural transition
known as “gating” that results in the opening of the channel. A further challenge has
been to define the regions involved in coupling agonist binding with gating. A recent
study (Kash et al., 2004) using site-directed mutagenesis and disulfide trapping
suggested that interactions between an acidic residue in loop 7 (Asp^{146}) and a basic
residue in pre-transmembrane domain-1 (Lys^{215}) were involved in coupling agonist
binding to channel gating.

The GABARs have multiple binding sites for positive and negative allosteric
modulators, including BZs, barbiturates and neurosteroids, as well as bicuculline,
pricrtoxin and zinc (Jones-Davis and Macdonald, 2003; Macdonald and Olsen,
1994) (Table II). Studies of recombinant GABARs have shown that different subtype
compositions determine their pharmacological and biophysical properties. The
ability of BZs to enhance GABAR currents depends on the presence of specific α
subunit subtypes (α1, α2, α3, α5), a β subunit and a γ2 subunit. Further BZ
specificity can be defined by sensitivity to the benzodiazepine-like agent, zolpidem.
Presence of α1 results in a receptor with high affinity for zolpidem, while the α2 or
Table II. Subunit-Specific Pharmacological Properties of Recombinant GABAR

<table>
<thead>
<tr>
<th>Pharmacological Property</th>
<th>Subtypes</th>
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<tr>
<td><strong>Benzodiazepines</strong></td>
<td></td>
</tr>
<tr>
<td>Benzodiazepine enhancement</td>
<td>γ2 with α &amp; β</td>
</tr>
<tr>
<td>High benzodiazepine affinity (nM BZ affinity)</td>
<td>α1, α2, α3 or α5 with γ2, β</td>
</tr>
<tr>
<td>BZ 1 Pharmacology (high zolpidem affinity)</td>
<td>α1 with γ2, β</td>
</tr>
<tr>
<td>BZ 2a,b Pharmacology (low zolpidem affinity)</td>
<td>α2, α3 with γ2, β</td>
</tr>
<tr>
<td>BZ 2c Pharmacology (zolpidem insensitive)</td>
<td>α5 with γ2, β</td>
</tr>
<tr>
<td>BZ 3 Pharmacology (BZR agonist insensitive)</td>
<td>α4 or α6 with γ2, β</td>
</tr>
<tr>
<td><strong>Barbiturates</strong></td>
<td></td>
</tr>
<tr>
<td>High sensitivity enhancement</td>
<td>α6 with β or β, γ</td>
</tr>
<tr>
<td>Low sensitivity enhancement</td>
<td>α1 with β or β, γ</td>
</tr>
<tr>
<td>High sensitivity and efficacy for direct activation</td>
<td>α6 with β or β, γ</td>
</tr>
<tr>
<td>Low sensitivity and efficacy for direct activation</td>
<td>α1 with β or β, γ</td>
</tr>
<tr>
<td><strong>Propofol</strong></td>
<td></td>
</tr>
<tr>
<td>High sensitivity direct activation</td>
<td>α6 with β or β, γ</td>
</tr>
<tr>
<td>Low sensitivity direct activation</td>
<td>α1 with β or β, γ</td>
</tr>
<tr>
<td><strong>Loreclezole</strong></td>
<td></td>
</tr>
<tr>
<td>High loreclezole sensitivity (EC50 = 1 μM)</td>
<td>β2 or β3 with α or α, γ</td>
</tr>
<tr>
<td>Low loreclezole sensitivity</td>
<td>β1 with α or α, γ</td>
</tr>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
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<tr>
<td>Enhancement</td>
<td>Phosphorylated γ2L subtype?</td>
</tr>
<tr>
<td><strong>Furosemide</strong></td>
<td></td>
</tr>
<tr>
<td>High furosemide sensitivity (IC50 = 10 μM)</td>
<td>α4 or α6 with β or β,</td>
</tr>
<tr>
<td>Low furosemide sensitivity (IC50 = 3 mM)</td>
<td>α1, α2, α3, or α5 with β or β, γ</td>
</tr>
<tr>
<td><strong>Zinc</strong></td>
<td></td>
</tr>
<tr>
<td>High zinc sensitivity (IC50 &lt; 10 μM)</td>
<td>α &amp; β without γ2</td>
</tr>
<tr>
<td>Moderate zinc sensitivity (IC50 10-100 μM)</td>
<td>α6 &amp; β without γ2</td>
</tr>
<tr>
<td>Low zinc sensitivity (IC50 &gt;100 μM)</td>
<td>α1 &amp; β with γ2</td>
</tr>
<tr>
<td><strong>Lanthanum</strong></td>
<td></td>
</tr>
<tr>
<td>Enhancement by lanthanum</td>
<td>α1 with β, γ</td>
</tr>
<tr>
<td>Inhibition by lanthanum</td>
<td>α6 with β, γ</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
</tr>
<tr>
<td>Enhancement by hydrogen ion</td>
<td>α1 &amp; β1 with or without δ</td>
</tr>
<tr>
<td>Inhibition by hydrogen ion</td>
<td>α1, β1, γ2 &amp; δ</td>
</tr>
</tbody>
</table>

This table is adapted from (Jones-Davis and Macdonald, 2003).
α3 subtypes, combined with β and γ subunits, result in receptors with moderate zolpidem affinity. The GABARs containing α5 are sensitive to the BZ, diazepam, but are insensitive to zolpidem, while GABARs with α4 or α6 are insensitive to most BZs. High zinc-sensitivity is conferred by the absence of an α1 subunit and/or a γ subunit, and the presence of an α4, α5 or α6 subtype. In contrast, enhancement of GABAR currents by the novel anticonvulsant, loreclezole, is determined by the presence of a β2 or β3 subunit (Jones-Davis and Macdonald, 2003), and is not found in β1-containing receptors due to a single amino acid difference (Wingrove et al., 1994). Changes in subunit composition may alter GABAR pharmacology, and thus affect inhibitory pathways resulting in an imbalance between inhibitory and excitatory systems.

**GABAR Trafficking**

The GABARs undergo constitutive receptor cycling between plasma membrane and intracellular compartments to maintain a stable cell surface receptor number (Barnes, 2000). Individual receptor subunits are synthesized de novo on the endoplasmic reticulum membrane and assembled into receptor complexes. Unassembled receptor subunits are degraded, while assembled receptors are targeted via the Golgi apparatus to the plasma membrane. The surface receptors may either aggregate to form synaptic clusters or cluster in clathrin-coated pits that produce coated vesicles by endocytosis. Via endosomal pathways, the internalized receptors can recycle to the surface or be degraded. Specific GABAR subunits and GABAR-
associated proteins have been implicated in the GABAR trafficking pathways. The protein gephyrin and γ2 subunit have been shown to play crucial roles in controlling GABAR synaptic targeting (Essrich et al., 1998). Knockout deletion of the γ2 subunit significantly decreases GABAR number and gephyrin at inhibitory synapses, while depletion of gephyrin results in a dramatic disruption of both GABAR clusters and synaptic targeting of the γ2 subunit (Essrich et al., 1998; Kneussel et al., 1999). Another protein, GABA$_A$ receptor associated protein (GABARAP), also has been identified to interact specially with γ2 subunit and play an important role in receptor clustering (Coyle et al., 2002). Recently, the ubiquitin-like protein Plic-1 has been identified as a GABAR associated protein using the yeast two-hybrid system (Bedford et al., 2001). Plic-1 contains an ubiquitin-like amino-terminus and a carboxy-terminal ubiquitin-associated (UBA) domain. The UBA domain of Plic-1 interacts with the major intracellular loop of the GABAR’s α and β subunits, and specifically enhances GABAR membrane insertion by increasing the half-life of intracellular receptor pools. Plic-1 appears to mediate GABAR stabilization by inhibiting GABAR poly-ubiquination and reducing receptor targeting to the proteasome (Bedford et al., 2001).

**GABAR Dysfunction in CNS Diseases**

Dysfunction of GABARs has been implicated in many CNS disorders such as epilepsy, encephalopathy and myoclonus. Drugs acting on the GABARs have been clinically useful in the treatment of anxiety, epilepsy, sleep disorders, alcohol
withdrawal and in the induction and maintenance of anesthesia. Further understanding of the cellular and genetic involvement of GABARs will be very helpful to prevent and treat these CNS disorders.

Epilepsy affects more than 0.5% of the population in the world (Kaneko et al., 2002). Epileptic seizures are associated with abnormally hyperactive neuronal discharges resulting from an imbalance between neuronal excitation and inhibition. The “GABA hypothesis” of seizure disorders suggests that disinhibition resulting from a deficit in GABAergic inhibitory synaptic transmission may contribute to the excessive excitatory activity of epileptic brain. Proof of this concept has come from the confirmation of epilepsy mutations involving GABAR subunit genes. Two groups (Baulac et al., 2001; Wallace et al., 2001) have reported distinct GABAR subunit mutations in two families with generalized epilepsy syndromes. In both families the mutation was in the GABARG2 gene that encodes the GABAR γ2 subunit. The GABAR γ2 subunit, however, is not the only GABAR subunit that has been implicated in the development of epilepsy and related syndromes. The evidence from the β3 subunit knockout mice suggested its importance in preventing the development of Angelman Syndrome: a disorder that is characterized by mental retardation, hyperactivity, sleep disorders and motor dysfunction, as well as epilepsy. Additionally, a mutation in the α1 subunit causing reduction of function has been found in a family with Juvenile Myoclonic Epilepsy (Cossette et al., 2002). These genetic studies clearly implicate the GABAR in the genesis of epilepsy and epilepsy syndromes.
**NT2-N Cells as a Neuronal Cell Model**

The NT2 cell line, derived from a human teratocarcinoma, differentiates terminally into neuron-like NT2-N cells when treated with RA (Pleasure et al., 1992). The NT2-N cells extend dendritic and axonal processes and express neuron-specific cell surface, cytoskeletal, and secretory markers and functional neurotransmitter receptors (Neelands et al., 1998; Pleasure et al., 1992), which include nicotinic and muscarinic acetylcholine receptors and choline acetyltransferase (Zeller and Strauss, 1995), N-methyl-D-aspartate (NMDA) (Munir et al., 1996, 1995) and non-NMDA (Younkin et al., 1993) glutamate channels, metabotropic glutamate receptors (Paterlini et al., 1998), dopamine receptors (Sodja et al., 2002), GABA\textsubscript{A} receptors (Matsuoka et al., 1997; Neelands et al., 1999, 1998), voltage-gated sodium channels (Greenfield et al., 1997), L- and N-type calcium channels (Gao et al., 1998), the $\text{K}^+$ and $\text{Cl}^-$ cotransporter 2 (KCC2) (Song et al., 2002) and the synaptic proteins SNAP25 and Rab-3a (Sheridan and Maltese, 1998). When plated on the primary astrocytes, NT2-N cells form both glutamatergic excitatory and GABAergic inhibitory synapses (Hartley et al., 1999) whose properties were similar to those of synapses of neurons in primary cultures. This system provides a model for further investigations into the neurobiological properties of receptors and synapses formed by human neurons.
The NT2-N cells express human GABARs with a consistent pharmacological profile and a pattern of GABAR subunit mRNA expression that develops over time in vitro (Neelands et al., 1999, 1998). Undifferentiated NT2 cells strongly expressed α5, β3, and π subtype mRNA and weakly expressed γ3, while NT2-N cells after 5 wk of RA treatment expressed high levels of α2, α3, α5, β3, γ3, and π subunit mRNAs (Neelands et al., 1999). The changes in GABAR subunit expression and pharmacology were similar to changes in mammalian CNS neurons during development, which may provide a useful model to explore the mechanisms underlying developmental regulation of GABAR subunit expression. The consistent expression of GABAR subunits at specific time points makes this model system nearly ideal for investigating the regulation of GABAR function and expression by PKC and hypoxia.

The NT2-N cells have been used as a reliable neuronal model in many experimental studies. In a study of cell death after hypoxia, one group (Rootwelt et al., 1998) found that NT2-N neurons died via an ionotropic glutamate receptor-mediated mechanism when exposed to hypoxia in the presence of glutamate; this was the first reported analysis of the mechanism of hypoxic cell death in cultured human neuronlike cells. Another group (Cao et al., 2001) reported that transfection of NT2-N neurons with pCMV-bcl2 or pHRE-bcl2 significantly reduced the numbers of apoptotic cells after hypoxia, which suggests a novel therapeutic strategy for alleviation of damage to ischemic brain areas by expression of anti-apoptotic or neurotrophic transgene proteins in hypoxic neurons. There are also very promising
results from experiments in which differentiated NT2-N cells as well as undifferentiated NT2 cells have been successfully grafted into rodent (Lee et al., 2000; Nelson et al., 2002; Trojanowski et al., 1997) and human CNS (Nelson et al., 2002; Newman et al., 2005) to aid recovery from neuron loss after ischemic insults. The NT2-N cells thus show potential for cell-mediated therapy to treat CNS diseases.

**GABAR and PKC Phosphorylation**

The major intracellular domains of many GABAR subunits, including α6, β subunits, and γ2L, contain a number of consensus sites for phosphorylation by serine/threonine and tyrosine protein kinases including protein kinase A (PKA), PKC, Ca$^{2+}$-calmodulin-dependent kinase II and Src family tyrosine kinases (Moss and Smart, 1996). Many studies have shown that PKC plays an important role in regulating GABAR trafficking and responses to GABA and allosteric modulators.

Protein kinase C is a family of some 12 serine-threonine kinases. The best-understood and most studied group is the conventional PKCs (cPKCs), which consist of α, β, and γ isotypes. The cPKCs are activated by phosphatidylserine (PS) in a Ca$^{2+}$-dependent manner; they also bind diacylglycerol (DAG), which increases enzyme affinity for PS and Ca$^{2+}$. The cPKCs can be activated by the tumor-promoting phorbol esters, phorbol myristate acetate (PMA) and phorbol dibutyrate (PDBu), by eliminating the requirement for DAG and decreasing the concentration of Ca$^{2+}$ needed for activation (Castagna et al., 1982). The novel PKCs (nPKCs)
comprise the \( \epsilon, \eta, \delta, \) and \( \theta \) isotypes. These kinases are \( \text{Ca}^{2+} \)-insensitive, but are still activated by DAG or phorbol ester in the presence of PS (Ono et al., 1988). The atypical PKCs (aPKCs) comprise the \( \iota \) and \( \zeta \) isotypes, which are insensitive to both \( \text{Ca}^{2+} \) and PMA/DAG (Ono et al., 1989). Finally, the recently discovered protein kinase C-related kinases (PRKs) define a fourth grouping consisting of at least three members, PRK1-3. The PRKs are insensitive to \( \text{Ca}^{2+} \), DAG and phorbol esters (Mukai et al., 1994; Palmer and Parker, 1995). Phorbol esters have been used extensively as pharmacological probes of PKC function. They bind to PKCs at the DAG site in the regulatory domain and stimulate prolonged activation of cPKCs and nPKCs (Nishizuka, 1992). It is important to note that phorbol esters can bind and modulate the function of other molecules besides PKC (Brose and Rosenmund, 2002). Therefore, responses to phorbol esters assumed to be due to PKC activation should be confirmed by blocking phorbol ester effects with PKC inhibitors.

Several laboratories have reported that activation of PKC by phorbol esters resulted in a significant inhibiton of GABA or muscimol-induced whole-cell currents (Kellenberger et al., 1992; Krishek et al., 1994; Leidenheimer et al., 1992; Sigel et al., 1991). An inhibitory effect of PKC is observed on receptors composed of \( \alpha 1\beta 1 \), \( \alpha 1\beta 1\gamma 2S \) or \( \alpha 1\beta 1\gamma 2L \) (Krishek et al., 1994; Sigel et al., 1991), \( \alpha 5\beta 2\gamma 2S \) or \( \alpha 5\beta 2\gamma 2L \) (Sigel et al., 1991), and \( \alpha 1\beta 2\gamma 2S \) (Kellenberger et al., 1992) subunits. Functional studies using site-directed mutagenesis determined that this down-regulation of receptor function is mediated by phosphorylation of \( \beta 1(S409) \), \( \beta 2(S410) \) (Kellenberger et al., 1992), \( \gamma 2S(S327) \) (Kellenberger et al., 1992; Krishek et al., 1991).
1994), and $\gamma_2L(S327)$ and S343 (Krishek et al., 1994). Moreover, these same sites, as well as S408 and S409 in $\beta_3$, can be phosphorylated by PKC \emph{in vitro} (McDonald and Moss, 1997; Moss et al., 1992a). These findings suggest that PKC can phosphorylate $\beta$ and $\gamma_2$ subunits to decrease activation of GABARs by GABA. In contrast, other studies using the intracellular dialysis of the active domain of PKC as a stimulus, have demonstrated enhanced GABA-induced Cl$^-$ currents (Lin et al., 1996, 1994; Poisbeau et al., 1999). The differences seen in the effects of PKC on GABAR function could be due to the different ways used to activate PKC (phorbol esters versus constitutively active PKC), or differences in the recording techniques or experimental models.

Several studies have tested the effects of PKC on GABAR sensitivity to allosteric modulators. Interestingly, PKC activation enhanced BZ potentiation of $\alpha_1\beta_1\gamma_2$ GABAR currents expressed in \emph{Xenopus laevis} oocytes (Leidenheimer et al., 1993). Similar enhancement of pentobarbital-induced potentiation of GABAR currents also has been reported in the same laboratory (Leidenheimer et al., 1993). However, others have found no PKC-induced change in sensitivity to diazepam or pentobarbital using the same expression system (Ghansah and Weiss, 2001). In specific PKC$\varepsilon$ isoform knockout mice, increased sensitivity to diazepam and pentobarbital has been observed (Hodge et al., 1999), while no changes in the sensitivity of GABARs to BZ or barbiturates have been seen in mice devoid of PKC$\gamma$ (Harris et al., 1995). The variable effects of PKC on GABAR function might result from differences in the neuron populations or expression systems, the GABAR
subunits expressed, the method and timing of PKC activation, the basal phosphorylation state of PKC targets, or the recording conditions.

The effects of PKC phosphorylation on GABAR function are also related to the GABAR recycling pathway. Activation of PKC has been shown to decrease the cell surface expression of GABARs (Chapell et al., 1998; Connolly et al., 1999). Activation of PKC promoted an increase in GABAR endocytosis in both HEK 293 cells and neurons that required the γ2 subunit, via a dynamin-dependent mechanism (Herring et al., 2005). This regulation was dependent on a dileucine motif within the receptor β2 subunit, as mutation of this sequence abolished PKC-mediated decreases in GABA-gated chloride currents in whole cell recording. Moreover, including a 10-amino acid peptide corresponding to the dileucine motif in the patch pipette solution prevented PKC modulation of receptor function. However, inhibition of PKC did not alter constitutive GABARs endocytosis, hence, PKC likely plays a regulatory role in receptor endocytosis.

**Hypoxia and GABAR Function**

The CNS is severely affected by hypoxic conditions such as cardiac arrest, major cerebral vessel thrombosis, pulmonary events and other situations. Prolonged hypoxia alters neurotransmitter systems causing an imbalance of excitatory and inhibitory neurotransmission leading to excitotoxicity (Yue et al., 1997), which results in a variety of neuropathological disorders such as encephalopathy (Low et
al., 1985), seizures (Bergamasco et al., 1984) and myoclonus (Hallett, 2000). The effect of hypoxia on N-methyl-D-aspartate receptor (NMDAR) expression and function has been studied in a number of systems (Aizenman et al., 2000; Bickler et al., 2000; Guerguerian et al., 2002; Gurd et al., 2002; Machaalani and Waters, 2002; Zanelli et al., 2002), and the role of NMDAR-mediated excitotoxicity in post-hypoxic neuronal apoptosis (Mishra et al., 2001) has previously been examined in NT2-N neurons (Rootwelt et al., 1998). Among the many neurotransmitter systems responding to hypoxia or ischemia, the GABAergic system may be particular important because GABA is the primary inhibitory neurotransmitter in mammalian brain and it functions in opposition to that of glutamate. Several GABAergic drugs have been shown some neuroprotective efficacy in animal models of cerebral ischemia (Galeffi et al., 2000; Inglefield et al., 1997; Madl and Royer, 2000; Schwartz-Bloom et al., 2000, 1998; Schwartz-Bloom and Sah, 2001). Understanding how hypoxia regulates inhibitory GABAergic systems may provide new insights for future treatment of ischemia/hypoxia induced neurological diseases.

Like most neurotransmitters, GABA has been shown to accumulate in the extracellular space in several ischemic models including the permanent middle cerebral artery occlusion model (Hagberg et al., 1985), the rat photochemical stroke model (Baldwin et al., 1994) and the gerbil global ischemia model (Mainprize et al., 1995; Shuaib et al., 1994). In the case of transient cerebral ischemia, the enhanced GABA concentration returns to normal levels within an hour of onset of reperfusion (Globus et al., 1991; Inglefield et al., 1995; Phillis et al., 1994; Schwartz et al.,
A similar change of GABA occurs in the ischemic human brain (Kanthan et al., 1995). Several mechanisms have been proposed for the increased extracellular GABA concentration during ischemia (Schwartz-Bloom and Sah, 2001), including both Ca$^{2+}$-dependent and -independent mechanisms. Other factors generated by ischemia could also cause the accumulation of extracellular GABA. For example, arachidonic acid and reactive oxygen species (ROS), generated during ischemia, have been shown to increase release and decrease uptake of GABA in many studies (Chan et al., 1983; Rego et al., 1996; Sah and Schwartz-Bloom, 1999). The accumulation of extracellular GABA during ischemia could be beneficial due to the increased GABA function, but one group has suggested that the accumulation of GABA could down-regulate GABA synthesis transiently (Green et al., 1992).

The GABARs, like many proteins, are affected by hypoxia or ischemic insults. Hypoxic regulation of GABAR function could occur at any of several levels including transcriptional regulation, translational modification, receptor recycling pathway regulation, or alteration of receptor phosphorylation status. Such regulation may be transient if the regulation involves changes in the receptor recycling pathway or phosphorylation status, or longer lasting if the regulation comes from transcriptional and translational modification.

