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Expression levels of the $\alpha 4$ subunit of the GABA_A receptor in differentiated neuroblastoma cells are correlated with GABA-gated current

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Abstract

The $\alpha 4$ subunit of the GABA_A receptor (GABAR) is capable of rapid plasticity, increased by chronic exposure to positive GABA modulators, such as the neurosteroid 3 α -OH-5 α [β]-pregnan-20-one (THP). Here, we show that 48 h exposure of differentiated neuroblastoma cells (IMR-32) to 100 nM THP increases $\alpha 4$ expression, without changing the current density or the concentration-response curve. Increased expression of $\alpha 4$ -containing GABAR was verified by a relative insensitivity of GABA (EC₂₀)-gated current to modulation by the benzodiazepine (BZ) lorazepam (0.01 – 100 μ M), and potentiation of current by flumazenil and RO15-4513, characteristic of $\alpha 4\beta 2$ pharmacology. In contrast to THP, compounds which *decrease* GABA-gated current, such as the BZ inverse agonist DMCM, the GABAR antagonist gabazine and the open channel blocker penicillin, *decreased* $\alpha 4$ expression after a 48 h exposure, without changing BZ responsiveness. However, pentobarbital, another positive GABA modulator, *increased* $\alpha 4$ expression, while the BZ antagonist flumazenil had no effect. In order to test whether changes in current were responsible for increased $\alpha 4$ expression, decreases in the Cl⁻ driving force were produced by chronic exposure to the NKCC1 blocker bumetanide (10 μ M). When applied under these conditions of reduced GABA-gated current, THP failed to increase $\alpha 4$ expression. The results of this study suggest that $\alpha 4$ expression is correlated with changes in GABA-gated current, rather than simply through ligand-receptor interactions. These findings have relevance for GABAR subunit plasticity produced by fluctuations in endogenous steroids across the menstrual cycle, when altered BZ sensitivity is reported.

Keywords

GABA; alpha-4; chloride current; neurosteroid; pregnanolone; benzodiazepine inverse agonist; depolarization; bumetanide

Introduction

The GABA_A receptor (GABAR) is a pentameric structure mediating a Cl⁻ conductance which is the major source of fast inhibition in the adult CNS. Of the most commonly expressed isoforms, the stoichiometry of this receptor is 2 α , 2 β and 1 γ or δ subunit (Chang et al.,

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1996), although other stoichiometries may exist with isoforms containing ϵ and θ subunits. Each subunit, in turn, can be comprised of varying sub-types (6α , 3β , 3γ , 1δ , 1ϵ , 1θ), which influences the receptor's biophysical and pharmacological properties (Lavoie et al., 1997; Smith and Gong 2005; Wafford et al., 2004). Variations in subunit isoform are observed in a regional manner across areas of the CNS (Wisden et al., 1992), but are also regulated in a dynamic manner by exposure to GABA-modulatory drugs (Holt et al., 1996; Mahmoudi et al., 1997; Smith et al., 1998). In particular, the GABAR $\alpha 4$ subunit is highly regulatable (Holt et al., 1996; Holt et al., 1997; Arnot et al., 2001; Mahmoudi et al., 1997; Devaud et al., 1997; Smith et al., 1998; Liang et al., 2007), although normally expressed at relatively low levels in most areas of the CNS (Wisden et al., 1992).

In vivo studies have indicated that fluctuations in circulating levels of the endogenous GABA-modulatory steroid 3α -OH- 5α [β]-pregnan-20-one (THP) produce significant, although transient, increases in $\alpha 4$ expression. *In vivo* administration of THP or its parent compound, progesterone, for 48–72 h to adult female rats causes a three-fold increase in $\alpha 4$ expression in CA1 hippocampus; however, the amplitude of the GABA-gated current is unchanged, suggesting that this is a subunit switch (Gulinello et al., 2001). This increase in $\alpha 4$ expression results in a relative insensitivity of pyramidal cell currents to modulation by benzodiazepine (BZ) agonists (Gulinello et al., 2001; Hsu and Smith, 2003), consistent with the characterization of $\alpha 4$ -containing GABAR as BZ-insensitive (Wafford et al., 1996). We have also recently demonstrated that 48 h administration of THP to differentiated neuroblastoma cells (IMR-32) is effective in increasing $\alpha 4$ expression *in vitro* (Zhou and Smith, 2007). Consistent with these findings, other *in vitro* studies have demonstrated that 7 d progesterone treatment upregulates $\alpha 4$ mRNA in NT2-N neurons (Pierson et al., 2005), while earlier studies suggested that 48 h administration of THP to cultured neurons reduces BZ binding (Friedman et al., 1996; Yu and Ticku, 1995), which may reflect increased expression of the BZ-insensitive $\alpha