There is specific evidence that hypoxia-induced GABAR regulation involves early short-term and later long lasting changes. Binding to GABARs in gerbil hippocampus and cerebral cortex was reduced within 30 min of the onset of...
reperfusion, which was thought to result from receptor internalization, but returned to normal after 2 h (Alicke and Schwartz-Bloom, 1995). Electrophysiological studies (Harata et al., 1997) also have demonstrated a down-regulation of hippocampal GABAR function early after the onset of reoxygenation. The actual mechanisms underlying these changes remain unknown, though several possibilities have been suggested. Increased intracellular calcium is a key mediator of neuronal injury produced by ischemia-reperfusion (Choi, 1995). The effects of calcium on GABAergic neurotransmission have been studied in many neuronal preparations. There was a significant reduction in muscimol-induced chloride uptake in cerebral cortical synaptoneurosomes after exposure to calcium (Schwartz et al., 1994). Increased intracellular calcium also decreased GABA currents in neuronal cultures (Martina et al., 1994; Stelzer et al., 1988). A major route for ischemia-induced Ca\(^{2+}\) influx is through glutamate receptors. In the immature brain, hypoxia-induced seizures induced immediate reduction in GABAR-mediated inhibition of hippocampal CA1 pyramidal neurons, and that this downregulation required the postsynaptic activation of Ca\(^{2+}\)-permeable AMPARs with secondary activation of the phosphatase calcineurin-A (CaN-A) (Sanchez et al., 2005). Hypoxia-induced CaN activation was associated with dephosphorylation of native GABARs, suggesting that altered phosphorylation status may be one mechanism by which GABARs may be regulated by hypoxia. Another explanation for the downregulation of GABAR function is the reduction of ATP levels during hypoxia. Several studies have shown that a reduction in intracellular ATP attenuated GABAR responses in neuronal cultures (Gyenes et al., 1988; Harata et al., 1997; Stelzer et al., 1988). Depletion of
ATP during hypoxia could contribute to the change in intracellular Cl⁻ accumulation or alteration of receptor phosphorylation state. The ischemia-induced increase in intracellular Cl⁻ has been implicated in the downregulation of GABAR function in many studies (Galeffi et al., 2004; Harata et al., 1997; Inglefield and Schwartz-Bloom, 1998); however, a few studies have shown that hypoxia leads to up-regulation of postsynaptic GABAR binding (Francis and Pulsinelli, 1983).

Other studies have demonstrated long-lasting hypoxia-induced changes in GABAR function. For example, a reduction in GABARs labeled by [³⁵S]TBPS in the hippocampal CA1 dendritic field and the striatum was seen several days after ischemia in gerbils and rats (Schwartz et al., 1995), and reductions in [³H]muscimol or [³H]flunitrazepam binding were seen in striatum 27 d after ischemia (Onodera et al., 1987). These later reductions in GABARs likely reflect loss of principal neurons on which they reside. One group (Li et al., 1993) has reported that hypoxia changed GABAR subunit expression in a transcriptional manner. They showed that there was a rapid decrease in expression of GABAR subunit (α1 and β2) mRNAs in hippocampal areas CA1, CA3 and dentate gyrus after hypoxia. Hypoxic regulation of GABAR gene expression could be another cause for the later changes in GABAR function after hypoxia.

Reactive oxygen species are another major factor in the regulation of GABAergic neurotransmission during and after hypoxia (Schwartz-Bloom and Sah, 2001). Such ROS as hydrogen peroxide, the superoxide anion, and the hydroxyl
radical are generated during reperfusion after hypoxia. Enhanced production of ROS, exceeding the antioxidant scavenging capacity of the cell, results in oxidative stress. The ROS have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA (Fridovich, 1995). There are several studies demonstrating that GABA neurotransmission is sensitive to ROS (Schwartz-Bloom and Sah, 2001). The effects of ROS on GABA neurotransmission can occur through either pre- or post-synaptic mechanisms. An increase of GABA release has been reported in hippocampal slices (Sah and Schwartz-Bloom, 1999) and cultured neurons (Rego et al., 1996) on exposure to ROS. In addition, peroxidation produced by iron ascorbate decreased synaptosomal GABA uptake (Palmeira et al., 1993). The ROS also have postsynaptic effects on GABARs. The generation of superoxide radicals decreased GABAR activity in cerebral cortical synaptoneurosomes (Schwartz et al., 1988). H₂O₂ reduced GABAR responses in hippocampal neurons from immature rats (Sah and Schwartz-Bloom, 1999). Exposure to ROS reduced GABA-gated Cl⁻ channel function in hippocampal slices (Sah et al., 2002). Moreover, exposure to H₂O₂ or oxygen free radicals significantly reduced inhibitory postsynaptic potentials (IPSPs) in hippocampal, cerebral cortical and thalamic neurons (Frantseva et al., 1998; Pellmar, 1995). In the later case, H₂O₂-induced free radical formation caused epileptiform activity in thalamocortical slices (Frantseva et al., 2000, 1998). The ROS can also influence gene expression by inducing several important transcription factors, which could be another mechanism by which GABARs are regulated.
Hypoxia and Transcription Factors

Evidence has been presented that hypoxia regulates GABAR subunit gene expression (Gao et al., 2004; Li H. et al., 1993), but the underlying mechanism is uncertain. Studies of hypoxic regulation of gene expression have focused on several transcription factors, such as AP-1 (Premkumar et al., 2000), EGR-1 (Yan et al., 1999), NFkB (Koong et al., 1994), C/EBPβ/NF-IL-6 (Yan et al., 2002) and HIF-1 (Semenza, 2001), all of which have been found to be involved in the modulation of gene expression by oxygen. Among them, HIF-1 has been shown to play a key role.

The HIF-1 is a heterodimeric transcription factor composed of two subunits, HIF-1α and ARNT (HIF-1β). Both subunits belong to the basic helix-loop-lelix-Per/ARNT/AhR/Sim (bHLH-PAS) transcription factor family (Wang and Semenza, 1995). The HIF-1β is constitutively expressed (Semenza, 2002) while the expression of HIF-1α subunit is tightly regulated by the cellular oxygen concentration (Wang et al., 1995; Wang and Semenza, 1995). In normoxia, HIF-1α is degraded by the proteasome 26S. The HIF-1α contains an oxygen-dependent degradation domain (aa 401-603) which is a highly conserved binding domain for the tumor suppressor von Hippel-Lindau protein (pVHL) (Tanimoto et al., 2000). The pVHL binds a complex containing elongins B and C, as well as cullin 2 and Rbx1 (Maxwell et al., 2001) and activates the E3 ubiquitin ligase, which then ubiquitinylates HIF-1α, targeting it for degradation (Figure 1). Prolyl hydroxylation of Pro564 and Pro 402 is required for
In normoxia, HIF-1α is modified by prolyl 4-hydroxylase and recognized by VHL, which targets it for proteasome degradation. B = elongin B; C = elongin C; CUL2 = cullin 2; VHL = von Hippel-Lindau protein.

This figure is adapted from (Michiels et al., 2002).
the interaction between HIF-1α and pVHL. This process is oxygen-dependent. In the absence of oxygen, prolyl hydroxylase is inactive, and the unmodified HIF-1α is unable to interact with pVHL and accumulates. In addition to hypoxia, HIF-1α also is induced by cobalt chloride, which directly interrupts the binding between HIF-1α and pVHL (Yuan et al., 2003), and by DFX, which chelates the free iron required for prolyl hydroxylase, as well as a number of other factors, including insulin, thrombin and AngII (Page et al., 2002; Semenza, 2002). After HIF-1α is activated, it dissociates from the chaperone protein, heat shock protein 90 (Hsp90) (Minet et al., 1999) and then translocates into the nucleus, where it dimerizes with HIF-1β to form the HIF-1 complex. To activate HIF-1 completely, several further steps are required. Phosphorylation probably plays an important role in this regulation (Minet et al., 2001). A cysteine residue in the C-terminal domain of the protein needs to be reduced for HIF-1α to interact with the coactivator CBP/p300. Interaction with thioredoxin and Ref-1 is required for maintaining this cysteine in a reduced form (Ema et al., 1999). The pathways leading to HIF-1 activation in hypoxia are summarized in Figure 2.

Due to its direct regulation by oxygen, HIF-1 has been viewed as a master regulator of cellular and physiological responses to hypoxia. HIF-regulated genes encode proteins involved in energy metabolism, cell proliferation/survival, erythropoiesis, angiogenesis/vascular remodeling, and vasomotor regulation (Semenza, 2002). The HIF-1 appears to be essential in a variety of biological
Figure 2. Graphic Depiction of the Pathways Leading to HIF-1 Activation in Hypoxia

Hsp90 = heat shock protein 90; pol II = polymerase II; Ref-1 = redox factor-1.

This figure is adapted from (Michiels et al., 2002).
processes, as HIF-1α expression and activity also are regulated by major signal transduction pathways including those involving phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK). Vascular endothelial growth factor (VEGF) is one of the downstream genes regulated by HIF-1α. Mazure et al. (1997) found that hypoxia-induced overexpression of VEGF was inhibited when they used a dominant negative mutant for the PI-3 kinase or for the Akt kinase, suggesting that the PI-3K/Akt pathway was involved in regulation of HIF-1 activation. Moreover, expression of a constitutively active Akt led to stabilization of the HIF-1α subunit (Zundel et al., 2000) and the use of PI-3K inhibitors such as wortmannin and LY294002 inhibited the stabilization of HIF-1α in hypoxic Hep3B cells (Zhong et al., 2000), which suggests that stabilization of the HIF-1α subunit during hypoxia is dependent on the PI-3K/Akt pathway.

Another important transcription factor related to hypoxic regulation of gene expression is NF-κB. The NF-κB exists in the cytoplasm in an inactive form bound with a specific regulatory protein, the inhibitor of κB (IκB). Activation of NF-κB is achieved by proteolytic degradation of IκB through certain signal pathways initiated at the cell surface, which eventually leads to IκB phosphorylation (on Ser32 and Ser36 for IκBα). Phosphorylated IκB is then selectively ubiquitinylated by an E3 ubiquitin ligase, which leads to the degradation of IκB by the 26S proteasome.
Dissociated NF-κB then translocates to the nucleus, where it binds to the promoter of its target genes to initiate transcription (Michiels et al., 2002). The NF-κB plays a central role in the inflammatory response. It can be activated by a variety of stimuli, including cytokines, bacterial and viral products, and oxidative stress. There is evidence that during hypoxia, NF-κB can be activated directly by ROS. Application of oxidizing agents (particularly H₂O₂) or ionizing radiation can directly activate members of the NF-κB family (Schreck et al., 1992). Hypoxia resulted in nuclear translocation and activation of NF-κB in several cell types (Cazals et al., 1999; Suzuki et al., 2000). Moreover, addition of antioxidant compounds or upregulation of cellular antioxidant systems prevented NF-κB activation by cytokines (Renard et al., 1997; Schmidt et al., 1995).

In summary, during and after hypoxia, there are many transcription factors that play different roles in the cellular and molecular adaptation to hypoxic stress. Some may be of benefit for neuronal survival, while others may be negative factors leading to apoptosis. There is likely a balance point among them that allows the neuron to adjust to hypoxic stress and perhaps prepare for subsequent episodes of hypoxia. Disrupting such a balance may result in CNS disorders. Future studies should focus on the detailed steps and pathways by which neurons autoregulate their responses to various environmental stimuli. We also should consider the whole brain as a complicated network where neurons connect to and influence each other. To solve problems in a single neuron is not our goal; our final target is the brain.
Activation of Protein Kinase C Reduces Benzodiazepine Potency at GABA$_A$ Receptors in NT2-N Neurons

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Running title: PKC Reduces Benzodiazepine Potency

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Phosphorylation of GABA<sub>A</sub> receptors (GABARs) by protein kinase C (PKC) modulates GABAR function and allosteric enhancement by benzodiazepines and barbiturates. However, the effects of phosphorylation have been inconsistent, possibly due to variability in neuron or GABAR populations. We used NT2-N neurons to address this issue in a more homogeneous cell population. Whole-cell and gramicidin “perforated-patch” recordings were used to analyze changes in GABAR currents induced by preincubation with 4β-phorbol-12,13-dibutyrate (PDBu), the inactive 4α-phorbol-didecanoate (4α-PDD) or bisindolylmaleimide (BIM, a PKC inhibitor). PDBu, but not 4α-PDD, caused a rightward shift of the concentration-response curve (C/R) for diazepam enhancement, without affecting maximal enhancement. BIM blocked the rightward shift of the diazepam C/R induced by PDBu. PDBu did not alter the GABA C/R or the current reversal potential. The PKC effect was specific to the benzodiazepine site, as PDBu did not alter potentiation of GABAR currents by pentobarbital. Exposure to diazepam (10 μM) for 7 days reduced maximal diazepam enhancement without affecting the EC<sub>50</sub>; PDBu also caused a small rightward shift of the diazepam EC<sub>50</sub> in these cells. PKC activation reduced the apparent affinity of diazepam at NT2-N GABARs without altering maximal enhancement, suggesting decreased allosteric coupling of the benzodiazepine and GABA sites.
INTRODUCTION

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian central nervous system. GABA_A receptors (GABAR) are composed of five subunits from seven different subunit families with multiple subtypes (α1-6, β1-3, γ1-3, δ, ε, θ, π) that assemble to form a pentomeric ligand-gated chloride ion channel (Olsen and Macdonald, 2002; Rudolph et al., 2001; Sieghart et al., 1999). GABAR currents can be modulated by positive and negative allosteric agents including benzodiazepines (BZs), barbiturates, neurosteroids, bicuculline and zinc. Studies of recombinant GABARs have shown that different subunit or subtype compositions influence their pharmacological and biophysical properties including agonist efficacy, channel kinetics, and allosteric properties (Olsen and Macdonald, 2002; Rudolph et al., 2001; Sieghart et al., 1999). BZs allosterically potentiate the actions of GABA at GABARs by binding to high-affinity binding sites at the interface of specific α- and γ-subunits (Rudolph et al., 2001; Smith, 2001).

GABAR function can also be regulated by phosphorylation by Ser/Thr and Tyr protein kinases at consensus phosphorylation sites in the major intracellular domains of several different GABAR subunits (Brandon et al., 2002; Moss et al., 1995; Moss et al., 1992b). Phosphorylation by calcium and phospholipid-dependent protein kinase (PKC) has frequently been reported to modulate GABAR function. Most results suggest that PKC activation decreases GABAR function (Filippova et al., 2000; Krishek et al., 1994; Leidenheimer et al., 1992) though in some systems PKC activation increases GABAR currents (Lin et al., 1994; Lin et al., 1996; Poisbeau et al., 1999). However, relatively few studies have focused on the effects of
PKC on BZ- and barbiturate-mediated potentiation of GABAR currents, and the results have been inconsistent or even contradictory. PKC activation enhanced BZ and pentobarbital potentiation of \( \alpha_1\beta_1\gamma_2 \) GABAR currents expressed in *Xenopus laevis* oocytes (Leidenheimer et al., 1993). However, others have found no PKC-induced change in sensitivity to diazepam or pentobarbital using the same expression system (Ghansah and Weiss, 2001). The variable effects of PKC on GABAR function might result from differences in the neuron populations or expression systems, the GABAR subunits expressed, the method and timing of PKC activation, the basal phosphorylation state of PKC targets, or the recording conditions. To address this issue in a more homogeneous population of neuronal cells, we used neuron-like NT2-N cells, derived from the human NTera2 teratocarcinoma cell line in response to retinoic acid (RA) (Andrews, 1984). NT2-N cells have a distinctly neuronal morphology and express neuron-specific cell surface, cytoskeletal, and secretory markers (Pleasure et al., 1992). NT2-N cells also express a full complement of excitatory and inhibitory neurotransmitter receptors, including GABARs with a consistent pharmacological profile composed from a distinct subset of GABAR subunits (Neelands et al., 1998) that develops over time *in vitro* (Neelands et al., 1999). At 11 weeks after the start of RA exposure, NT2-N cells express the \( \alpha_1, \alpha_2, \alpha_3, \) and \( \alpha_5, \beta_2 \) and \( \beta_3, \gamma_2S \) and \( \gamma_3, \varepsilon \) and \( \pi \) subunits (Greenfield et al., 2001). While the GABAR composition of NT2-N cells is not indicative of any specific neuron population, this combination occurs in many CNS regions and is broadly representative of GABARs in central neurons.
In the present study, we evaluated the effects of PKC activity on GABAR sensitivity to BZs by comparing GABA currents in NT2-N cells before and after exposure to different PKC modulators. Using whole-cell and gramicidin “perforated-patch” voltage-clamp recording techniques, we found that PKC activation reduced diazepam potency to enhance GABAR currents without changing maximal enhancement. In cells exposed to diazepam for 7 days, maximal enhancement of GABAR currents by diazepam was decreased with no change in the EC$_{50}$. In these diazepam-treated cells, PKC activation also shifted the EC$_{50}$ rightward, suggesting that the effects of PKC to reduce diazepam potency are distinct from the reduction in maximal efficacy induced by chronic diazepam exposure. PKC may reduce diazepam affinity at its GABAR binding site by decreasing the allostERIC coupling between BZ and GABA sites on NT2-N GABARs. Some of these results have previously been presented in abstract form (Gao et al., 2003).

METHODS

Reagents. Drugs and chemicals used in these studies included: 4β-phorbol 12,13-dibutyrate (PDBu), 4α-phorbol didecanoate (4α-PDD), 5-fluoro-2’-deoxyuridine (FUDR), bisindolylmaleimide X hydrochloride (BIM), diazepam, GABA, gramicidin, pentobarbital and retinoic acid (RA), all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

NT2-N cell culture NT2 immortalized precursor cells (Stratagene, La Jolla, CA) were maintained in Dulbecco’s modified Eagle’s medium–high glucose (DMEM-HG,
Invitrogen, Grand Island, NY) with 10% fetal bovine serum and penicillin / streptomycin in a 37 °C incubator with 95% air / 5% CO₂ (Neelands et al., 1998; Pleasure et al., 1992). Medium was changed at least twice per week. Cells were plated at $2 \times 10^6$ cells / 75 cm$^2$ flask and treated 1 μM retinoic acid (RA) in DMEM-HG complete medium with for 5 weeks. Each flask of cells was then replated into two 75 cm$^2$ flasks (1:2 dilution) with DMEM-HG medium containing mitotic inhibitors (10 μM FUDR + 10 μM uridine and 1 μM cytosine arabinoside) to select for non-dividing NT2-N neurons. NT2-N cells were maintained with mitosis inhibitors for 3 weeks, at which point few undifferentiated NT2 precursor cells remained. After another 3 weeks in DMEM-HG, cells were plated onto 35 mm culture dishes for PKC modulator treatments and subsequent electrophysiological recording. We define the “age” of NT2-N cells from the onset of RA treatment, hence the cells used in these experiments were considered 11-week NT2-N cells.

**Drug treatment**

In most experiments, 11-week NT2-N cells were incubated with either PDBu (100 nM), 4α-PDD (100 nM), or BIM (1 μM) for 10 min before electrophysiological recording and then continuously bathed with these drugs during recordings (30-40 min). To determine the effects of PKC on maximal GABAR current amplitudes within individual cells, in some experiments GABA-activated currents (100 μM GABA) were recorded in single cells before and after PDBu (100 nM) exposure for 10 min.
Electrophysiology

*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). Signals were low-pass filtered at 1 kHz and digitized at 2 kHz using a Digidata 1200A Data Acquisition System (Axon Instrument, Foster City, CA) and subsequently analyzed off-line using Clampfit 8.0 software (pClamp 8.0, Axon Instruments). Patch-clamp electrodes were pulled from micro-hematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) using a P-97 Flaming-Brown micropipette puller (Sutter Instrument Co., Novato, CA). Electrodes of 5-10 MΩ were filled with internal micropipette solution containing (in mM): 153.3 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, 4 MgATP, 300-305 mOsm, PH 7.3. NT2-N cells were removed from the 5% CO₂ incubator and the feeding medium was replaced with external recording solution containing (in mM): 142 NaCl, 1 CaCl₂, 8.1 KCl, 6 MgCl₂, 10 Glucose, 10 HEPES, 315-325 mOsm, pH 7.4. GABA and other drugs were sequentially applied in increasing concentrations using a gravity-driven, modified U-tube “multipuffer” system (Greenfield and Macdonald, 1996). To avoid accumulation of desensitized channels, drugs were applied at least 2 min apart. Peak GABAR 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:

\[
I = \frac{I_{\text{max}}}{1 - 10^{(\log(\text{EC50} - [\text{drug}]) \times \text{Hill slope})}}
\]

where \(I\) is the peak GABAR current at a given GABA concentration, and \(I_{\text{max}}\) is the maximal GABAR current. Curve fitting was performed using Prism 3.0 software.
Drug enhancement of GABAR current was measured by dividing the peak amplitude of GABAR currents elicited in the presence of drug by the average of control currents elicited by GABA alone before and after co-application. Cells with marked rundown (> 20% reduction over 10 minutes) were excluded from analysis.

**Gramicidin perforated-patch recording**

Perforated-patch recordings (Abe et al., 1994; Owens et al., 1996) were obtained using similar methods as whole-cell recordings, except the plasma membrane was not mechanically ruptured after gigaseal formation. Gramicidin, an antibiotic which forms Cl\(^{-}\)-impermeable pores, was dissolved in dimethylsulfoxide (DMSO) (1 mg/ml) then diluted in the internal micropipette solution to a final concentration of 10 \(\mu\text{g/ml}\). The tip of the electrode was loaded with a small volume of gramicidin-free internal solution in order to avoid interference with seal formation. After seal formation, the progress of gramicidin pore formation was evaluated by monitoring the decrease in membrane resistance. Drugs were applied to the cell after the membrane resistance had stabilized to 50-100 M\(\Omega\); this usually took about 5 to 10 min. GABA (30 \(\mu\text{M}\)) was applied with the membrane held at a series of potentials from \(-70\) mV to \(+50\) mV. The GABAR reversal potential was defined as the x-intercept value of a linear regression through the plot of peak GABA-evoked currents vs. membrane holding potential at voltages just above or below the reversal potential, approximating the chord conductance of the GABA reversal potential. In some experiments, perforated-patch recordings were converted to whole-cell
recordings by applying suction to rupture the underlying plasma membrane. In each case, the GABA current reversal potential shifted to ~0 mV after the plasma membrane was ruptured, consistent with the chloride reversal potential between the pipette solution and extracellular solution due to nearly equivalent chloride concentrations.

Chronic diazepam treatment

Diazepam was dissolved in DMSO and then diluted in DMEM-HG medium to a final concentration of 10 μM. NT2-N cells were used 10 weeks after onset of retinoic acid treatment, as described previously (Gao et al., 2004), and treated with DMEM-HG containing 10 μM diazepam for one week, so that cells were the same “age” in vitro at the end of treatment as untreated cells. The medium was then aspirated and NT2-N cells were washed four times with external recording solution, including one wash at 37 °C for 30 min to ensure complete dissociation of bound diazepam, prior to patch-clamp recording as above.

Statistical analysis

Data are presented as mean ± S.E.M. of n experiments throughout. Differences between groups were compared by Student's t-tests, and p<0.05 was taken as the limit of statistical significance.

RESULTS

Effects of PDBu on GABAR currents
GABA-activated currents were obtained by whole-cell patch clamp recordings from 11-week NT2-N cells voltage clamped at –70 mV. There was no significant change in the amplitude of GABAR currents (100 µM) before and after 10 min exposure to PDBu (100 nM at room temperature, Fig. 1A). Slight reductions in current amplitude due to rundown were not different from that seen in cells exposed to external recording solution for the same duration. To assess whether preincubation with PDBu alters GABA sensitivity, GABA C/R curves were obtained by brief applications of increasing GABA concentration (1 µM - 1 mM). PDBu (100 nM) did not change the GABA C/R curve (Fig. 1B). There was no significant change in maximal current or the GABA EC$_{50}$ (Control: I$_{\text{max.}}$ = 868±96 pA, EC$_{50}$ = 27±5.4 µM, n=22; PDBu: I$_{\text{max.}}$ = 772±149 pA, EC$_{50}$ = 36±8.9 µM, n=6). Preincubation with PDBu (100 nM) for 10 or 15 min at 37°C (n=3-4), addition of PDBu (100 nM) to the internal recording solution (n=3-4), or use of 1 µM PDBu (n=3) similarly had no effect on maximal or EC$_{50}$ GABA currents (data not shown).

**Effect of PDBu on GABA Current Reversal Potential**

To determine whether PDBu altered the GABA current reversal potential, we used the gramicidin perforated-patch recording technique to allow whole-cell recording without disturbing the chloride reversal potential. Gramicidin forms a stable density of Cl$^-$-impermeant pores within 10-15 min, and maintains physiological [Cl$^-$]$_{\text{int}}$ for >3h (Kyrozis and Reichling, 1995). After gigaseal formation and development of stable membrane resistance (50-100 MΩ), I-V curves were plotted from peak currents obtained by applications of 30 µM GABA at multiple holding potentials.
The reversal potential for perforated-patch recordings was approximately –40 mV; when converted to conventional whole-cell recording configuration by applying suction, the reversal potential shifted to ~0 mV as expected for symmetrical chloride concentrations (n=4, see Fig. 2A). In prior studies, the resting membrane potentials in NT2-N cells ranged from –49 to –33 mV (average –40.9 ± 0.4 mV, n = 118) (Neelands et al., 1998), measured by determining the holding potential requiring zero current soon after rupturing the cell membrane to establish whole-cell mode. These data suggest that NT2-N neurons have relatively high internal chloride concentration ([Cl\(^-\)]\(_i\)) as observed with immature neurons in the developing CNS (Leinekugel et al., 1999; Owens et al., 1996), hence factors influencing [Cl\(^-\)]\(_i\) will alter the GABA current reversal potential. To assess whether brief PDBu exposure changes [Cl\(^-\)]\(_i\), we measured the GABA reversal potential in NT2-N cells preincubated with or without PDBu (100 nM). Preincubation with PDBu (10-15 min at room temperature) did not change the GABA current-voltage (I-V) relationship or reversal potential (Fig. 2B; Control $E_{GABA} = -32\pm6$ mV, n=6; PDBu $E_{GABA} = -33\pm4$ mV, n=9).

**Effects of PKC activity on diazepam enhancement of GABAR currents**

The BZ, diazepam, enhanced whole-cell GABAR currents in a concentration-dependent manner. Diazepam C/R curves were obtained by co-application of increasing concentrations of diazepam with 10 μM GABA. To determine whether PKC activity affects BZ sensitivity, we obtained diazepam C/R curves in NT2-N cells following preincubation with different PKC modulators. PDBu (100 nM) preincubation caused a slight but significant rightward shift of the C/R curve for
diazepam enhancement of 10 μM GABA currents and did not affect the maximal enhancement by diazepam (Fig. 3A). The relative enhancement of GABA currents in control and PDBu-treated cells differed significantly (p<0.001) at 30 nM, 100 nM and 300 nM diazepam. The EC\textsubscript{50} for diazepam with all cells pooled increased from 63 nM (95% CI: 41 – 93 nM) to 207 nM (95% CI: 128 – 335 nM). Although the means of EC\textsubscript{50}s from individual cell C/Rs were somewhat different from the fit of pooled responses (Control: 96 ± 5 nM, n=11; PDBu: 126 ± 7 nM, n=8), the difference was significant (p<0.01). The biologically inactive phorbol ester, 4α-phorbol didecanoate (4α-PDD, 100 nM, Fig. 3B) and the selective PKC inhibitor (Davies et al., 2000), bisindolylmaleimide X hydrochloride (BIM, 1 μM, Fig. 3C) each individually had no effect on the diazepam C/R curve. However, BIM (1 μM) blocked the right shift of the diazepam C/R induced by 100 nM PDBu (Fig. 3D). These data indicate that the diazepam sensitivity of NT2-N cell GABARs was reduced by PDBu-mediated activation of PKC.

No Effect of PDBu on allosteric modulation by pentobarbital

To determine whether the effect of PKC on diazepam enhancement was specific to the BZ site, we tested enhancement of GABAR currents by pentobarbital (PB) before and after treatment with PDBu. PB (1 -1000 μM) enhanced 10 μM GABAR currents in a concentration-dependent manner (Fig. 4). There was no significant difference in maximal enhancement (Control: 3.39 ± 0.46 fold, n=9, PDBu: 3.44 ± 0.34 fold, n=8), EC\textsubscript{50} for enhancement (Control: 62±14 μM; PDBu: 36±4 μM) or Hill slope (Control: 1.66 ± 0.69; PDBu: 1.77 ± 0.62) between control NT2-N cells and cells
treated with 100 nM PDBu (Fig. 4). Thus, PDBu exposure did not change the PB sensitivity for GABARs in NT2-N cells, suggesting that its effect on diazepam enhancement may be specific to BZs.

Effects of PDBu on diazepam enhancement in chronic diazepam treated cells

Chronic BZ treatment, both in vivo and in vitro, results in decreased responsiveness of GABARs to subsequent BZ exposure (Mohler et al., 1978; Primus and Gallager, 1992; Rosenberg et al., 1985; Zeng and Tietz, 1999). Our preliminary studies of the effects of chronic BZs on GABARs in NT2-N cells suggested a slight decrease in potency after 7-day exposure to 10 μM diazepam and 1-day withdrawal (Greenfield, 2000). To determine whether the changes induced by PDBu in GABAR BZ sensitivity were similar to those caused by chronic exposure, we treated NT2-N cells for 1 week with 10 μM diazepam, but without a withdrawal period in order to maximize the likelihood of detecting diazepam-induced changes. In contrast to our preliminary findings associated with a 1-day withdrawal, 7-day diazepam (10 μM) treatment significantly reduced the maximal diazepam potentiation of 10 μM GABA currents with no significant shift in EC50 of the C/R for diazepam enhancement compared to control cells (Fig. 5). In 7-day diazepam-treated cells, PDBu (100 nM) caused a significant (p<0.05) rightward shift of the C/R curve for diazepam enhancement of 10 μM GABA currents (DZP-7d EC50: 77 ± 27 nM, n=6; DZP-7d+PDBu EC50: 699 ± 330 nM, n=4) without affecting the (reduced) maximal enhancement by diazepam (Fig.5), suggesting that PKC activation reduced
diazepam’s potency while prolonged diazepam exposure reduced subsequent efficacy.

**DISCUSSION**

PKC appears to regulate GABAR activity and membrane trafficking, but the effects have varied in different systems. A constitutively active fragment of PKC (PKM) increased peak α1β1γ2L recombinant GABAR currents in L929 cells (Lin et al., 1994), requiring phosphorylation of both β1 and γ2L (Lin et al., 1996). However, PKC activation decreased α5β2γ2 (Sigel et al., 1991) or α1β2γ2S (Kellenberger et al., 1992; Krishek et al., 1994) GABAR currents in *Xenopus* oocytes (Leidenheimer et al., 1992) and HEK293 cells, as well as native GABARs in cortical neurons (Brandon et al., 2000). PKC can phosphorylate conserved serine (S) residues in the GABAR β- and γ2-subunits, including S409 in β1, S410 in β2, both S408 and S409 in β3, S327 in γ2S and γ2L and S343 in γ2L, hence some of this variability may be due to the specific subunits or sites phosphorylated. PKC constitutively phosphorylated the β3 subunit at S408 and S409, which reduced GABAR currents (Brandon et al., 2000), but curiously, phosphorylation of the same sites by PKA was reported to increase GABAR currents (McDonald et al., 1998), hence other phosphorylation sites are probably involved in the effects of one or both kinases. PKA and PKC had different effects on synaptic GABAR function depending on the location of the synapse. In hippocampal granule cells, PKC increased mIPSC peak amplitudes, while in CA1 neurons, PKA reduced mIPSC
amplitudes, and PKC had no effect (Poisbeau et al., 1999). Phosphorylation effects at synaptic GABARs may thus depend on both subunit composition and cell-specific factors.

The present experiments were designed to assess whether PKC activation alters responses to GABA and allosteric modulators in an *in vitro* neuronal cell system with well-defined GABAR subunit expression and pharmacology. In our system, brief application of the PKC activator, PDBu, did not alter the maximal currents or EC$_{50}$ of the GABA C/R, or the reversal potential of GABAR currents. NT2-N cells express a consistent profile of subunits in RT-PCR studies, including mRNAs for $\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$ (with lower expression of $\alpha_4$), $\beta_2$ and $\beta_3$ (with lower expression of $\beta_1$), $\gamma_2S$ and $\gamma_3$, $\varepsilon$ and $\pi$ subunits (Gao et al., 2004). Pharmacological studies suggest that assembled NT2-N GABARs likely contain the $\alpha_2$ and/or $\alpha_3$, $\beta_2$ and/or $\beta_3$, and $\gamma_2$ subunits (Neelands et al., 1998). Hence, possible PKC phosphorylation sites would include S410 in $\beta_2$, both S408 and S409 in $\beta_3$, and S327 in $\gamma_2S$. Failure to detect an effect on GABA maximal currents or EC$_{50}$ could be due to a high basal phosphorylation state (from which additional stimulation of PKC would have no effect), insufficient concentration of PKC modulators, inadequate duration of stimulation or other conditions of incubation. Indeed, PKC mRNA, protein and activity are dramatically upregulated during differentiation from NT2 precursor cells to neuronal NT2-N cells (Abraham et al., 1991). However, the variability of responses seen in other systems suggests that direct GABAR phosphorylation by PKC may not explain the changes in maximal current.
In *Xenopus* oocytes, phorbol myristate acetate (PMA) reduced $\alpha_1\beta_1\gamma_2$ GABAR currents by up to 90% without altering the GABA EC$_{50}$ or enhancement by diazepam (300 nM) (Ghansah and Weiss, 2001), though the relatively high diazepam concentration may have obscured a more subtle effect on EC$_{50}$. The dramatic reduction in GABAR current may have resulted from PKC-stimulated receptor internalization, which has previously been demonstrated in *Xenopus* oocytes (Filippova et al., 2000) but has not been observed in neurons. Inhibition of PKC prevented BZ-induced GABAR internalization (Filippova et al., 1999; Johnston et al., 1998), while PKC stimulation reduced expression of $\alpha_1\beta_2\gamma_2$ receptors in HEK293 cells, apparently by blocking receptor recycling to the cell surface (Connolly et al., 1999). This phenomenon may be temperature-dependent in mammalian cells. PMA decreased surface expression of $\alpha_1\beta_2\gamma_2$L GABARs by stimulating endocytosis when patch-clamp recordings were performed at 35 °C, but not at room temperature (Herring et al., 2003). Mutation of the $\beta_2$ subunit dileucine AP2 recognition motif abolished both constitutive (Herring et al., 2003) and PKC-mediated (Herring et al., 2003) GABAR endocytosis, as well as the reduction in GABAR current induced by PKC, consistent with a clathrin-mediated mechanism dependent on the $\beta_2$ subunit.

To determine whether a potential PKC effect was missed due to incubation and recording at room temperature, we performed a series of recordings with PDBu preincubation at 37 °C, as well as experiments in which PDBu was included in the recording pipette. Again, no change in maximal current or EC$_{50}$ for GABA was observed. These data suggest that PKC-mediated GABAR internalization may be
less significant in NT2-N neurons than in HEK293 cells (at 37 °C) or oocytes (at room temperature), and may not be significant in native neurons.

The lack of change in GABA responsiveness was not due to failure of PDBu to activate PKC, as PDBu shifted the C/R for diazepam to the right, an effect inhibited by BIM and not produced by the inactive phorbol ester, 4a-PDD, clearly consistent with PKC mediation. There was no effect on pentobarbital potentiation of GABAR currents, hence the effect appears to be BZ-specific. PKC has been shown to affect in vivo BZ sensitivity in other systems as well, though again with variable results. Mutant mice lacking the PKCε isoform were behaviorally more sensitive to diazepam and showed increased enhancement of muscimol-stimulated Cl− flux by flunitrazepam, suggesting a negative role for that PKC isozyme in modulating GABA/BZ allosteric coupling (Hodge et al., 1999). However, mice lacking the PKCγ isoform had no change in BZ sensitivity (Harris et al., 1995), hence the effect may be isoform-specific. The rightward shift in the diazepam C/R curve observed in our experiments with PKC activation is consistent with the loss of a negative effect of PKC on GABA/BZ allosteric coupling seen in the PKCε null mutant. In rat brain slices, PDBu enhanced BZ binding but decreased muscimol binding, suggesting an alteration in GABA/BZ site coupling (Oh et al., 1999) consistent with our findings. In contrast, PKC activation enhanced BZ potentiation of α1β1γ2S GABAR currents in Xenopus oocytes (Leidenheimer et al., 1993), though others have found no PKC-induced change in sensitivity to diazepam or pentobarbital using the same expression system (Ghansah and Weiss, 2001). It should be noted that the prominent PKC effect on receptor recycling in oocytes may have altered the population of GABARs
at the cell surface, which may explain the variable effects on BZ potentiation between these studies.

Like PKC activation, chronic BZ treatment of cortical neurons in culture shifts a fraction of BZ binding from the surface membrane to clathrin-coated vesicles (Tehrani and Barnes, 1994). BZ-evoked downregulation of surface membrane binding was blocked by PKC inhibitors (Brown and Bristow, 1996; Johnston et al., 1998), suggesting that phosphorylation may be involved in this process. However, in insect Sf9 cells (Ali and Olsen R.W., 2001), inhibition of PKA activity promoted allosteric uncoupling and stimulation of PKA favored coupling of the GABA and BZ binding sites on α1β2γ2 GABARs, while stimulation or inhibition of PKC had no effect. Mutation of the S410 PKA phosphorylation site in the β2 subunit did not block these PKA effects, suggesting that PKA may phosphorylate sites on other GABAR subunits or other proteins.

Our initial experiments with chronic BZ treatment, using NT2-N cells exposed to diazepam (10 μM) for 7 days followed by 1 day washout, suggested a slight reduction in BZ potency for GABA current enhancement and a downregulation of α5 subunit expression (Greenfield, 2000; Greenfield et al., 2001). The recovery of diazepam efficacy and reduced potency associated with this protocol could be attributed to subtle changes in GABAR subunit populations (suggested in our initial studies) resulting in new GABARs with altered BZ pharmacology, though post-translational modifications of existing GABARs were also possible.

In the present experiments without a 1-day washout, however, we observed a significant reduction in maximal efficacy of diazepam potentiation after 7-day
diazepam exposure, with no shift in EC$_{50}$ for enhancement. Independent of the reduction in efficacy, PDBu treatment caused a small but significant rightward shift of the diazepam C/R curve for GABA current enhancement that was very similar to that seen in control neurons, suggesting that PKC activation had the same effect on the potency of diazepam potentiation despite the downregulation of efficacy induced by chronic BZ exposure. Hence, changes in BZ enhancement of GABA currents may involve distinct and separable mechanisms, one involving PKC phosphorylation with a reduction in BZ potency, the other involving loss of efficacy, possibly related to altered subunit composition with a reduction in BZ-sensitive GABARs. It will be important to measure possible changes in subunit mRNA and protein after chronic BZ exposure using semiquantitative RT-PCR and immunoblotting, respectively. Binding studies exploring the ability of GABA to increase BZ binding will also be useful in determining whether these different treatments result in “uncoupling” of GABA and BZ binding.

In summary, we have found that PDBu reduced diazepam potency for enhancing GABAR currents without changing efficacy, and had no effect on the potency or efficacy of GABA for activating GABAR currents. The targets of PKC activation are unknown, and could include both GABAR subunits and other proteins. Chronic diazepam treatment reduced the efficacy of diazepam to enhance GABAR currents without affecting potency, and PKC activation reduced diazepam potency in chronic diazepam-treated cells just as it did in control neurons. The reduction in BZ potency may represent one form of “uncoupling” of the GABA and BZ sites, though this should be confirmed with binding studies. The reduction in diazepam efficacy
after 7-day diazepam exposure may be related to a change in GABAR subunit composition, which will also be explored in future experiments.
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Figure 1. PKC activation by 4β-phorbol 12,13-dibutyrate (PDBu) did not change the GABA sensitivity. (A) Whole-cell GABAR currents were evoked by brief application of 100 μM GABA. There was no significant change in GABA-activated currents before and after 10 minute exposure to 100 nM PDBu at room temperature (RT). Slight reductions in currents due to rundown were not different from cells exposed to external solution for the same duration. (B) Peak GABAR currents elicited by increasing GABA concentrations were fitted to a 4 parameter logistic function. Preincubation with 100 nM PDBu for 10 to 15 minutes at RT (closed circles) did not change the GABA concentration-response curve compared to control (open circles). There was no significant change in maximal current or the EC₅₀ for GABA.
Figure 2. PDBu did not change GABAR reversal potential. (A) Sample I-V relationship of GABA currents in gramicidin perforated-patch and whole-cell patch mode in a single cell. With perforated patch recording, the GABA reversal potential ($E_{\text{GABA}}$) was approximately $-40$ mV (open diamonds), whereas in whole-cell mode for the same cell, the reversal potential shifted to 0 mV (open circles) as expected for symmetrical chloride concentrations. (B) Preincubation with 100 nM PDBu did not significantly change the GABA current voltage relationship or reversal potential in perforated patch recordings (Control (open diamonds): $E_{\text{GABA}} = -32 \pm 6$ mV, n=6; PDBu (closed diamonds): $E_{\text{GABA}} = -33 \pm 4$ mV, n=9).
Figure 3. PKC activation altered the GABAR sensitivity to diazepam (DZP). (A) PDBu (100 nM, closed circles, n=7-10) caused a slight but significant (p < 0.05) rightward shift of the C/R curve for diazepam enhancement of 10 μM GABA currents relative to control (n=7-9) but did not affect maximal enhancement by diazepam. (B) 4α-PDD (100 nM, closed triangles, n=3) and (C) bisindolylmaleimide (BIM, 1 μM, closed squares, n=7) had no effect on the diazepam C/R curve. (D) BIM (1 μM, closed inverted triangles, n=5-6) blocked the right shift of the diazepam C/R induced by 100 nM PDBu.
Figure 4. PDBu did not alter the GABAR sensitivity to pentobarbital (PB). Preincubation with 100 nM PDBu did not affect PB-mediated potentiation of GABAR currents. There were no significant changes in maximal enhancement, EC$_{50}$ and Hill slope between control and PDBu treatment (Control maximal enhancement=3.39±0.46, EC$_{50}$=62±14 μM n=9; PDBu maximal enhancement = 3.44±0.34, EC$_{50}$=36±4 μM, n=8).
Figure 5. PDBu reduced GABAR sensitivity to diazepam in 7-day diazepam-treated cells. A. Chronic (7-d) diazepam (DZP, 10 μM)) exposure (closed triangles, n=7) had no significant effect on the EC$_{50}$ of GABA-evoked currents (20 ± 3.1μM) relative to control cells (open circles, 30±4.4 μM, p>0.05, n=10-11) or on maximal GABA currents (p>0.05), hence the same GABA concentration was used in both cases. B. Chronic (7-d) DZP treatment (10 μM, closed triangles, n=6-7) reduced the maximal enhancement of GABA currents by DZP relative to control (open circles, n=7-9) without changing the EC$_{50}$ of the DZP C/R curve. PDBu (100 nM, open triangles, n=4-6) caused a significant (p < 0.05) rightward shift of the DZP C/R curve without affecting maximal enhancement in 7-d DZP-treated cells (p >0.05).
Manuscript 2

Hypoxia Alters GABA\textsubscript{A} Receptor Function and Subunit Expression in NT2-N Neurons

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ABSTRACT

Hypoxia causes dysfunction of excitatory and inhibitory neurotransmission, often resulting in encephalopathy, seizures or myoclonus. We evaluated the effects of hypoxia on GABA$_A$ receptor (GABA$_A$R) function and expression in an *in vitro* model of neuronal hypoxia. NT2-N cells, derived from the human NT2 teratocarcinoma cell line, were exposed to $\leq$1% O$_2$ for 8 hours and then used immediately for experiments or allowed to recover under normoxic conditions (95% air/5% CO$_2$) for 24, 48 or 96 h. Hypoxic treatment did not cause obvious morphological changes or cell death. In whole-cell patch clamp recordings, the GABA current EC$_{50}$ was unchanged, however, maximal GABA-evoked currents changed in a biphasic manner. Maximal GABA currents were significantly increased immediately after hypoxia, but were significantly reduced after 48 h normoxic recovery, and then returned to baseline after 96 h recovery. Maximal potentiation of 10 $\mu$M GABA currents by diazepam was increased 48 h after hypoxia, but potentiation by zolpidem was decreased. Barbiturate enhancement and zinc inhibition of GABA currents were unchanged. Semiquantitative reverse transcriptase (RT)-PCR showed decreased $\alpha_1$, $\alpha_5$, $\beta_2$ and $\gamma_2$ subunit mRNA after hypoxia. Hypoxic exposure altered GABA$_A$R physiology and subunit mRNA expression, which may correlate with symptoms observed after hypoxia *in vivo*. 
INTRODUCTION

Hypoxia can cause severe central nervous system (CNS) dysfunction including encephalopathy (Low et al., 1985), seizures (Bergamasco et al., 1984) and myoclonus (Hallett, 2000). During and after hypoxia, extracellular levels of amino acid neurotransmitters increase in many brain regions (Hagberg et al., 1987), causing an imbalance of excitatory and inhibitory neurotransmission leading to excitotoxicity (Yue et al., 1997). The effect of hypoxia on N-methyl-D-aspartate receptor (NMDAR) expression and function has been studied in a number of systems (Aizenman et al., 2000; Bickler et al., 2000; Guerguerian et al., 2002; Gurd et al., 2002; Machaalani and Waters, 2002, Zanelli et al., 2002), and the role of NMDAR-mediated excitotoxicity in post-hypoxic neuronal apoptosis (Mishra et al., 2001) has previously been examined in NT2-N cells (Rootwelt et al., 1998). However, relatively little attention has been focused on inhibitory neurotransmitter systems.

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the mammalian CNS. GABA$_A$ receptors (GABA$_A$Rs) are composed of five subunits from seven different subunit families with multiple subtypes ($\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, $\delta$, $\varepsilon$, $\theta$, $\pi$) that form a ligand-gated chloride ion channel. Studies of recombinant GABA$_A$Rs have shown that different subtype compositions determine their pharmacological and biophysical properties (Macdonald and Olsen, 1994). For example, benzodiazepine (BZ) augmentation of GABA$_A$R currents requires a $\gamma$ subunit, and BZ specificity is determined by the $\alpha$ subtype (Korpi et al., 2002). Presence of $\alpha_1$ results in a receptor with high affinity for zolpidem, while the $\alpha_2$ or $\alpha_3$ subtypes, combined with $\beta$ and $\gamma$ subunits, result in receptors with moderate
zolpidem affinity. GABA\(_{A}\)Rs containing \(\alpha 5\) are sensitive to the BZ, diazepam, but are insensitive to zolpidem, while GABA\(_{A}\)Rs with \(\alpha 4\) or \(\alpha 6\) are insensitive to most BZs. Specific GABA\(_{A}\)R \(\alpha\) subunits also appear to mediate the different clinical properties of the BZs, with \(\alpha 1\) responsible for sedative, amnestic and anticonvulsant actions and \(\alpha 2\), \(\alpha 3\) and \(\alpha 5\) responsible for anxiolytic and other properties (Rudolph et al., 2000). Changes in subunit composition may thus alter GABA\(_{A}\)R pharmacology (Macdonald and Olsen, 1994) and affect the inhibitory pathways involved in encephalopathy and seizures.

Like the excitatory neurotransmitter receptors, GABA\(_{A}\)Rs are affected by hypoxia. GABA\(_{A}\)R binding in gerbil hippocampus was reduced after hypoxia, which was thought to result from receptor internalization (Alicke and Schwartz-Bloom, 1995). Electrophysiological studies (Harata et al., 1997) have also demonstrated a down-regulation of hippocampal GABA\(_{A}\)R function, which were attributed to changes in the transmembrane Cl\(^-\) gradient (Inglefield and Schwartz-Bloom, 1998) resulting from decreased ATP or increased intracellular Ca\(^{2+}\) (Harata et al., 1997). However, the actual mechanisms underlying changes in GABA\(_{A}\)R function remain unknown. Studies using primary culture or acutely dissociated CNS neurons may be complicated by the presence of multiple neuron types expressing different GABA\(_{A}\)R subunit compositions. The marked difference in vulnerability to hypoxia between CA1 and CA2/CA3 hippocampal pyramidal neurons (Petito et al., 1987) illustrates the variation in neuronal response even among morphologically similar neurons within a single brain structure.
To circumvent these difficulties, we have used neuronal cells derived from a human tumor cell line. NTera2 cells (NT2), derived from a human teratocarcinoma, terminally differentiate into neuron-like NT2-N cells when treated with retinoic acid (RA) (Andrews, 1984). NT2-N cells extend dendritic and axonal processes and express neuron-specific markers (Pleasure et al., 1992) and functional neurotransmitter receptors (Beczkowska et al., 1997). NT2-N cells express human GABA<sub>A</sub>Rs with a consistent pharmacological profile and a pattern of GABA<sub>A</sub>R subunit mRNA expression (Neelands et al., 1998) that develops over time in vitro (Neelands et al., 1999), and thus offer a potentially useful model in which to study changes in human GABA<sub>A</sub>R function and subunit expression in response to hypoxia.

In the present study, we have used whole-cell patch clamp recordings and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) to investigate the effects of hypoxia (8 h at O<sub>2</sub> ≤ 1%) on GABA<sub>A</sub>R pharmacology and subunit mRNA expression in NT2-N cells in vitro. Hypoxic exposure altered GABA<sub>A</sub>R function with a biphasic time-course, accompanied by changes of GABA<sub>A</sub>R subunit mRNA. The regulation of GABAR subunit gene expression by hypoxia may be a novel mechanism underlying changes in inhibitory neurotransmission after hypoxia. Some of these results were previously presented in abstract form (Gao et al., 2002).
METHODS

**Cell Culture.** NT2 stem cells (Stratagene, La Jolla, CA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Grand Island, NY) high glucose (HG) with 10% fetal bovine serum and penicillin/streptomycin in a 37 °C incubator with 95% air/5% CO₂ (Neelands et al., 1998; Pleasure et al., 1992). Media was changed at least twice per week. Cells were plated at 2 X 10⁶ cells / 75 cm² flask and treated with DMEM-HG complete medium with 1 μM retinoic acid (RA) for 5 weeks. Each flask of cells was then replated into two 75 cm² flasks (1:2 dilution) with DMEM HG media containing mitotic inhibitors (10 μM 5-fluorodeoxyuridine (FUDR) + 10 μM uridine and 1 μM cytosine arabinoside) to select for non-dividing NT2-N cells. NT2-N cells were maintained with mitosis inhibitors for 3 weeks, at which point few undifferentiated NT2 precursor cells remained, and then replated onto 35 mm Corning dishes for hypoxic exposure and electrophysiological recording. NT2-N cells used for RNA isolation were not replated, but remained in 75 cm² flasks during exposure to hypoxia and subsequent recovery until RNA was harvested.

**Hypoxia and reoxygenation.** NT2-N cells were used 8 weeks after onset of RA treatment. On the day of experiment, cells were examined microscopically before use to confirm viability. DMEM HG was bubbled for 15 min with 95% N₂/5% CO₂ and warmed to 37 °C. Cells were washed twice with extracellular recording solution (see below), followed by deoxygenated DMEM HG. Cells were then placed in a pre-warmed, humid anaerobic chamber (M.I.C.-101, Billups-Rothenberg, Del Mar, CA). The air inside the chamber was replaced by perfusing
with the 95% N₂/5% CO₂ mixture at 25 L/min for 4 min. After atmosphere replacement, a digital oxygen meter (Traceable Model # 06-662-66, Control Co., Friendswood, TX) connected to the chamber showed an O₂ concentration of 0.8-1.0%, which was unchanged after 8 h exposure. All hypoxic exposures were performed at [O₂] < 1%. The chamber was then sealed and placed in a 37°C incubator for 8 h. After hypoxic exposure, the medium was replaced with fresh DMEM HG (pre-equilibrated with air) and cells were either studied immediately (as described below), or returned to the normoxic incubator (95% air/5% CO₂, 37°C) for 24, 48 or 96 h prior to recording or RNA harvesting. For the “0 h” recovery time point, cells were recorded or harvested for mRNA within 1-2 h of termination of hypoxia; for subsequent time points, recordings or RNA harvesting occurred within ±2 h of the stated time.

Cytotoxicity Assays. Cytotoxic effects of hypoxia were assessed by trypan blue exclusion and by measuring release of lactate dehydrogenase (LDH) into the culture medium using the Cytotox96 Non-radioactive Assay kit (Promega, Madison, WI). The red formazan LDH enzyme product was detected at 490 nm wavelength on a Spectramax Plus 96 well plate reader (Molecular Devices, Sunnyvale, CA). NT2-N cells aged 8 weeks from onset of retinoic acid treatment were plated onto 35 mm dishes 2 days prior to study. On the day of hypoxic exposure, the routine DMEM-HG medium was exchanged with DMEM-HG without phenol red (to reduce background red absorption), in which the concentration of FBS was reduced to 5% to minimize background LDH activity. A standard volume of 1.0 ml was used for each of 3 dishes under control conditions (no hypoxic exposure) and 3 dishes exposed to
the hypoxic conditions described above for 8 h. Immediately after hypoxic exposure, and after 24, 48 or 96 h normoxic recovery, three 100 μl samples were removed from each dish and placed in identified wells of a 96-well-plate, and stored in the dark at 4 °C until all samples were collected. The sample volume was replaced with fresh phenol red-free medium (with 5% serum) and dishes were returned to the normoxic incubator until the next sample time point. After the 100 μl samples were removed at the final (96h) time point, 70 μl of 10X Lysis Solution (9% Triton X-100) was added to each dish to assess maximal LDH activity. Samples were placed in 0.5 ml Eppendorf tubes, briefly centrifuged to remove cellular debris, and subsequently transferred to the 96-well plate. Reconstituted Substrate Mix (50 μl) was then added to each well at room temperature (23 °C) in timed fashion, and the reaction was stopped with 1M acetic acid after 30 minutes. LDH activity was measured as absorbance at 490 nm within one hour after the addition of acetic acid. Fresh medium (without phenol red) was used as the blank from which all absorbances were subtracted. Cytotoxicity was determined as the percent LDH activity released into the medium relative to maximal activity. Absorbances were corrected for the amount of activity present from the preceding time point, and total LDH activity in each dish was estimated by adding the incremental LDH activities at each time point to the activity after cell lysis. Percent activities at each time point were determined by dividing the adjusted absorbance at that time point by the total LDH activity and multiplying by 100.

**Electrophysiology.** Whole-cell recordings were obtained using standard patch-clamp technique (Hamill et al., 1981) with an Axopatch 200B amplifier (Axon
Instruments, Union City, CA). NT2N cells were removed from the 5% CO₂ incubator and the feeding medium was replaced with external recording solution containing (in mM): 142 NaCl, 1 CaCl₂, 6 MgCl₂, 8.1 KCl, 10 glucose, 10 HEPES, 315-325 mOsm, pH 7.4. Patch clamp electrodes of 5-10 MΩ were filled with internal micropipette solution containing (in mM): 153.3 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, 4 MgATP, 300-305 mOsm, pH 7.3. Signals were low-pass filtered at 1000 Hz using the onboard 4-pole Bessel filter in the Axopatch 200B, then digitized on line at 1000 Hz using a Digidata 1200A Data Acquisition System (Axon Instruments, Foster City, CA) and subsequently analyzed off-line using Clampfit 8.0 software (pClamp 8.0, Axon Instruments). Patch-clamp electrodes were pulled from Fisher Micro-hematocrit capillary tubes (Fisher Scientific) using a P-97 Flaming-Brown micropipette puller (Sutter Instrument Co.). Membrane potential at the onset of recording was assayed by shifting briefly to current clamp mode with current set to zero immediately upon attaining whole cell configuration, before significant mixing of cellular and pipette contents had occurred. For GABA current recordings, membrane potential was held at –70 mV unless otherwise indicated. GABA and other drugs were sequentially applied in increasing concentrations using a gravity-driven, modified U-tube “multipuffer” system (Greenfield and Macdonald, 1996) with 10-90% solution exchange time of < 60 ms (Neelands et al., 1998). To avoid accumulation of desensitized channels, drugs were applied at least 2 min apart. Peak GABA₃R 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: 

\[ I = \frac{I_{\text{max}}}{1 - 10^{\left(\log(EC_{50} - [\text{drug}])\right) \times \text{Hill slope}}} \]

where I is the peak current at
a given GABA concentration, and $I_{\text{max}}$ is the maximal GABA$_A$R current. Curve fitting was performed using Prism 3.0 software (Graph Pad Software Inc., San Diego, CA). Maximal current and C-R curve fits were obtained from individual cells, then averaged and compared by ANOVA with post-hoc t-tests using Bonferroni’s correction for multiple comparisons. Cell capacitance was measured using the “Membrane Test” function of Clampex 8.0 shortly after gaining intracellular access. Rise times were measured from normalized currents, and desensitization rates were taken from single exponential decay curve fits using Prism 3.0 software. The percent enhancement or inhibition produced by co-application of a drug or modulator was determined by dividing the peak amplitude in the presence of drug by the average of control currents elicited by GABA alone before and after co-application, and multiplying by 100. Cells with significant rundown (> 20% reduction over 10 minutes) were excluded from analysis.

**Reverse Transcriptase PCR.** Total RNA was isolated from 75 cm$^2$ culture flasks of NT2-N cells using the Trizol$^\text{TM}$ method (Invitrogen / Life Technologies, Grand Island, NY). Two μg of total RNA were treated with amplification grade DNase I (Invitrogen) for 15 min at 25 °C, which was inactivated by addition of 25 mM EDTA followed by heat inactivation at 70 °C for 10 min, then the mixture was chilled on ice. First strand cDNA synthesis was performed using Superscript II (Invitrogen, Carlsbad, CA) with oligo (dT)$_{12-18}$ primer (0.5 mg/ml) according to the manufacturer’s protocol. As a negative control, 1 μl of diethylpyrocarbonate (DEPC)-treated water was substituted for Superscript II in some experiments. The reverse transcription enzyme was then heat inactivated for 15 min at 70°C. PCR
was subsequently performed with gene-specific primers for each of the GABA_AR subunits, β-actin, neurofilament-1 and GABA_A receptor associated protein (GABARAP), as listed in Table 1, using Platinum Taq polymerase (Invitrogen) on a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Boston, MA). Primers were selected with the assistance of Primer3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to maximize selectivity between subunits, with the 5’ primer derived from the region encoding the cytosolic loop between transmembrane domains 3 and 4, and the 3’ primer from the 3’ non-coding region. The cycling protocol consisted of 2 min at 94°C followed by 35 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1 min, with a final 7 min extension at 72 °C. PCR reactions were performed individually for each subunit in 50 μl of the following mixture: 1 μl of a 20 μM primer mixture, 1 μl of the reverse transcription product, 5 μl of 10X PCR buffer, 1.5 μl of 50 mM MgCl₂, 1 μl of 10 mM dNTP mixture, 0.5 μl Platinum Taq polymerase (5 units/μl) and 40 μl of DEPC-treated water. For each primer pair, PCR produced no visible product bands in the absence of template cDNA, with DNAse-treated RNA that was not reacted with Superscript II, or in the absence of Platinum Taq polymerase. Saturation experiments using control NT2-N cell mRNA confirmed linear increases in product density for each primer pair through at least 37 cycles of amplification. PCR products were mixed with gel loading solution type I (Sigma-Aldrich Chemicals, St. Louis, Mo.) and electrophoresed at 120V for 30-40 min on a 1.5% agarose gel prestained with ethidium bromide (0.05 mg/100 ml agarose) and then digitally imaged using a Typhoon 8600 Fluorescence imager (Biorad, Hercules, CA). Molecular weights of PCR products were calculated from a
100 bp ladder (Invitrogen) and band density was measured using UN-SCAN-IT gel 5.1 software (Silk Scientific Co., Orem, UT). The density of the PCR product for each GABA<sub>A</sub>R subunit was divided by the density of the actin PCR product band for that RNA sample, run in a parallel PCR reaction at the same time, to yield a relative product density ratio. No consistent change in actin PCR product densities was observed at any time point after hypoxia. In some experiments, primers for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were used in addition to actin primers; no differences in the ratio of actin to GAPDH products were observed. However, the greater relative abundance of GAPDH mRNA required a lower cycle number to remain in the linear amplification range, hence actin was used as the normalizing product. Relative product densities for multiple experiments were averaged and compared to controls by one-way ANOVA; post-test analysis was performed by unpaired t-test for selected subunits and time points, with Bonferroni’s correction for multiple comparisons, using Prism 3.0 software (Graphpad).

**RESULTS**

**Effects of hypoxia on NT2-N cell viability and morphology.** After 5 weeks exposure to RA and 3 additional weeks treatment with mitosis inhibitors, a population consisting mostly of neuron-like NT2-N cells was produced, with few remaining NT2 precursor cells, as previously observed (Neelands et al., 1998; Pleasure et al., 1992). NT2-N cells had a distinctly neuronal morphology with branching, tapered dendrites and uniform diameter axons (Figure 1A), and when
plated at moderate density tended to cluster in ganglion-like groups as observed with primary neuronal cultures (data not shown). Preliminary experiments showed no obvious NT2-N cell loss or morphological changes after hypoxia when cells were maintained in standard (high glucose) DMEM medium and exposed to ≤ 1% O₂ for up to 8 h; in all subsequent experiments, 8 h was used as the standard hypoxic exposure time. Preservation of control NT2-N morphology (Figure 1A) is evident in sample photomicrographs of living cells taken immediately after hypoxic treatment (Figure 1B) or after 48 h recovery under normoxic conditions (95% air/5% CO₂, 37 °C, Figure 1C). Some loss of background NT2 precursor cells was apparent at the 48 h timepoint. To assess cell integrity after hypoxia, cells were stained with 0.05% trypan blue (Sigma Chemical Co.) as a marker of cell death. There was no difference in the number of trypan blue-stained NT2-N cells between control and hypoxic groups (data not shown), with less than 5% of cells stained in either condition. No obvious changes in morphology were observed at recovery time points up to 96 h after hypoxia.

Trypan blue staining may not adequately account for hypoxic cell death if dead cells do not remain adherent to the bottom of the dish. As an independent means to determine whether the 8 h hypoxic exposure caused significant cell death or injury, we also measured release of lactate dehydrogenase (LDH) into the medium. Prior to hypoxic exposure, the standard medium was replaced with DMEM-HG of identical composition except lowered serum concentration and absence of phenol red indicator dye, which would otherwise contribute to the background activity. Samples of medium were assayed for LDH release at 24, 48 and
96 h after exposure. After hypoxic exposure, LDH release was consistently less than 5% of maximal LDH, and there was no difference compared to control cells at any of the time points measured (n=3 dishes per condition with results repeated twice; a sample experiment is shown in Figure 2). Slightly lower values were observed at the initial time point, likely due to the shorter incubation (8 h vs. 24 h), though this difference was not significant.

Another index of cell injury is altered morphology, as injured cells may swell or retract dendritic processes during involution. Since membrane capacitance (Cm) is closely correlated with cell membrane surface area (Gentet et al., 2000), retraction of proximal dendrites or somatic swelling after hypoxia could result in altered Cm, given adequate space clamping. During whole-cell recording (see below), we measured Cm of individual cells shortly after gaining access to the intracellular space. There was no significant difference in Cm between control cells (Control, 19.7 ± 2.0 pF, n = 11) and hypoxia-treated groups recorded within 1 h after 8 hours hypoxia (20.4 ± 2.5 pF, n = 11), or after normoxic recovery periods of 24 h (21.6 ± 2.6 pF, n = 10), 48 h (20.2 ± 2.3 pF, n = 6) or 96 h (17.0 ± 1.0 pF, n = 9), by one-way ANOVA (p>0.05). Together, these data suggest that 8 h of hypoxia (at ≤ 1% O₂) did not alter cell size or increase cell death in NT2-N cells.

**Effects of Hypoxia on GABAₐR currents.** Whole-cell patch-clamp recordings from control and hypoxia-treated NT2-N cells showed no significant differences in resting membrane potential, access resistance, or membrane capacitance (see above). However, hypoxia changed maximal GABAₐR currents with a biphasic time course. Brief (≤ 10 sec) applications of increasing GABA
concentrations (1 μM to 1 mM) evoked concentration-dependent inward currents from both control and hypoxia-treated NT2-N cells voltage clamped at −70 mV (Figure 3A). Peak currents values were fitted to a sigmoidal logistic function (Figure 3B) showing no significant change in the GABA EC$_{50}$, (by one-way ANOVA). A small but significant reduction in Hill slope was observed at 24 h and 48 h after hypoxia compared with control (both p<0.05 by t-test with Bonferroni’s correction after ANOVA, see Table 2), which could indicate a change in the cooperativity of GABA$_A$R binding after hypoxia, though other explanations (including mixed populations of receptors with different EC$_{50}$s) are also possible.

A more prominent change was observed in maximal GABA-evoked currents. Maximal currents were significantly increased in cells recorded within 1 h after hypoxia (0 h), but were significantly reduced below baseline after 48 h recovery (p<0.001 by t-test with Bonferroni’s correction after ANOVA) and then returned to baseline after 96 h recovery (see Table 2). The changes in maximal current were not due to altered cell size, as membrane capacitance was not significantly different between time-points (Figure 3C). Moreover, maximal GABA$_A$R current normalized to membrane capacitance (control: 36.4 ± 4.6 pA/pF, n=14) was significantly increased at 0 h after hypoxia (63.3 ± 8.3 pA/pF, n=13, p<0.05, Bonferroni’s test after ANOVA), but was significantly reduced after 48 h recovery (19.6 ± 4.1 pA/pF, n=12, p<0.001) and returned to baseline at 96 h (49.5 ± 10.2 pA/pF, n=9, p>0.05, Figure 3D). Normalized to control, GABA$_A$R current density was increased 174 ± 11% immediately after hypoxia and reduced to 54 ± 11% at 48 h.
The kinetics of GABA-evoked currents also changed after hypoxia. GABA currents were normalized to the maximal current for each cell and then averaged to assess changes in the concentration-dependent activation rates and desensitization rates at GABA concentrations greater than 10 μM (Figure 4A-C). Normalized current traces demonstrate slowing of concentration-dependent activation at 0 h (Fig. 4B) and 48 h (Fig. 4C) after hypoxia, compared to control currents (Fig. 4A). Current activation rates, measured as the 10-90% rise time (Fig. 4D), were slowed at 0 h and 48 h after hypoxia at GABA concentrations greater than 30 μM. This slowing was significant (p<0.05) at 0 h for 100 μM GABA currents and at 48 h for 100 μM (p<0.05), 300 μM (p<0.05), and 1000 μM (p<0.05). There was also slowing of desensitization rates at 0 h and 48 h after hypoxia (Fig. 4E), which was significant at 0 h for 30 μM (p<0.05) and at 48 h for 30 μM (p<0.05), 100 μM (p<0.001), and 300 μM (p<0.01). This slowing did not result from a shift in GABA concentration-response relationship, as no significant change in the EC\textsubscript{50} for GABA was observed at either time point after hypoxia, and suggests that changes in gating kinetics for channel opening and desensitization may have occurred.
Effects of hypoxia on GABA_\text{A}R pharmacology. Individual control cells and hypoxia-treated cells allowed to recover for 0, 24, 48 or 96 h were tested for their responsiveness to the positive GABA_\text{A}R allosteric modulators diazepam, zolpidem, and pentobarbital, and the negative allosteric modulator, Zn^{+2}. A summary of concentration-response parameters for these modulators is presented in Table 2.

Diazepam. The benzodiazepine, diazepam, enhanced whole-cell GABA_\text{A}R currents in a concentration-dependent manner (Figure 5A). Diazepam concentration-response curves were obtained by co-application of increasing concentrations of diazepam with 10 μM GABA. There was no significant difference in EC_{50} or Hill slope (p > 0.05 by one-way ANOVA) for diazepam enhancement of 10 μM GABA-evoked currents at any time-point after hypoxia. Maximal enhancement by diazepam tended to be increased after hypoxia (Con: 229 ± 14 %, n=13; 0 h: 294 ± 44%, n=4; 24 h: 263 ± 23%, n=9); this difference was significant 48 h after hypoxia (283 ± 19%, n=7, p< 0.05 by unpaired t-test compared to control).

Zolpidem. The imidazopyridine, zolpidem, is a benzodiazepine site agonist with relatively high affinity for GABA_\text{A}R\text{s} containing α1 subtype, moderate affinity at α2 and α3-containing receptors, and very low affinity at receptors with the α5 subtype (Korpi et al., 2002). Zolpidem enhanced whole-cell GABA_\text{A}R currents in a concentration-dependent manner (Figure 4B). Zolpidem concentration-response curves were obtained by coapplication of increasing concentrations of zolpidem with 10 μM GABA (Figure 5B). After hypoxia, there was an initial small but significant
reduction in the EC$_{50}$ for zolpidem enhancement of GABA$_{A}$R currents (Con: 396 ± 71 nM, n=6; 0 h: 147 ± 16 nM, n=3, p < 0.05, see Table 2), associated with a significant reduction in maximal zolpidem potentiation of 10 μM GABA currents compared to control cells (Con: 283 ± 13%, n=8; 0 h: 219 ± 19%, n=3). The reduction in maximal zolpidem potentiation persisted after 48 h normoxic recovery (48 h: 209 ± 8.7%, n=8, p < 0.001), though the EC$_{50}$ reverted to control values (409 ± 125 nM, n=6, p> 0.05).

**Pentobarbital.** Pentobarbital also enhanced 10 μM GABA$_{A}$R currents in a concentration-dependent manner (Figure 5C). There was no significant difference in maximal enhancement, EC$_{50}$ for enhancement, or Hill slope between control NT2-N cells and cells 48 h after hypoxic treatment.

**Zinc.** The divalent cation, Zn$^{2+}$, is a noncompetitive antagonist of GABA$_{A}$R currents (Macdonald and Olsen, 1994). Zn$^{2+}$ reduced whole-cell currents elicited by 30 μM GABA in a concentration-dependent manner (Figure 5D). There was no significant difference in maximal inhibition, IC$_{50}$ or Hill slope for Zn$^{2+}$ inhibition of GABA$_{A}$R currents between control cells (n=7) and cells recorded immediately (n=6) or 48 h (n=4) after hypoxia (see Table 2).

**Effects of hypoxia on GABA$_{A}$R subunit expression.** Total RNA was isolated from NT2-N cells at the same developmental stage (8 weeks after the onset of RA treatment) at which electrophysiological studies were performed, under control conditions or at the same time points after 8 h hypoxia. After first strand cDNA synthesis, reverse transcription polymerase chain reaction (RT-PCR) was
performed using primers specific for human \( \alpha 1-6, \beta 1-3, \gamma 1-3, \delta, \epsilon, \pi \) and \( \theta \) subunit subtypes (see Table 1), to determine whether GABA\(_A\)R subunit mRNA expression was altered relative to the expression of actin. The relative expression of GABA\(_A\)R subunits changed in a specific, time-dependent fashion after hypoxic exposure, as demonstrated in Figure 6. NT2-N cells 8 weeks after onset of retinoic acid treatment expressed a consistent profile of subunits including \( \alpha 1, \alpha 2, \alpha 3, \) and \( \alpha 5 \) (with less consistent expression of \( \alpha 4 \)), \( \beta 2 \) and \( \beta 3 \) (with less expression of \( \beta 1 \)), \( \gamma 2 \) and \( \gamma 3 \), and the more recently discovered subunits, \( \epsilon \) and \( \pi \) (see Figure 6A). There was no consistently detectible expression of the \( \alpha 6, \gamma 1, \delta \) or \( \theta \) subtypes under control conditions. These results are consistent with an intermediary stage of \textit{in vitro} development between NT2N cells at 5 weeks (Neelands et al., 1998) and 13 weeks (Neelands et al., 1999) after the onset of RA exposure, with the presence of mRNAs for \( \alpha 1, \alpha 4, \beta 2 \) and \( \gamma 2 \), which were detected in the prior studies at 13 weeks but not at 5 weeks, when only \( \alpha 2, \alpha 3, \alpha 5, \beta 3 \) and \( \gamma 3 \) were consistently detected (Neelands et al., 1998).

After hypoxia, there was an overall trend toward decreased expression of GABA\(_A\)R subunits relative to actin (Figure 6B), which was significant at 48 h recovery compared to control (\( p<0.01 \) for average of all GABA\(_A\)R subunit/actin ratios). Expression of the \( \alpha 1 \) subunit was significantly reduced (\( p < 0.05, n=4 \) replicates at each time-point) immediately after hypoxia and remained low to absent at subsequent time-points up to 96 h after hypoxia. The reduction in \( \alpha 5 \) was also significant (\( p < 0.05 \)) at the 0 h and 48 h recovery time-points. Significant
reductions in $\beta_2$ and $\gamma_2$ (p < 0.05) relative expression were also observed after 48 h recovery. Since changes in GABA$_A$R number might be related to interactions with cytoskeletal structural proteins, we also measured the relative expression of the GABA$_A$ receptor-associated protein (GABARAP) after hypoxia. This protein was strongly expressed in control NT2N cells (1.25 ± 0.11 relative to actin, n=8), but was unchanged at any time-point after hypoxia (0 h: 1.13 ± 0.11; 24 h: 1.15 ± 0.05; 48 h: 1.23 ± 0.05; 96 h: 1.11 ± 0.11, n=4 each, all p>0.05). However, the neuronal structural protein, neurofilament 1 (NF), which was consistently expressed at moderate levels in control cells (0.46 ± 0.08 relative to actin, n=8), was reduced 48 h after hypoxia (0.23 ± 0.08, n=4, p < 0.05, unpaired t-test). The significance of this reduction, in the absence of obvious morphological changes, was uncertain.

**DISCUSSION**

The present experiments were designed to assess whether hypoxia triggers modulation of GABA$_A$R subunit mRNA expression and pharmacology in an *in vitro* neuronal cell system, independent of brain regional context or connectivity. The absence of synapse formation in this system allows investigation of receptor regulation without presynaptic influences. NT2-N cells provide an excellent model in which to test this hypothesis, due to the relative homogeneity of their responses to GABA and GABA$_A$R modulators (Neelands et al., 1998) and their expression of a consistent subset of GABA$_A$R subunit mRNAs at each stage of *in vitro* development (Neelands et al., 1999). The profile of GABA$_A$R subunit mRNA expression
observed here in NT2-N cells 8 weeks after the onset of retinoic acid treatment was intermediate between that observed at 5 weeks (Neelands et al., 1998) and 13 weeks (Neelands et al., 1999). It is possible that individual cells may not express the complete pattern of subunits observed in mRNA obtained from an entire flask of cells. To determine the individual mRNA expression patterns of single NT2-N cells would require single cell RT-PCR or amplified RNA techniques (Van Gelder et al., 1990), and would be subject to possible errors based on the small amount of mRNA that can be obtained from single cells. However, the consistency of pharmacological responses between cells, and the overall consistency of the PCR profile of controls (evidenced by small standard errors) suggests that GABA<sub>A</sub>R subunit mRNA expression in individual NT2-N cells may be quite similar.

We found that 8 h hypoxic exposure did not increase immediate or delayed cell death. A prior study of the effects of hypoxia on NT2-N cells (Rootwelt et al., 1998) showed a significant increase in delayed cell death, with LDH release increased from 14 ± 4% to 24 ± 6% at 48 h after 6 h of hypoxia. Although the duration of hypoxia was similar, those experiments used an oxygen-scavenging catalyst to create nominal hypoxia (0.0% O<sub>2</sub>), which may account for the increased LDH release. In our experiments, the reduction in oxygen tension to ~ 1% of ambient gas corresponds to a PO<sub>2</sub> of ~ 8 mm Hg (at sea level), consistent with levels obtained during ischemia. Below 6 mm Hg, oxygen deprivation results in marked hydrolysis of creatine phosphate and decreased ATP levels (Folbergrova et al., 1990). Although some brain regions, such as area CA1 of hippocampus (Schmidt-Kastner and Freund, 1991), are particularly vulnerable to even brief hypoxic insult,
other areas tolerate moderate hypoxic exposure, and may undergo compensatory physiological changes. Our model, in which the reduction in PO$_2$ does not result in significant cell death, may thus mimic the microenvironment of the ischemic penumbra, and could reflect the type of changes in gene expression and receptor function that may occur in surviving brain neurons. Such changes could provide an opportunity for therapeutic intervention to protect the ischemic or hypoxic brain (Schwartz et al., 1995).

Hypoxia affected maximal GABA$_A$R currents in a biphasic manner. Within 2 h after hypoxic treatment, GABA$_A$R current (normalized to cell capacitance) was significantly increased, associated with a decrease in maximal zolpidem potentiation and the EC$_{50}$ for zolpidem. The early change in zolpidem sensitivity is unlikely to have resulted from transcriptional regulation, due to the short duration of hypoxia (8 h) which was likely insufficient for replacement of existing GABA$_A$Rs with newly transcribed and translated receptors. Moreover, the observed reduction in $\alpha$1 subunit expression seen at the 0 h time point would be expected to cause an increase the EC$_{50}$ for zolpidem, not a decrease as observed. The transient increase in maximal currents immediately after hypoxia could have resulted from increased insertion of internalized GABA$_A$Rs into the membrane, a decrease in constitutive internalization of GABA$_A$Rs during hypoxia, or a change in the phosphorylation state of GABA$_A$R subunits or associated proteins, among other possibilities. Slowing of GABA current rise times and desensitization rates, as observed here, was associated with dephosphorylation of the GABA$_A$R $\beta$1 or $\beta$3 subunit in HEK293T cells (Hinkle and Macdonald, 2002); such dephosphorylation might be expected in the presence of
lowered ATP levels during hypoxia. It is unclear whether a change in phosphorylation could explain the altered zolpidem responsiveness or increased maximal current. Teleologically, a transient increase in GABA\(_A\)R function immediately after hypoxia could serve as a compensatory mechanism to combat depolarization-induced excitotoxicity.

The subsequent reduction in maximal GABA\(_A\)R currents after 48 h recovery was also not associated with any obvious alteration of cell size or morphology. Both the increase in maximal current shortly after hypoxia and the subsequent decrease after 48 h recovery likely reflect altered channel density, as equal chloride concentrations were used to obviate any changes in the chloride or bicarbonate ion concentrations, and there was no change in cell capacitance, an indicator of cell size. Small changes in cell size at the distal dendrites might not be detected, however, due to space clamp issues. A change in single channel conductance or open time properties is also possible. Further studies using single channel recordings and more detailed morphometric investigation may help to resolve these questions.

The pharmacology of GABA\(_A\)R currents was also altered after 48 h recovery, with slightly increased maximal diazepam enhancement but decreased zolpidem enhancement. These changes appeared to be specific to the BZ site, as the GABA EC\(_{50}\) was unchanged, and neither pentobarbital enhancement nor zinc inhibition of GABA\(_A\)R currents was altered by hypoxia. Coincident with the altered BZ pharmacology, we found changes in the expression pattern of GABA\(_A\)R subunits, with marked reduction of \(\alpha_1\) subunit mRNA expression, as well as smaller but significant reductions in \(\alpha_5\), \(\beta_2\) and \(\gamma_2\) mRNA expression, 48 h after hypoxia. These
changes in BZ pharmacology could have resulted from reduced $\alpha_1$ expression if other BZ-sensitive $\alpha$ subtypes ($\alpha_2$, $\alpha_3$ or $\alpha_5$) were assembled in its place. The observed slowing of current rise times and desensitization rates 48 h after hypoxia could be seen with substitution of $\alpha_3$ for $\alpha_1$, as prior studies with recombinant receptors demonstrated slower activation and desensitization rates with $\alpha_3$-containing receptors (Gingrich et al., 1995). However, $\alpha_3$-containing receptors had a 10-fold higher $EC_{50}$ for GABA than $\alpha_1$-containing receptors, which was not observed in our studies. Inclusion of the $\alpha_5$ subtype would create a GABA$_A$R with diazepam sensitivity but little response to zolpidem. However, a transient reduction in $\alpha_5$ mRNA expression was observed at the 48 h recovery time-point, making such a substitution less likely. The lack of change in zolpidem $EC_{50}$ for enhancing GABA$_A$R currents is also perplexing, since loss of $\alpha_1$ would be expected to shift the $EC_{50}$ to the right. However, increased diazepam and decreased zolpidem enhancement, and the slowing of activation and desensitization kinetics without a change in GABA $EC_{50}$, might also result from combinations of different $\alpha$ subunits, either within single GABA$_A$R holoreceptors or in mixed populations of GABA$_A$Rs. Confirmation of this hypothesis would require pharmacological testing of defined combinations of recombinant $\alpha$ subunits with appropriate $\beta$ and $\gamma$ subunits.

**Hypoxia and GABA$_A$Rs.** Hypoxia has been shown to affect GABAergic neurotransmission and GABA$_A$R function (Schwartz-Bloom and Sah, 2001). However, studies of post-synaptic GABA$_A$Rs have shown conflicting results. In rat cerebral cortex (Ninomiya et al., 1982), a short, acute hypoxic exposure led to up-
regulation of postsynaptic GABA\(_\alpha\)R binding, attributed to decreased presynaptic GABA release; GABA\(_\alpha\)R binding returned to control values after a sustained 24 h hypoxic period. In the ischemic gerbil brain, however, GABA\(_\alpha\)R downregulation occurred within 30 min after reperfusion, possibly due to receptor internalization, but returned to normal after 2 hours (Alicke and Schwartz-Bloom, 1995). In other studies, hypoxia-induced changes were long-lasting; a reduction in GABA\(_\alpha\)Rs labeled by \(^{35}\text{S}\) t-butylbicyclopentosphorothionate (TBPS) in the CA1 dendritic field and the striatum was seen several days after ischemia in rats (Schwartz et al., 1995), and reductions in \(^{3}\text{H}\)muscimol or \(^{3}\text{H}\)flunitrazepam binding were seen in gerbil striatum 27 days after ischemia (Onodera et al., 1987).

Although the reduction in GABA\(_\alpha\)R binding after hypoxia \textit{in vivo} primarily reflects loss of principal neurons, changes in GABA\(_\alpha\)R subunit mRNA expression have also been observed in surviving neurons. mRNA levels of the \(\alpha1\) and \(\beta2\) subunits decreased within 30 minutes after reperfusion in hippocampal areas CA1, CA3 and dentate gyrus (Li et al., 1993). These levels normalized within 4 hours in CA3 and dentate gyrus, areas less sensitive to hypoxia. We observed similar subunit mRNA changes (reduction of \(\alpha1, \alpha5, \beta2\) and \(\gamma2\)), suggesting that NT2-N cells may provide a valid model of neurotransmitter receptor transcriptional regulation by hypoxia. The restoration of maximal GABA\(_\alpha\)R currents to control values after 96 h, associated with increases in mRNAs of most subunits back to control levels, indicates that the reduction in GABA\(_\alpha\)R currents in NT2-N cells is transient. However, the reduction in \(\alpha1\) mRNA was still present at 96 h after hypoxia. It will be important to determine, using immunohistochemistry and Western blot analysis,
whether the subunit proteins also change after hypoxia. Further studies at later time points will help determine whether this represents a long lasting phenotypic “switch” in GABA$_A$R subunit mRNA transcription, and whether the observed changes alter GABA$_A$R subunit composition. Such a switch \textit{in vivo} might account for a prolonged or permanent increase in seizure susceptibility, since the $\alpha_1$ subunit is associated with the anticonvulsant actions of the BZs (Rudolph et al., 2000) implying localization of $\alpha_1$-containing receptors at sites important for seizure regulation. The modification of GABA$_A$R subunit mRNAs by hypoxia may thus be a novel mechanism of neuronal plasticity that could partially account for the clinical effects of hypoxia \textit{in vivo}. However, changes in GABA$_A$R expression and function may or may not occur in actual neurons within the brain, and these findings should be replicated in other neuronal systems \textit{in vitro} and \textit{in vivo} to determine their clinical relevance.
REFERENCES


Greenfield, L. J. Jr., Macdonald, R. L., 1996. Whole cell and single channel $\alpha$1 $\beta$1 $\gamma$2S GABA\textsubscript{A} receptor currents elicited by a "multipuffer" drug application device. Pflügers Archives 432, 1080-1090.


Hagberg, H., Andersson, P., Kjellmer, I., Thiringer, K., Thordstein, M., 1987. Extracellular overflow of glutamate, aspartate, GABA and taurine in the cortex...
and basal ganglia of fetal lambs during hypoxia-ischemia. Neuroscience Letters 78, 311-317.


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Figure 1. Photomicrographs of NT2-N cells. Hoffmann modulation contrast images of NT2-N neurons under control (normoxic) conditions (A), immediately following 8 h of hypoxia (B) and 48 h after hypoxia (C). No obvious differences in cell size or morphology were observed. The scale bar in C represents 20 μm and applies to all three images.
Figure 2. Lactate dehydrogenase (LDH) release after hypoxia. Bars represent normalized LDH release relative to total LDH after cell lysis for that condition (n=3 dishes per condition, means of 3 replicate samples from each dish at each time point were averaged). Closed bars signify hypoxia-treated cultures; open bars were control dishes not exposed to hypoxia, processed in parallel. Medium was replaced just prior to the 8 h hypoxic exposure, and samples were obtained immediately afterward (0 h), and after 24 h, 48 h or 96 h recovery. No significant differences in LDH release were observed between control and hypoxia-treated cells (p>0.05 by one-way ANOVA).
Figure 3. **GABA$_A$R currents after hypoxia.** A. Whole-cell GABA$_A$R currents evoked by increasing concentrations of GABA (1 μM – 1 mM) in a control cell (left traces) and 48 h after hypoxia (right traces). Maximal current amplitudes were normalized to demonstrate similarities in time-course, but note the difference in vertical scale (pA). B. Peak currents evoked by increasing GABA concentrations in control cells (filled circles) and immediately (0 h, open circles, n=12-14), 24 h (open squares, n = 8), 48 h (open triangles, n=7-9) or 96 h (open diamonds, n=9) after 8 h of hypoxia. Symbols represent means of peak currents evoked at that GABA concentration. For clarity, error bars are not shown for the 24 h and 96 h time points, which were not significantly different from control. C. Membrane capacitance (Cm) measured shortly after attaining whole-cell recording configuration, for control recordings and at each recovery time point. No significant difference from control was observed at any time point (n shown in each column, p>0.05 by one way ANOVA). D. Maximal GABA currents normalized to cell capacitance for control cells and at selected times after hypoxia. Bars represent mean ± S.E.M.; the n for each condition is shown in the appropriate bar. Asterisks show columns significantly different from each other as marked (* p<0.05, *** p<0.001 by t-test with Bonferonni correction after ANOVA).
Figure 4. Changes in the kinetics of GABA$_A$R currents after hypoxia. Normalized averaged GABA$_A$R currents (3-6 cells each condition) elicited by GABA (10 – 1000 μM) in control NT2-N cells (A) and cells recorded within 1 h after hypoxia (B) or 48 h after hypoxia (C). Currents obtained after hypoxia show slowing of activation and desensitization kinetics. D. Activation times, measured as 10%-90% rise time for each GABA concentration for control cells (n=7-9), 0 h after hypoxia (n=7-9), and 48 h after hypoxia (n=5-7), were significantly increased at concentrations of 30 μM and above (* p<0.05, ** p < 0.01, *** p<0.001). E. Desensitization time constants ($\tau$) from single exponential fits of current decays during continued application of GABA for control cells (n=5-6), 0 h after hypoxia (n=4-7), and 48 h after hypoxia (n=3-5), showing significant slowing of desensitization after hypoxia.
Figure 5. Modulation of GABA$_A$R currents after hypoxia. A. Enhancement of 10 μM GABA currents by increasing concentrations of diazepam (DZP) in control cells (closed circles, n=13) and 48 h (open triangles, n=7) after 8 h of hypoxia, normalized to control current amplitude (i.e. showing fold-change relative to control). A significant increase was observed at 48 h after hypoxia (p < 0.05) compared to control, with no change in EC$_{50}$ or Hill slope (see Table 2). B. Enhancement of 10 μM GABA currents by zolpidem (ZOL) in control cells (closed circles, n=8) and 48 h after hypoxia (open triangles, n=6). The EC$_{50}$ for enhancement was unchanged, but maximal enhancement was significantly reduced after hypoxia (p < 0.01). C. Enhancement of 10 μM GABA currents by pentobarbital (PB) in control cells (closed circles, n=3) or and 48 h (open triangles, n=2) after hypoxia. Maximal enhancement and EC$_{50}$ were not significantly different (p>0.05). D. Inhibition of 30 μM GABA currents by increasing concentrations of Zn$^{2+}$ in control cells (n=5) and 48 h after hypoxia (open triangles, n=3). No differences in zinc inhibition were observed (p>0.05).
Figure 6. Reverse transcriptase PCR of GABA<sub>A</sub>R subunits and other gene products. A. Agarose gel electrophoresis of PCR products of reverse-transcribed RNA from control cells and 24 h or 48 h after hypoxia. Lanes show individual reactions with primers specific for GABA<sub>A</sub>R subunits (α1-α6, β1-β3, γ1-γ3, δ, ε, π, θ), neurofilament-1 (NF), GABARAP (GR), and β-actin (Ac). Molecular weight markers (M) are in 100 bp increments; the darker band indicates 600 bp. B. Averaged ratios of PCR product densities relative to actin for each GABA<sub>A</sub>R subunit from control and post-hypoxic NT2-N cells at indicated times after hypoxia. N=9 independent reactions for control cells, 4-5 at each post-hypoxic time-point. Asterisk denotes a significant difference from control (p < 0.05 by t-test with Bonferroni correction after ANOVA).
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Table 2. GABA pharmacology of control NT2-N cells and at stated recovery times after hypoxia

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<td>875±167(9)</td>
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<td>9.9±2.1(4)</td>
<td>12±2.8(3)</td>
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<td>( IC_{50} ) (μM)</td>
<td>65±12(7)</td>
<td>73±16(6)</td>
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<td>63±8.6(4)</td>
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* \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) compared to control

<sup>a</sup> 0 h, 24 h, 48 h, 96 h = 8 h hypoxia followed by 0 h, 24 h, 48 h, or 96 h normoxic recovery
Manuscript 3

Hypoxia-inducible factor-1α alters GABA_A receptor expression and function in NT2-N neurons:
Different effects of hypoxic and chemical stimulation

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Running title: HIF-1α alters GABA_A receptor function

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ABSTRACT

We have previously demonstrated that hypoxia (1% O2, 8 h) altered GABA_A receptor (GABAR) function and subunit expression in NT2-N neuronal cells with a biphasic time course. Maximal GABAR currents were increased immediately after hypoxia, but reduced 48h later, associated with reduced mRNA levels of the α1, α5, β2 and γ2 GABAR subunits. We now report that this hypoxic stimulus induced significant accumulation of hypoxia-inducible factor 1α (HIF-1α), a major transcription factor regulating cellular responses to hypoxic stress. To determine whether HIF could mediate hypoxia-induced GABAR changes, we used two chemical inducers of HIF-1α, Co^{2+} and deferoxamine, to stimulate HIF-1α in the absence of hypoxia. Exposure to Co^{2+} (100 – 1000 μM, 8 h) caused concentration-dependent accumulation of HIF-1α protein immediately after treatment, which faded 24 h later. In whole-cell patch-clamp recordings, maximal GABA-evoked currents were unchanged immediately after 100 μM Co^{2+} exposure but significantly increased 48 h later, in contrast to increased GABAR current immediately after hypoxia and decreased current 48 h afterward. Semiquantitative reverse transcriptase (RT)-PCR performed 24 h after Co^{2+} treatment showed increased α5, β3 and ε subunit mRNA expression. Similar induction of HIF-1α and changes in GABAR function and expression with the same time course were induced by exposure to the iron chelator, deferoxamine (100 μM). These data demonstrate that chemical induction of HIF-1α alters GABARs differently than hypoxia, suggesting that hypoxia triggers additional mechanisms that occlude or override HIF-1α effects on GABAR function.
Hypoxia/ischemia can cause seizures (Bergamasco et al., 1984) or myoclonus (Hallett, 2000) which may be related to hypoxia-induced dysfunction of GABA (γ-aminobutyric acid) type A receptors. GABA, the major inhibitory neurotransmitter in the mammalian CNS, binds to GABARs to regulate gating of the chloride ion channel (Olsen and Macdonald, 2002). Like other members of the ligand-gated ion channel superfamily, GABARs are pentameric hetero-oligomers assembled from 7 subunit families with multiple subtypes: α(1-6), β(1-3), γ(1-3), δ, ε, π, and θ that determine their kinetics and pharmacology (Olsen and Macdonald, 2002). GABARs play a key role in the control of cell and network activity, hence modification of GABAR function by hypoxia has important consequences for neural excitation and communication (Schwartz-Bloom and Sah, 2001). After hypoxia, significant changes in GABAR number and function have been observed (Alicke and Schwartz-Bloom, 1995; Mileson et al., 1992). In a rat model of focal ischemic stroke, immunohistochemical studies demonstrated reductions in the α1, α2, α5 and γ2 subunits at 1, 7 and 30 days after infarct, peaking at the 7d time point; these changes were specific, as the α3 subunit was unchanged ipsilateral to the infarct but upregulated at homotopic contralateral cortex (Redecker et al., 2002). In NT2-N cells, we observed reductions in the same subunits 48h after 8h hypoxia (α1, α5, β2 and γ2 mRNA (Gao et al., 2004)), suggesting that NT2-N cells are indeed a valid model of GABA_AR regulation by hypoxia, though they do not correlate directly with any particular class of neurons.
The underlying mechanisms responsible for post-hypoxic regulation of GABAR subunit expression remain to be elucidated. Several transcription factors have been linked to the modulation of gene expression by oxygen, including AP-1 (Premkumar et al., 2000), NFκB (Koong et al., 1994), EGR-1 and C/EBPβ/NF-IL-6 (Yan et al., 2002) and hypoxia-inducible factor 1 (HIF-1) (Maxwell and Salnikow, 2004). HIF-1 plays a key role in the initiation of hypoxic responses due to its unique ability to act as an oxygen sensor. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β. HIF-1α is basic helix-loop-helix (bHLH) protein that is constitutively expressed but degraded in normoxia after oxygen-dependent hydroxylation of specific proline residues by prolyl hydroxylases. Proline hydroxylation facilitates interaction with the tumor suppressor von Hippel Lindau (VHL) protein and subsequent ubiquitination and degradation in the proteasome. HIF-1β, also known as arylhydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed (Maxwell and Salnikow, 2004). It combines with HIF-1α and binds to hypoxia response element (HRE) genomic DNA domains to promote transcription of hypoxia-regulated genes. HIF-1α can also be induced by exposure to cobalt chloride, deferoxamine (DFX) and other factors including insulin, thrombin and angiotensin II (Page et al., 2002). DFX prevents HIF-1α proline hydroxylation by chelating iron, a required cofactor for this enzyme (Wang and Semenza, 1993); cobalt may either substitute for Fe(II) with less efficacy (Maxwell and Salnikow, 2004) or bind directly to HIF-1α to prevent O₂-dependent degradation (Yuan et al., 2003). Activated HIF-1 stimulates transcription of genes involved cellular and physiological responses to hypoxia, particularly those involved in glycolysis,
angiogenesis and erythropoiesis. HIF-stimulated genes like vascular endothelial growth factor (VEGF) have been shown to promote neuronal survival (Wick et al., 2002), but it is not known whether HIF is involved in neuroprotection against hypoxia or regulates GABAR subunit expression in NT2-N cells.

NT2-N neuronal cells provide a useful model for studies of hypoxic effects on GABAR function and subunit expression (Gao et al., 2004). NT2 (Ntera2) cells, derived from a human teratocarcinoma, differentiate into the neuronal NT2-N phenotype when treated with retinoic acid (Pleasure et al., 1992). NT2-N cells express human GABARs with a consistent pharmacological profile and a pattern of GABAR subunit mRNA expression that develops over time in vitro (Neelands et al., 1998). Here we report that exposure to 1% O₂ for 8 h, which altered GABAR function without increasing cell death (Gao et al., 2004), caused induction of HIF-1α. Both cobalt and deferoxamine exposure also induced HIF-1α accumulation in NT2-N neurons. Unlike hypoxia, however, these agents increased maximal GABAR currents and subunit mRNA expression 48 h after exposure. HIF-1α stimulation thus regulated GABAR function, but in a fashion very different from that induced by hypoxia, suggesting that hypoxia likely triggers additional mechanisms that occlude or override the effects of HIF-1α activation.
METHODS

Reagents. Cobalt chloride, deferoxamine, GABA, retinoic acid (RA) and other fine chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) at the highest available purity.

NT2-N cell culture. NT2 immortalized precursor cells (Stratagene, La Jolla, CA) were maintained in Dulbecco’s modified Eagle’s medium–high glucose (DMEM-HG, Invitrogen, Grand Island, NY) with 10% fetal bovine serum and penicillin/streptomycin in a 37 °C incubator with 95% air/5% CO₂ (Neelands et al., 1998; Pleasure et al., 1992). Medium was changed at least twice per week. Cells were plated at 2 × 10⁶ cells/75 cm² flask and treated with retinoic acid (RA, 1 μM) in DMEM-HG complete medium with for 5 weeks. Each flask of cells was then replated into two 75 cm² flasks (1:2 dilution) with DMEM-HG medium containing mitotic inhibitors (10 μM FUDR, 10 μM uridine and 1 μM cytosine arabinoside) to select for non-dividing NT2-N neuronal cells. NT2-N cells were maintained with mitosis inhibitors for 3 weeks, at which point few undifferentiated NT2 precursor cells remained. Cells were then plated onto 35 mm culture dishes for subsequent electrophysiological recording. We define the “age” of NT2-N cells from the onset of RA treatment; hence the cells used in these experiments were 8-week NT2-N cells.

Drug and hypoxia treatment. On the day of experiment, cells were examined microscopically before use to confirm viability. NT2-N cells were treated with 100 μM cobalt chloride or 100 μM deferoxamine in DMEM HG for 8 hours in the
normoxic incubator (95% air/5% CO₂, 37°C). After chemical exposure, the medium was replaced with fresh DMEM HG and cells were either studied immediately (as described below), or returned to the normoxic incubator (95% air/5% CO₂, 37°C) for 24, 48 or 96 h prior to recording or RNA harvesting. For the “0 h” recovery time point, cells were recorded or harvested for mRNA within 1-2 h of termination of chemical exposure; for subsequent time points, recordings or RNA harvesting occurred within ± 2 h of the stated time.

Hypoxic exposure was performed as previously described (Gao et al., 2004). Briefly, cells were placed in a pre-warmed, humid anaerobic chamber (M.I.C.-101, Billups-Rothenberg, Del Mar, CA), which was then perfused with a 95% N₂/5% CO₂ mixture at 25 L/min for 4 min, producing an O₂ concentration of 0.8-1.0% which was unchanged after 8 h exposure. The chamber was then sealed and placed in a 37°C incubator for 8 h. At the end of hypoxic exposure, the medium was replaced with fresh DMEM HG (pre-equilibrated with air) and cells were either studied immediately or returned to the normoxic incubator (95% air/5% CO₂, 37°C) for 24, 48 or 96 h prior to experiments.

**Cytotoxicity Assays.** Cytotoxic effects of hypoxia, cobalt or DFX were assessed by trypan blue exclusion and by measuring release of lactate dehydrogenase (LDH) into the culture medium, as previously reported (Gao et al., 2004). Trypan blue (0.05%) was applied to cultures at selected times after hypoxia, cobalt or DFX treatment and the percentage of cells stained in 5 high power fields was obtained in triplicate for each condition. LDH release was measured in 8-week NT2-N cells plated onto 35 mm dishes 2 days prior to study. On the day of treatment, the routine
DMEM-HG medium was exchanged with medium lacking phenol red (to reduce background red absorption), in which the concentration of FBS was reduced to 5% to minimize background LDH activity. A standard volume of 1.0 ml was used for each of 3 dishes for each condition. Immediately after treatment, and after 24, 48 or 96 h recovery, three 100 µl samples were removed from each dish and placed in identified wells of a 96-well-plate, parafilm sealed and stored in the dark at 4 °C until all samples were collected. After the 100 µl samples were removed, 70 µl of 10X Lysis Solution (0.8% Triton X-100) was added to each dish to assess maximal LDH activity. Samples were placed in 0.5 ml Eppendorf tubes, briefly centrifuged to remove cellular debris, and subsequently transferred to the 96-well plate. Reconstituted Substrate Mix (50 µl) was then added to each well at room temperature (23 °C) in timed fashion, and the reaction was stopped with 1M acetic acid after 30 minutes. LDH activity was measured within one hour as absorbance of the formazan LDH product at 490 nm on a Spectramax Plus 96 well plate reader (Molecular Devices, Sunnyvale, CA). Fresh medium lacking phenol red was used as the blank from which absorbances were subtracted. Cytotoxicity was determined as the percent LDH activity released into the medium relative to maximal activity after cell lysis. Percent activities at each time point were determined by dividing the adjusted absorbance by the total LDH activity and multiplying by 100.

**Reverse Transcriptase PCR** Total RNA was isolated from 75 cm² culture flasks of NT2-N neurons using the Trizol™ method (Invitrogen/Life Technologies, Grand Island, NY). Two μg of total RNA were treated with amplification grade
DNase I (Invitrogen) for 15 min at 25 °C, which was inactivated by addition of 25 mM EDTA followed by heat inactivation at 70 °C for 10 min, then the mixture was chilled on ice. First strand cDNA synthesis was performed using Superscript II (Invitrogen) with oligo (dT)$_{12-18}$ primer (0.5 mg/ml) according to the manufacturer’s protocol. As a negative control, 1 μl of diethylpyrocarbonate (DEPC)-treated water was substituted for Superscript II in some experiments. The reverse transcription enzyme was then heat inactivated for 15 min at 70°C. PCR was subsequently performed with gene-specific primers for each of the GABA$_A$R subunits and β-actin as previously reported (Gao et al., 2004), using Platinum Taq polymerase (Invitrogen) on a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Boston, MA). The cycling protocol consisted of 2 min at 94°C followed by 35 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 1 min, with a final 7 min extension at 72 °C. PCR reactions were performed individually for each subunit in 50 μl of the following mixture: 1 μl of a 20 μM primer mixture, 1 μl of the reverse transcription product, 5 μl of 10X PCR buffer, 1.5 μl of 50 mM MgCl$_2$, 1 μl of 10 mM dNTP mixture, 0.5 μl Platinum Taq polymerase (5 units/μl) and 40 μl of DEPC-treated water. For each primer pair, PCR produced no visible PCR product bands in the absence of template cDNA, with DNase-treated RNA that was not reacted with Superscript II, or in the absence of Platinum Taq polymerase. Saturation experiments using control NT2-N cell mRNA confirmed linear increases in product density for each primer pair through at least 37 cycles of amplification. PCR products were mixed with gel loading solution type I (Sigma-Aldrich Chemicals, St. Louis, Mo.) and electrophoresed at 120V for 30-40 min on a 1.5% agarose gel.
prestained with ethidium bromide (0.05 mg/100 ml agarose) and then digitally imaged using a Typhoon 8600 Fluorescence imager (Biorad, Hercules, CA). Molecular weights of PCR products were calculated from a 100 bp ladder (Invitrogen) and band density was measured using UN-SCAN-IT gel 5.1 software (Silk Scientific Co., Orem, UT). The density of the PCR product for each GABA<sub>A</sub>R subunit was divided by the density of the actin PCR product band for that RNA sample, run in a parallel PCR reaction at the same time, to yield a relative product density ratio. Relative product densities for multiple experiments were averaged and compared to controls by one-way ANOVA; post-test analysis was performed by unpaired t-test for selected subunits and time points, with Bonferroni’s correction for multiple comparisons, using Prism 4.0 software (Graphpad).

*Immunoblots* 8-week NT2-N cells were grown in T75 flasks and exposed to hypoxia, cobalt- or deferoxamine (DFX)-containing medium or control for 8 h as described. For whole cell protein extracts, cells were rinsed twice in cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 4 °C) and then lysed either immediately or 24 h after 8 h hypoxia, in lysis buffer containing 100 mM NaCl, 50 mM NaF, 50 mM β-glycerol-phosphate, 2 mM EDTA, 1% Triton X-100, with 200 μM Na<sub>3</sub>VO<sub>4</sub> and 200 μM PMSF freshly added just prior to use. Control cells underwent the same solution changes and lysis. Cells were scraped and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was stored at -70 °C until subsequently analyzed as described below.

For nuclear protein extracts, NT2-N cells in T75 flasks were rinsed with cold PBS (4 °C), then scraped into 1 ml cold PBS, collected in a 1.5 ml microcentrifuge
tube and centrifuged at 3000 rpm for 10 min in an Eppendorf 5415C centrifuge at 4 °C. The cell pellet was washed in five packed cell volumes ( ~ 400 μl) of Buffer A (10 mM TrisHCl (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, freshly supplemented with 2 mM dithiothreitol (DTT), 1 mM sodium vanadate, 50 μl protease and phosphatase inhibitor cocktail (Sigma P8340), 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml leupeptin, and 10 μg/ml aprotinin) and incubated in Buffer A on ice for 10 min. Cells were homogenized with 12 strokes in a glass Dounce homogenizer. Nuclei were pelleted by centrifugation at 11,000 rpm (10,000 g) for 10 min at 4 °C. The supernatant was discarded, and nuclei were resuspended in 100 μl of Buffer B (0.42 M KCl, 20 mM TrisHCl (pH 7.5), 20% glycerol, 1.5 mM MgCl₂, freshly supplemented with 2 mM DTT, 1 mM sodium vanadate, 50 μl inhibitor cocktail). The suspension was incubated on a rotator at 4 °C for 30 min and then centrifuged in an Eppendorf 5415C at 4 °C for 30 min at 14,000 rpm (15,800 X g), and the supernatant containing nuclear proteins was collected.

Nuclear and whole cell extract proteins were analyzed by SDS-PAGE using an 8% polyacrylamide resolving gel through a 4% stacking gel. Equivalent amounts of protein (25 μg/lane for nuclear extracts, 50 μg/lane for whole cell extracts, determined by modified Bradford assay (Zor and Selinger, 1996) against BSA standards) were loaded into each lane. Molecular weight marker proteins were from Biorad (#161-0325). Proteins were then transferred by electrophoresis to a nitrocellulose membrane (95V, 4 h). The gel was subsequently counterstained with Coomassie blue (0.1%) and destained (in 40% methanol, 10% acetic acid) to confirm complete transfer of proteins to nitrocellulose. The membrane was blocked with 5%
dry milk at room temperature for 1 h, then incubated in a monoclonal anti-HIF-1α primary antibody directed against amino acids 432-528 of human HIF-1α (NB100-123, Novus Biologicals, Littleton, CO) at 1:500 dilution overnight at 4 °C. The membrane was then rinsed 5 times 5 min in TBS-T (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween20) followed by incubation in goat anti-mouse IgG-HRP secondary antibody (sc-2031, Santa Cruz Biotechnology, 1:10,000 dilution in 5% milk solution). The membrane was then incubated with chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology, Rockford, IL) for 1 min and exposed to Pierce CL-XPosure film (#34090) at room temperature for 1 min, then developed. The membrane was subsequently stripped and re-incubated with a rabbit IgG β-actin primary antibody (Sigma A5060, 1:3000 dilution) followed by anti-rabbit IgG-HRP secondary antibody (1:10,000 dilution), chemiluminescence reaction and development as above to quantify actin protein to ensure equal protein loading.

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). NT2-N cells were removed from the 5% CO₂ incubator and the feeding medium was replaced with external recording solution containing (in mM): 142 NaCl, 1 CaCl₂, 6 MgCl₂, 8.1 KCl, 10 glucose, 10 HEPES, 315-325 mOsm, pH 7.4. Patch clamp electrodes of 5-10 MΩ were filled with internal micropipette solution containing (in mM): 153.3 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, 4 MgATP, 300-305 mOsm, pH 7.3. Signals were low-pass filtered at 1000 Hz using the onboard 4-pole Bessel filter in the Axopatch 200B, then
digitized on line at 1000 Hz using a Digidata 1200A Data Acquisition System (Axon Instruments, Foster City, CA) and subsequently analyzed off-line using Clampfit 9.0 software (pClamp 9.0, Axon Instruments). Patch-clamp electrodes were pulled from Fisher Micro-hematocrit capillary tubes (Fisher Scientific) using a P-97 Flaming-Brown micropipette puller (Sutter Instrument Co.). For GABA current recordings, membrane potential was held at –70 mV unless otherwise indicated. GABA and other drugs were sequentially applied in increasing concentrations using a gravity-driven, modified U-tube “multipuffer” system with 10-90% solution exchange time of < 60 ms (Greenfield and Macdonald, 1996; Neelands et al., 1998). To avoid accumulation of desensitized channels, drugs were applied at least 2 min apart. Peak GABAR 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: $I = (I_{\text{max}})/(1-10^{(-(\log(EC_{50}) - [\text{drug}]) \times \text{Hill slope})})$, where $I$ is the peak current at a given GABA concentration, and $I_{\text{max}}$ is the maximal GABA$_A$R current. Curve fitting was performed using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). Maximal current and C-R curve fits were obtained from individual cells, then averaged and compared by ANOVA with post-hoc t-tests using Bonferroni’s correction for multiple comparisons. Cell capacitance was measured using the “Membrane Test” function of Clampex 9.0 shortly after gaining intracellular access. The percent enhancement or inhibition produced by co-application of a drug or modulator with GABA was determined by dividing the peak amplitude in the presence of drug by the average of control currents elicited by GABA alone before
and after co-application, and multiplying by 100. Cells with significant rundown (>20% reduction over 10 minutes) were excluded from analysis.

Statistical analysis Data are presented as mean ± S.E.M. of n experiments throughout. Groups were compared by Student's t-test, and \( p<0.05 \) was considered significant.
RESULTS

HIF-1α is induced by hypoxia. To determine whether exposure to 1% O2 would induce HIF-1α protein accumulation in NT2-N neurons, we probed immunoblots of NT2-N cell whole cell protein extracts with a monoclonal anti-HIF-1α antibody (NB100-123, Novus Biologicals, Littleton, CO). The density of the HIF-1α protein band dramatically increased immediately after 2, 8 or 24 hours hypoxic treatment (Fig. 1A, B). Marked induction of HIF-1α was observed after 2h hypoxia, and band density increased with longer durations of hypoxia up to 24 h. After 24 h of normoxic recovery, HIF-1α band density was significantly reduced, consistent with restoration of O2-dependent degradation. Subcellular fractionation of nuclear vs. cytoplasmic proteins demonstrated HIF-1α in both whole-cell and nuclear protein extracts, confirming that the induced protein is translocated to the nucleus (Fig. 1C). As with the whole cell fraction, the nuclear fraction returned to baseline after 24 h normoxic recovery (Fig. 1C).

NT2-N cell viability after hypoxia. To assess the effects of hypoxic exposure on cellular integrity and survival, we measured trypan blue staining and lactate dehydrogenase (LDH) release after 2, 8 or 24 h of hypoxia. Staining with 0.05% trypan blue, a marker of cell death, showed no difference in the number of stained neurons after 2 or 8 h hypoxia (Fig. 2A), but increased cell death 48 h after a 24 h hypoxic exposure. We also measured release of LDH into the medium as a marker of cell injury. There were no differences in LDH activity at 0, 24, 48 or 96 h after 2 or 8 h hypoxia compared to control cells, with less than 15% of maximal LDH
released per 24 h period. Exposure to hypoxia for 24 h significantly increased immediate LDH release from 15.3 ± 0.4% to 40.4 ± 14.3% (n=3, p<0.05), with further increases at 24 and 48 h after hypoxia, consistent with delayed/apoptotic cell death (Fig. 2B). In most subsequent experiments, 8h was used as a standard exposure time, as this treatment was sufficient to induce robust HIF-1α expression without increasing cell death.

*Cobalt and deferoxamine induce HIF-1α.* In addition to hypoxia, cobalt chloride and deferoxamine have been shown to stimulate HIF-1α accumulation. To determine whether these agents increase HIF-1α in NT2-N cells, we exposed cells to selected concentrations of each for 8 h in an effort to reproduce the time course of HIF-1α induction by hypoxia. Significant accumulation of HIF-1α was observed in NT2-N cell whole cell lysates immediately after exposure to cobalt chloride (100 μM, 8 hr), which increased with increasing cobalt concentrations (Fig. 3A). As observed with hypoxia, little or no residual HIF-1α protein was detected 24 h after exposure, suggesting that cobalt exposure mimics the time course of HIF-1α induction by hypoxia. Stimulation of HIF-1α protein accumulation was also produced by exposure to deferoxamine (100 μM, 8 h, Fig. 3B).

*Cobalt and deferoxamine effects on NT2-N cell morphology.* NT2-N cells 8 weeks after the onset of RA treatment had neuronal morphology with branching, tapered dendrites and uniform diameter axons (Fig. 4A, C), as previously observed (Gao et al., 2004; Neelands et al., 1998; Pleasure et al., 1992). Exposure to CoCl₂ (100 μM for 8 h, Fig. 4B) resulted in no immediate or delayed changes in neuronal
morphology or cell density, as shown in sample photomicrographs of live neurons taken 48 h after exposure. Similarly, deferoxamine (100 μM for 8 h, Fig. 4D) had no appreciable effect on neuronal morphology or survival. Additionally, since membrane capacitance (Cm) is closely correlated with cell membrane surface area, and injured cells often retract processes during involution, Cm can be used to assess for altered morphology or injury. There was no significant difference in Cm between control cells and cobalt or deferoxamine-treated groups immediately or 48 h after treatment (data not shown). Treatment with either cobalt (100 μM) or deferoxamine (100 μM) for 8-hours was thus well tolerated by NT2-N neurons.

Cobalt exposure increased maximal GABAR currents. GABA-activated currents were obtained by whole-cell patch-clamp recordings from 8-week NT2-N cells voltage clamped at –70 mV. There were no significant differences between control and cobalt-treated groups in resting membrane potential, access resistance, or membrane capacitance (data not shown). GABA concentration-response (C/R) curves were obtained by brief applications of increasing GABA concentration (1 μM - 1 mM, Fig. 5). Our previous studies demonstrated an initial increase in maximal GABA-evoked currents within 1 h after hypoxic exposure (1% O2, 8 h), followed by a reduction to 60% of control 48 h after hypoxia (Gao et al., 2004). Since cobalt induced HIF-1α to a similar extent and with the same time course as hypoxia, we hypothesized that cobalt exposure would have a similar effect on maximal GABAR currents. However, unlike hypoxia, exposure to cobalt chloride (100 μM, 8 hr) did not affect maximal GABAR current immediately after exposure, suggesting that the hypoxia-induced early increase in GABAR currents is not related to HIF-1α
induction. More surprisingly, instead of a reduction in maximal GABAR currents 48 h after exposure, cobalt treatment caused a significant increase in GABA maximal currents after 48 h recovery, which returned to baseline 96 h after cobalt treatment (Fig. 5, insert). Cobalt did not significantly alter the GABA EC$_{50}$ or Hill slope at any measured time point up to 96 h after exposure (data not shown).

*Cobalt allosterically inhibited GABAR currents.* Although cobalt-containing solutions were removed and the cells thoroughly rinsed prior to patch-clamp recording, it was possible that cobalt, like other divalent cations including Zn$^{2+}$ and Cd$^{2+}$ (Fisher and Macdonald, 1998), could act as a noncompetitive antagonist of GABAR function, obscuring an early increase in GABAR currents. To assess whether it has a direct allosteric effect on GABAR currents, cobalt was coapplied at increasing concentrations with 30 μM GABA. Cobalt caused a concentration-dependent inhibition of peak GABAR current (Figure 6) with an IC$_{50}$ of $5.3 \pm 1.3$ mM (n=6). Given the relatively low affinity of this inhibition compared to the cobalt concentrations used to stimulate HIF-1α (100 – 1000 μM), it was very unlikely that residual cobalt obscured an early increase in GABAR currents induced by HIF-1α or had other significant acute effects on GABAR function.

*Deferoxamine exposure increased GABAR currents.* The marked discrepancy between the delayed effects of hypoxia and cobalt on GABAR currents 48 h after treatment could have resulted from additional effects of cobalt not produced by hypoxia, for example, ineffective substitution for calcium-dependent processes. Alternatively, hypoxia might trigger other regulatory processes, in addition to stimulating HIF-1α, which could negate HIF-1α–induced GABAR
upregulation. To distinguish between these possibilities, we used deferoxamine as an alternative means to stimulate HIF-1α that should not affect Ca^{2+}-dependent processes. Deferoxamine (100 μM, 8 h) stimulated HIF-1α accumulation in NT2-N neurons, and we thus hypothesized that deferoxamine exposure should mimic the effects of cobalt on GABAR function if those effects were predominantly due to HIF-1α stimulation. GABA C/R curves were obtained by sequential brief applications of increasing GABA concentration (1 μM - 1 mM) to control and deferoxamine-treated NT2-N cells. As observed with cobalt-treated cells, there was a significant increase in GABA maximal current 48 h after exposure to deferoxamine (100 μM, 8 h) with no change in the GABA EC_{50} (Fig. 7; Control I_{max}=852±206 pA, n=7; DFX 48h-re I_{max}=1901±308 pA, n=11, p<0.05). These data together suggest that induction of HIF-1α by cobalt or deferoxamine results in positive modulation of GABAR function 48 h after exposure, and that induction of HIF-1α in the absence of hypoxia has a markedly different effect on GABAR function than when induced by hypoxia.

Effect of cobalt and deferoxamine on GABAR subunit expression. The reduction in GABAR maximal currents 48 h after hypoxia was associated with reductions in GABAR subunit mRNA expression (relative to actin) for the α1, α5, β2 and γ2 subunits (Gao et al., 2004). If these changes in GABAR subunit expression resulted from HIF-1α stimulation, then induction of HIF-1α by cobalt or deferoxamine might also lower expression of the same subunits despite the overall increase in GABAR current 48 h after exposure, presumably mediated by other
mechanisms. Conversely, the increase in GABAR current stimulated by Co$^{2+}$ and
deferoxamine might result from HIF-induced increases in specific subunits. To
distinguish between these possibilities, we used reverse transcriptase polymerase
chain reaction (RT-PCR) to measure GABAR subunit expression after Co$^{2+}$ or
deferoxamine exposure. Total RNA was isolated from NT2-N cells under control
conditions or at the same time points after 8 h cobalt (or DFX) treatment used after
hypoxia in prior studies (Gao et al., 2004). After first strand cDNA synthesis, RT-
PCR was performed using primers specific for human $\alpha$1-6, $\beta$1-3, $\gamma$1-3, $\delta$, $\varepsilon$, $\pi$ and $\theta$
subunit subtypes and normalized to the actin RT-PCR product band, as previously
described (Gao et al., 2004). As previously observed, 8 week NT2-N cells
expressed a consistent profile of subunits including $\alpha$1, $\alpha$2, $\alpha$3, $\alpha$5, $\beta$2, $\beta$3, $\gamma$2, $\gamma$3, $\varepsilon$
and $\pi$ (Fig. 8). At the 24 and 48 h time points after CoCl$_2$ exposure (100 $\mu$M, 8h) we
observed specific changes in GABAR subunit gene expression. Relative expression
of $\alpha$2, $\alpha$3, $\alpha$5, $\beta$2, $\beta$3, $\gamma$2, $\varepsilon$ and $\pi$ subunit mRNAs were significantly increased 24 h
after Co$^{2+}$ exposure (p<0.05, control n=8, Co 24 h n=5) and returned to baseline at
48 h recovery. These data suggested that the increase in maximal GABAR currents
48 h after exposure was likely due to an increase in the expression of these specific
GABAR subunits, and temporally linked to induction of HIF-1$\alpha$.

DFX exposure (100 mM, 8 h) followed by 24 h recovery caused significant
increases in $\alpha$3 and $\beta$3 subunit mRNAs (p<0.05, n=6 in each group). There were
non-significant trends toward increases in $\alpha$4, $\alpha$5 and $\varepsilon$ mRNA. Although the
specific subunits significantly increased by DFX were different from those induced
by Co$^{2+}$, perhaps reflecting differences in the amount or duration of HIF1\(\alpha\) activation or additional non-HIF-mediated effects of either Co$^{2+}$ or DFX, the overall trend toward increased subunit expression was similar between DFX and Co$^{2+}$, and consistent with the increased maximal current recorded with both agents 48 h after exposure. Additionally, the effect on GABAR subunit mRNAs occurred 24 h after exposure, while maximal current was increased at 48 h, suggesting a delay in surface expression of functional GABARs. The increased maximal current and subunit mRNA expression induced by these treatments is markedly different from the reductions in GABAR current and subunit mRNA expression after hypoxia, suggesting that induction of HIF-1\(\alpha\) is not sufficient to reproduce the effects of hypoxia on GABAR expression and function.
DISCUSSION

Our previous investigation demonstrated specific changes in GABAR expression and function in NT2-N neurons with a biphasic time course after 8 h exposure to 1% O$_2$. After an initial increase in maximal GABAR currents in the first hour of normoxia, a reduction in GABAR currents to 60% of control 48 h after hypoxia was associated with lower mRNA levels of the $\alpha_1$, $\alpha_5$, $\beta_2$ and $\gamma_2$ subunits. These findings correlated well with GABAR subunit changes observed in a rat model of focal ischemic stroke, in which immunohistochemical studies demonstrated reductions in the $\alpha_1$, $\alpha_2$, $\alpha_5$ and $\gamma_2$ subunits at 1, 7 and 30 days after infarct, peaking at the 7d time point (Redecker et al., 2002). Due to the prominent role of HIF-1$\alpha$ in regulating cellular responses to hypoxic stress, and the presence of a hypoxia response element (HRE) sequence 5’ to the $\alpha_1$ subunit promoter (Kang et al., 1994), we hypothesized that HIF-1$\alpha$ might mediate some of the delayed effects of hypoxia on GABAR expression. To test this hypothesis, we first demonstrated that the hypoxic exposure triggered induction of HIF-1$\alpha$ protein, and that HIF-1$\alpha$ protein was translocated to the nucleus. However, induction by the same hypoxic stimulus that induces GABAR changes does not establish whether HIF-1$\alpha$ is actually involved in hypoxic regulation of GABAR function. To address this question, we studied the effects of two other known HIF-1$\alpha$ inducers, cobalt chloride and deferoxamine, on GABAR subunit expression and function. Both of these agents induced HIF-1$\alpha$ protein by Western blot immediately after exposure, with reduction or loss of expression 24 h afterward, mimicking the time course of HIF-1$\alpha$ induction.
by hypoxia. Unlike hypoxia, however, neither cobalt nor deferoximine had an immediate effect on maximal GABAR currents. Moreover, the effect of both of these agents to increase both maximal currents and mRNA levels of specific GABAR subunits 48 h after exposure was opposite that of hypoxia. There are several possible explanations for these differences.

One possibility is that cobalt, deferoxamine or both have additional effects on cellular function beyond stimulation of HIF-1α accumulation, which are responsible for the late increases in maximal current and subunit expression. However, Co²⁺ and deferoxamine induce HIF-1α by distinct mechanisms, and any non-specific effects should differ between them. Cobalt may either substitute for Fe(II) in the proline hydroxylase enzyme with less efficacy (Maxwell and Salnikow, 2004) or directly bind to HIF-1α and inhibit the interaction between HIF-1α and VHL to prevent O₂-dependent degradation (Yuan et al., 2003). Co²⁺ depresses cellular respiratory activity and interferes with heme-dependent metabolism involving the cytochrome P450 system (Maines and Kappas, 1977). It can displace Zn²⁺ from zinc finger domains in transcription factors, and can cause DNA damage or carcinogenesis (Sarkar, 1995). Co²⁺ induced a hypoxic transcriptional response and mitochondrial DNA damage related to reactive oxygen species (ROS) induction in PC12 cells (Wang et al., 2000). Co²⁺ can also take the place of Mg²⁺, Ca²⁺ or Zn²⁺ in enzymatic reactions, altering their function in unpredictable ways (Jennette, 1981). We found that that Co²⁺, like other divalent cations, allosterically inhibited GABAR currents at relatively high concentration (EC₅₀ = 5.3 mM), but it is unlikely that Co²⁺ obscured an early increase in GABAR function, as Co²⁺-containing medium was thoroughly
removed by 4 rinses of control medium prior to recording. The Co$_{2}^{+}$ effect on GABAR currents was thus likely due primarily to the induction of HIF and downstream effects.

DFX prevents HIF-1$\alpha$ proline hydroxylation by chelating iron (Wang and Semenza, 1993), which is a required cofactor for this enzyme. DFX has multiple effects on cellular function, most related to iron chelation. It is used clinically for this purpose and is relatively non-toxic (Kontoghiorghes et al., 2000). It prevents the Fe$_{2}^{2+}$-mediated conversion of H$_2$O$_2$ to OH$^{+}$ + OH$^{-}$ (the Fenton reaction), and hence might reduce cellular injury due to reactive oxygen species (ROS). It strongly induces expression of the transferrin receptor (TfR) through HIF acting at the TfR promoter, decreases N-myc expression, induced iNOS via the transcription factor NF-IL6, and induces cyclooxygenase 2 expression, possibly via ERK (Templeton and Liu, 2003). It induces the expression of HIF-related genes in cortical cultures including LDH-A, enolase-1, p21$^{waf1/cip1}$ and erythropoietin, all of which have HIF-1 and ATF-1/CREB binding sites in their promoters (Zaman et al., 1999). Hence, the effects of deferoxamine on neuronal expression appear to be the same as those of HIF or hypoxia. The finding that both DFX and Co$_{2}^{+}$ caused similar upregulation of maximal GABAR currents and subunit expression strongly suggests that their ability to upregulate HIF-1$\alpha$ is involved in this effect.

A more likely explanation for the differences between hypoxia and HIF-1$\alpha$ induction on GABAR function is that hypoxia induces additional cellular effects. Hypoxia triggers a host of stress-activated regulatory cascades, some mediated by
the oxygen-sensing transcription factor, HIF-1α (Maxwell and Salnikow, 2004), but other signaling pathways include cAMP-dependent protein kinase A (PKA), the p42 and p44 mitogen-activated protein kinase (MAPK/ERK) (Conrad et al., 1999), the p38 or stress-activated protein kinase (SAPK) pathway, the phosphoinositol 3-kinase (PI3K)-Akt pathway (Beitner-Johnson et al., 2001), and calcium-mediated pathways due to the opening of voltage-gated Ca\(^{2+}\) channels (VGCCs) by hypoxia-induced depolarization. Co\(^{2+}\) and DFX stimulate HIF and possibly other downstream pathways, but should not activate Ca\(^{2+}\) signaling (Jennette, 1981). Hence, the downregulation of GABA\(_A\)R maximal current and specific subunit levels after hypoxia may be due to Ca\(^{2+}\)-dependent or alternative processes, while stimulation of HIF in isolation increases GABA\(_A\)R currents and subunit levels.

An additional issue is the possibility of hypoxic or reoxygenation injury. Reactive oxygen species (ROS) generated during reperfusion can lead to membrane peroxidation (Feng et al., 1998) causing loss of cell or organelle integrity and other effects. Generation of superoxide radicals inhibited GABAR activity in cerebral cortical synaptoneurosomes in a Ca\(^{2+}\)-dependent manner (Sah et al., 2002). When area CA1 pyramidal neurons were exposed to H\(_2\)O\(_2\), elevation of intracellular Cl\(^{-}\) and reduced GABA responses have been observed (Sah et al., 2002; Sah and Schwartz-Bloom, 1999). However, other studies in rat brain mitochondria (Cino and Del Maestro, 1989) and NT2-N cells (Almaas et al., 2003) have questioned whether ROS-induced changes are related to reoxygenation. We were not able to maintain hypoxia during recording, hence all recovery experiments were performed at atmospheric [O\(_2\)]. Reoxygenation injury was unlikely, as we did not detect increased
LDH release or trypan blue staining of injured neurons either acutely after reoxygenation or at subsequent recovery time points (Gao et al., 2004). However, ROS-mediated effects on GABAR processing or function might explain differences between the effects of hypoxia (with reoxygenation) and chemically induced HIF-1α.

The effects of hypoxia or HIF-1α on GABAR regulation may occur not only at the level of subunit gene expression, but also in translation, membrane insertion, the recycling pathway or receptor phosphorylation status. Prior studies of the effects of hypoxia on GABAR function in other systems may reflect such changes. A reduction in binding of the GABAR antagonist, [3H]SR-95531, in gerbil hippocampus 30 or 60 min after 5 min of ischemia, with no change in [3H]flunitrazepam binding, was thought to result from an increase in receptor internalization due to a transient increase in extracellular GABA concentration (Alicke and Schwartz-Bloom, 1995). Hypoxia-induced loss of GABA<sub>A</sub>R binding can be long-lasting (Mileson et al., 1992) likely reflecting loss of principal neurons. However, changes in GABA<sub>A</sub>R subunit mRNA expression have been observed. mRNA levels of the α1 and β2 subunits decreased within 30 minutes after reperfusion in hippocampal areas CA1, CA3 and dentate gyrus (Li et al., 1993). In a photothrombotic stroke model, increases in α1 and γ2 and decreased β1 mRNA were seen in the hemispheres with infarcts, but since the entire hemisphere was pooled, the change may represent a reaction of non-hypoxic areas to hyperexcitable peri-infarct regions (Liu et al., 2002). Electrophysiological studies have also demonstrated down-regulation of hippocampal GABA<sub>A</sub>R function, which was
attributed to changes in the transmembrane Cl− gradient (Sah and Schwartz-Bloom, 1999). The variability of these results suggests that GABAR changes after hypoxic exposure may be model- or system-dependent.

It is tempting to speculate that HIF-1α induction of GABAR expression could be neuroprotective by countering excitotoxicity (due to depolarization-induced release of glutamate) in the post-hypoxic state. Newborn rat brains exposed to transient or moderate hypoxia were able to adapt to hypoxic conditions through activation of HIF-1α (Bergeron et al., 2000). HIF-regulated genes encode proteins involved in energy metabolism, erythropoiesis, angiogenesis, and vasomotor regulation, which tend to promote neuronal survival. Our finding that chemical induction of HIF-1α upregulates GABAR function while hypoxic exposure (with similar HIF-1α activation) reduces GABAR expression suggests that hypoxia triggers additional mechanisms that occlude or override the HIF-1α effect. Understanding the molecular mechanisms involved in hypoxia-induced occlusion of HIF-1α signaling could provide new strategies for intervention in the setting of acute stroke or other hypoxia-related CNS disorders to prevent neuronal damage.
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FOOTNOTES

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Figure 1. HIF-1α immunoblots after hypoxia. A. Immunoblots of whole-cell extracts from NT2-N cells in control atmosphere and immediately (0 h, R0) or 24h (R24) after 2, 8 or 24h (H2, H8, H24) of hypoxia, probed with a primary antibody to HIF-1α, showing protein bands at 120 kDa. Faint bands were observed after 24h recovery. B. Density ratio of HIF/actin for hypoxia and recovery shows increased HIF-1α with longer hypoxia times. C. Whole cell and nuclear protein extracts show accumulation of HIF-1α in the nucleus and loss of protein after 24 h recovery.
Figure 2. Cell viability after hypoxia. A. Trypan blue-stained cells (%) after 2, 8 or 24 h hypoxia at 0 h or 48 h recovery. Increased cell death was seen only 48 h after 24 h hypoxia. B. LDH release (% of total) after 2, 8 or 24 h hypoxia at 0 h, 24 h or 48 h recovery. LDH release was unchanged after 2 or 8 h hypoxia but increased immediately after 24 hypoxia, with continued increases after 24 or 48 h normoxic recovery.
Figure 3. HIF-1α immunoblots after cobalt or deferoxamine. A. Immunoblots of whole-cell extracts from NT2-N cells after 8 h exposure to CoCl₂ (0, 100, 300 or 1000 μM) at 0 o 24 h of recovery, probed with a primary antibody to HIF-1α, showing bands at 120 kDa at 0 h that are lost at 24 h recovery. B. Immunoblot of NT2-N cell proteins after 8 h exposure to DFX (100 μM) showing induction of HIF-1α protein at 0 h recovery.
Figure 4. Photomicrographs of NT2-N cells after Co2+ or DFX. Hoffman modulation contrast images of NT2-N neurons under control condition (A and C) and 48 h after 8 h exposure to CoCl₂ (100 μM, C) or deferoxamine (100 μM, D). No differences in cells size or number were observed. Scale bars: A, 20 μm; B: 100 μm.
Figure 5. Effect of CoCl$_2$ on GABA-evoked currents. A. Whole-cell GABAR currents evoked by increasing concentrations of GABA (1 μM – 1 mM), immediately (0 h, filled circles), 48 h (filled triangles) or 96 h (filled squares) after exposure to cobalt (100 μM, 8 h), compared to control (open circles), each fitted to a sigmoidal logistic function. There was no significant difference in EC$_{50}$ or Hill slope. Inset: Maximal GABA current was increased 48 h after exposure and returned to baseline after 96 h (Con $I_{\text{max}}$=972±154 pA, n=8; 0h $I_{\text{max}}$=1016±186 pA, n=9; 48h $I_{\text{max}}$=1490±153 pA, n=5, *p<0.05; 96h $I_{\text{max}}$=964±222 pA, n=4).
Figure 6. CoCl$_2$ inhibition of GABAR currents. Whole cell currents elicited by 30 μM GABA were allosterically inhibited by CoCl$_2$ (IC$_{50}$=5.3±1.3 mM, n=6). No inhibition was observed at concentrations used to stimulate HIF-1α accumulation.
Figure 7. Effect of DFX on GABAR currents. Whole-cell GABAR currents evoked by increasing concentrations of GABA (1 μM – 1 mM) 48 h (filled squares) after DFX (100 μM, 8 h) exposure, compared to control (open squares), fitted to sigmoidal logistic functions. There was no significant difference in EC₅₀ or Hill slope. Inset: Maximal GABA current was increased 48 h after exposure (Con Iₘₐₓ=852±205 pA, n=7; DFX 48h Iₘₐₓ=1901±308 pA, n=11, *p<0.05).
Figure 8. GABAR subunit RT-PCR after CoCl₂ exposure. Reverse transcriptase PCR (RT-PCR) of individual GABAR subunit mRNAs using gene-specific primers for human α1-6, β1-3, γ1-3, δ, ε, π and θ subunit subtypes was performed on RNA samples isolated 24 h (n=5) or 48 h (n=4) after CoCl₂ (100 μM, 8 h) exposure, compared to control (n=8). Bars show ratio of GABAR subunit PCR product density to actin product from the same RNA sample. Significant increases in α2, α3, α5, β2, β3, γ2, ε and π subunit mRNAs were observed 24 h after Co²⁺ exposure, which returned to baseline at 48 h recovery.
Figure 9. GABAR subunit RT-PCR after DFX exposure. RT-PCR of individual GABAR subunit mRNAs performed on RNA samples isolated 24 h (n=6) after DFX (100 μM, 8 h) exposure, compared to control (n=6). Bars show ratio of GABAR subunit PCR product density to actin product from the same RNA sample. Significant increases in α3 and β3 subunit mRNAs were observed (p<0.01).
DISCUSSION/SUMMARY

Ionotropic GABA(A) receptors mediate most fast inhibitory synaptic transmission in the mammalian brain, and play a crucial role in controlling neuronal excitation and communication. Modifications of GABAR function are implicated in some forms of epilepsy, and are strongly associated with anxiety, depression and chronic substance abuse. This dissertation examines the regulatory pathways that modulate GABAR expression and function. Such regulation could occur at multiple levels involved in the synthesis, processing and degradation of receptor/channel proteins including transcription, translation, post-translational processing, covalent modification (e.g., by phosphorylation), receptor trafficking, recycling and degradation. Changes in any of these processes could influence the number, composition or function of the receptors expressed at the cell surface. In the first manuscript, we investigated changes in GABAR function in response to activation of PKC, which likely alters the phosphorylation status of GABAR or related proteins, and found that stimulation of PKC altered GABAR sensitivity to diazepam without changing the maximal GABA-induced current or EC$_{50}$ for activation by GABA. The second manuscript demonstrates that hypoxia regulates GABAR function and expression with a biphasic time course. The third paper shows that hypoxia stimulates accumulation of the transcription factor, HIF-1α, in NT2-N cells, but that this regulation is complex, as chemical stimulation of HIF-1α causes different changes in GABAR expression and function than hypoxia.
Protein kinase C appears to regulate GABAR activity and membrane trafficking, but the effects have varied in different systems. Most results suggest that PKC activation decreases GABAR function (Filippova et al., 2000; Krishek et al., 1994; Leidenheimer et al., 1992) though in some systems PKC activation increases GABAR currents (Lin et al., 1996, 1994; Poisbeau et al., 1999). However, relatively few studies have focused on the effects of PKC on BZ- and barbiturate-mediated potentiation of GABAR currents, and the results have been inconsistent or even contradictory (Ghansah and Weiss, 2001; Leidenheimer et al., 1993). The variable effects of PKC on GABAR function might result from differences in neuron populations or expression systems, the GABAR subunits expressed, the method and timing of PKC activation, the basal phosphorylation state of the receptor, or the recording conditions. To address this issue in a more homogeneous population of neuronal cells, we used neuron-like NT2-N cells, derived from the human NTera2 teratocarcinoma cell line in response to RA (Andrews, 1984). The present experiments were designed to assess whether PKC activation alters responses to GABA and allosteric modulators in an in vitro neuronal cell system with well-defined GABAR subunit expression and pharmacology. In this system, brief application of the PKC activator, PDBu, did not alter the maximal currents or EC$_{50}$ of the GABA concentration-response (C/R), or the reversal potential of GABAR currents. Failure to detect an effect on GABA maximal currents or EC$_{50}$ could be due to a high basal phosphorylation state (from which additional stimulation of PKC
would have no effect), insufficient concentration of PKC modulators, inadequate
duration of stimulation or other conditions of incubation. Indeed, PKC mRNA,
protein and activity are dramatically upregulated during differentiation from NT2
precursor cells to neuronal NT2-N cells (Abraham et al., 1991). We also performed a
series of recordings with PDBu preincubation at 37 °C, as well as experiments in
which PDBu was included in the recording pipette, to determine whether a potential
PKC effect was missed due to incubation and recording at room temperature. Again,
no change in maximal current or EC$_{50}$ for GABA was observed.

Exposure to PDBu shifted the C/R for diazepam to the right, an effect
inhibited by bisindolylmaleimide (BIM) and not produced by the inactive phorbol
ester, 4α-phorbol didecanoate (4α-PDD), clearly consistent with PKC mediation.
There was no effect of PKC modulators on pentobarbital potentiation of GABAR
currents, hence the effect appears to be BZ-specific. Furthermore, in experiments
with chronic BZ treatment, we observed a significant reduction in maximal efficacy
of diazepam potentiation after 7 d diazepam exposure, with no shift in EC$_{50}$ for
enhancement. Independent of the reduction in efficacy, PDBu treatment caused a
small but significant rightward shift of the diazepam C/R curve for GABA current
efficiency that was very similar to that seen in control neurons, suggesting that
PKC activation had the same effect on the potency of diazepam potentiation despite
the downregulation of efficacy induced by chronic BZ exposure. Hence, changes in
BZ enhancement of GABA currents may involve distinct and separable mechanisms,
one involving PKC phosphorylation with a reduction in BZ potency, the other
involving loss of efficacy, possibly related to altered subunit composition with a reduction in BZ-sensitive GABARs.

Protein kinase C is a superfamily composed of some 12 serine-threonine kinases which are divided into four classes. Phorbol esters like PDBu bind to PKCs at the DAG site in the regulatory domain and stimulates prolonged activation of two classes: cPKCs (α, β, and γ isotypes) and nPKCs (ε, η, δ, and θ isotypes) (Nishizuka, 1992). Mutant mice lacking the PKCε isoform were behaviorally more sensitive to diazepam (Hodge et al., 1999), while mice lacking the PKCγ isoform had no change in BZ sensitivity (Harris et al., 1995). Therefore, the effect of PKC on GABAR is isotype-dependent. It would be useful to determine which specific PKC isotypes are dominantly expressed in NT2-N cells and how each isotype affects GABAR function. Since PKC activation reduced BZ potency, this is consistent with a dominant effect of PKCε in NT2-N cells.

In summary, we found that PDBu reduced diazepam potency for enhancing GABAR currents without changing efficacy, and had no effect on the potency or efficacy of GABA for activating GABAR currents. The specific targets of PKC activation are unknown, and could include both GABAR subunits and other proteins. Chronic diazepam treatment reduced the efficacy of diazepam to enhance GABAR currents without affecting potency, and PKC activation reduced diazepam potency in chronic diazepam-treated cells just as it did in control neurons. The reduction in BZ potency may represent one form of “uncoupling” of the GABA and BZ sites, though
this should be confirmed with binding studies. The reduction in diazepam efficacy after 7 d diazepam exposure may be related to a change in GABAR subunit composition, which also will need to be explored in future experiments.

**Regulation of GABAR by Hypoxia**

Hypoxia has been shown to affect GABAergic neurotransmission and GABAR function (Schwartz-Bloom and Sah, 2001). The experiments presented in the second and third manuscripts were designed to assess whether and how hypoxia triggers modulation of GABAR subunit expression and pharmacology in an *in vitro* human neuron system, independent of brain regional context or connectivity. The absence of synapse formation in this system allows investigation of receptor regulation without presynaptic influences. The NT2-N cells provide an excellent model in which to test this hypothesis due to the relative homogeneity of their responses to GABA and GABAR modulators (Neelands et al., 1998), and their expression of a consistent subset of GABAR subunits at each stage of *in vitro* development (Neelands et al., 1999).

Exposure to 1% O₂ for 8 h did not increase immediate or delayed cell death in NT2-N cells. Using whole-cell patch clamp recording, we found that hypoxia affected maximal GABAR currents in a biphasic manner. The GABAR current (normalized to cell capacitance) was significantly increased within 2 h after hypoxic treatment, but was significantly reduced below baseline after 48 h recovery and then
returned to baseline after 96 h recovery. The increase in GABAR current immediately after hypoxia was associated with a decrease in maximal zolpidem potentiation and the EC$_{50}$ for zolpidem. The early change in GABAR function and pharmacology is unlikely to have resulted from transcriptional regulation, due to the short duration of hypoxia (8 h) which was likely insufficient for replacement of existing GABARs with newly transcribed and translated receptors. Moreover, the observed reduction in $\alpha_1$ subunit expression seen at the 0 h time point would be expected to cause an increase the EC$_{50}$ for zolpidem, not a decrease as observed. The transient increase in maximal currents immediately after hypoxia could have resulted from increased insertion of internalized GABARs into the membrane, a decrease in constitutive internalization of GABARs during hypoxia, or a change in the phosphorylation state of GABAR subunits or associated proteins, among other possibilities. Slowing of GABA current rise times and desensitization rates was associated with dephosphorylation of the GABAR $\beta$1 or $\beta$3 subunit in HEK293T cells (Hinkle and Macdonald, 2002), which might be expected in the presence of lowered ATP levels during hypoxia. It is unclear whether a change in phosphorylation could explain the altered zolpidem responsiveness or increased maximal current. However, our previous study of PKC effects on diazepam sensitivity showed a reduction in apparent affinity for diazepam with PKC activation, which is consistent with the reduction in zolpidem sensitivity after hypoxia. Teleologically, a transient increase in GABAR function immediately after hypoxia could serve as a compensatory mechanism to combat depolarization-induced excitotoxicity.
Along with the subsequent reduction in maximal GABAR currents after 48 h recovery, the pharmacology of GABAR currents also was altered after 48 h recovery, with slightly increased maximal diazepam enhancement but decreased zolpidem enhancement. In addition, we used RT-PCR to study the GABAR subunit gene expression. We found there were changes in the expression pattern of GABAR subunits 48 h after hypoxia, with marked reduction of α1 subunit expression, as well as smaller but significant reductions in α5, β2 and γ2 mRNA expression. The changes in BZ pharmacology could have resulted from reduced α1 expression if other BZ-sensitive α subtypes (α2, α3 or α5) were assembled in its place. These data together suggested that the late reduction of GABAR function could be due to the alteration of GABAR subunit gene expression by hypoxia.

It is tempting to speculate whether hypoxic exposure could directly induce gene expression changes affecting GABAR composition and function. We found that hypoxic exposure induced the accumulation of HIF-1α, which is an important transcription factor and has been shown to be a key regulator in cellular and physiological responses to the stress of hypoxia. In the third manuscript, we report that exposure to both cobalt and DFX, two other known HIF-1α inducers, increased HIF-1α protein levels in NT2-N cells. Furthermore, maximal GABA currents were increased 48 h after cobalt or DFX treatment, associated with increases in mRNA expression for specific GABAR subunits in a similar pattern 24 h after exposure and
induction of HIF-1α protein. The finding that chemical induction of HIF-1α protein resulted in specific changes in GABAR expression and function suggests that HIF-1α is involved in regulating GABARs.

However, the changes we observed after chemical induction of HIF-1α were somewhat different from those found in the hypoxia experiments. There are at least three likely explanations for this contradiction.

First, hypoxic regulation of GABARs could occur at different regulatory steps, which result not only in alteration of gene expression but also in changes in translation pathways, recycling pathways or receptor phospholation status. The GABAR binding in gerbil hippocampus was reduced after hypoxia, which was thought to result from receptor internalization (Alicke and Schwartz-Bloom, 1995). It is not known whether changes in the receptor-recycling pathway occurred in our experimental model. The subcellular distribution of GABARs can be studied directly by using specific GABAR subunit immunostaining techniques to examine for post-hypoxic changes in subunit protein in the intracellular and surface membrane compartments. GABAR-associated proteins such as gephyrin and GABARAP are essential in receptor insertion and trafficking. Altered expression or function of such proteins would affect GABAR membrane expression. Experiments to determine the effects of hypoxic on gephyrin and GABARAP should also be performed to determine whether such proteins play a role in post-hypoxic GABAR regulation. In addition, specific evidence has shown that GABAR function can be
affected by phosphorylation status after hypoxia. One recent study showed that hypoxia-induced activation of calcineurin, a calcium-dependent phosphatase, was associated with reduced GABAR-mediated inhibition, suggesting a mechanism by which GABAR may be regulated by altered phosphorylation status in response to hypoxia (Sanchez et al., 2005). Further studies of hypoxia-induced changes in phosphorylation and their effects on GABAR regulation will be needed to determine the potential roles of different protein kinases and phosphatases at distinct time points after hypoxia.

Changes in the ionic driving force for the channel also may be important. Electrophysiological studies (Harata et al., 1997) also have demonstrated a down-regulation of hippocampal GABAR function, which was attributed to changes in the transmembrane Cl\(^-\) gradient (Inglefield and Schwartz-Bloom, 1998) resulting from decreased ATP or increased intracellular Ca\(^{2+}\) (Harata et al., 1997). Hence, many other factors besides HIF-1\(\alpha\) signaling pathways could affect the findings of the hypoxia experiments.

Second, a major issue is the possibility of reperfusion injury. Reactive oxygen species generated during reperfusion contribute to the pathogenesis of hypoxia and cause a number of cellular and molecular changes. Generation of superoxide radicals inhibited GABAR response in cerebral cortical synaptoneuroses in a Ca\(^{2+}\)-dependent manner (Schwartz-Bloom et al., 1998). When area CA1 pyramidal neurons were exposed to H\(_2\)O\(_2\), elevation of intracellular
Cl\(^-\) and reduced GABA responses were observed in several studies (Sah et al., 2002; Sah and Schwartz-Bloom, 1999). Another important transcription factor related to hypoxic regulation of gene expression, NF-κB, can be directly activated by ROS. It is not known whether NF-κB can regulate GABAR expression. Specific inducers for NF-κB could be used to test its effects on GABAR expression and function. In the present study, we used chemical induction of HIF-1α protein, which excluded possible reperfusion effects that could contribute to the changes found in the hypoxia experiments.

Lastly, hypoxia not only induces HIF-1α protein, but also activates several other specific signaling pathways that have been suggested to detect hypoxia, leading to the activation of additional specific transcription factors and expression of appropriate target genes. For example, hypoxia-induced depolarization also may open voltage-gated calcium channels (VGCCs) which activate calcium-dependent signaling. Cobalt and DFX stimulate HIF-1α protein accumulation, but do not activate calcium signaling. Hence, the downregulation of GABAR maximal current and specific subunit levels after hypoxia may be due to calcium-dependent processes, while activation of HIF by cobalt and DFX causes increased GABAR currents and subunits level. To support this concept, we have used nifedipine, a L-type calcium channel blocker, to block VGCCs during hypoxic treatment. In a preliminary study, we found nifedipine (1 μM), present during 8h hypoxia, blocked hypoxia-induced changes in GABAR subunit expression (n=6, data not shown). It will be necessary to determine whether nifedipine also prevents the effects of
hypoxia on GABAR maximal current by using whole cell recording technique. If nifedipine prevents the decrease in GABAR currents 48 h after hypoxia, this would suggest that calcium signaling activated by hypoxia plays a key role in post-hypoxic downregulation of GABAR expression and function. Other transcription factors, such as NF-κB, AP-1, SP-1 and p53, could be activated by hypoxia and they have been reported to be actively involved in hypoxia response pathways. Furthermore, there might have been cross talk between these signaling pathways, creating further complexity in the hypoxic regulation of GABARs.

Finally, there is an interesting question brought out by our findings – whether HIF-1 could be a neuroprotection factor. Evidence has linked GABAR dysfunction with many CNS disorders induced by hypoxia or ischemia. Drugs acting on the GABARs have been clinically useful in the treatment of epilepsy and some other neuronal disorders because these drugs can enhance GABAR function. Our results showed that induced HIF-1 increased GABAR expression and function, which could overcome the imbalanced excitotoxicity caused by further hypoxic exposure. Bergeron and colleagues (Bergeron et al., 2000) showed that newborn rat brain exposed to transient or moderate hypoxia were able to adapt to hypoxic conditions through activation of HIF-1α. HIF-regulated genes encode proteins involved in energy metabolism, cell survival, erythropoiesis, angiogenesis, and vasomotor regulation. We suggest that, in many instances, the induction of HIF-1 target genes such as GABAR subunits in the brain may be beneficial. Our studies thus provide new insights into the molecular mechanisms by which HIF-1 regulates GABAR gene
expression and function. A better understanding of the pathways involved in HIF-1 regulation of GABAR function may lead to consideration of HIF-1α as a therapeutic target for diverse CNS disorders associated with hypoxia.


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ABSTRACT

GABA_A receptors (GABARs) mediate fast inhibitory neurotransmission in the human brain. Since GABARs play a key role in controlling neuronal and network activity, modulating their function will have important consequences for neural excitation and communication. In this dissertation, using human NT2-N cells as a neuronal model system, we evaluated the effects of protein kinase C (PKC) and hypoxia on the regulation of GABAR function. In the first manuscript, we found that PKC, activated by phorbol dibutyrate (PDBu), reduced the apparent affinity of the benzodiazepine, diazepam, to enhance GABAR currents without affecting maximal enhancement; this effect could be blocked by the PKC inhibitor, bisindolylmaleimide. This PKC effect was specific to the benzodiazepine site, as PDBu did not alter potentiation of GABAR currents by the barbiturate, pentobarbital. Reducing the apparent affinity of diazepam at NT2-N GABARs by activation of PKC could be one important mechanism by which GABAR function can be modified by phosphorylation. In the second manuscript, we demonstrated that hypoxic exposure altered GABAR function with a biphasic time-course, accompanied by changes in specific GABAR subunit mRNA levels. We also found that hypoxic exposure induced the accumulation of the oxygen-dependent transcription factor, hypoxia-inducible factor 1α (HIF-1α), a key regulator of cellular and physiological responses to hypoxic stress. To assess whether hypoxic exposure could directly regulate GABAR gene expression, we studied the effect of two chemical HIF-1α inducers, cobalt chloride and deferoxamine. Both of these agents...
transiently increased HIF-1α levels. In contrast to increased GABAR current immediately after hypoxia and decreased current 48 h afterward, exposure to cobalt or deferoxamine resulted in increased maximal GABA currents 48 hours after exposure, with increases in specific GABAR subunit mRNA levels after 24 hours. These data demonstrate that chemical induction of HIF-1α alters GABARs differently than HIF-1α induction by hypoxia, suggesting that hypoxia triggers additional mechanisms that oppose HIF-1α effects on GABAR function. Understanding the mechanisms of GABAR regulation by protein kinas and hypoxia will ultimately improve the treatment of epilepsy and other CNS disorders associated with GABAR dysfunction.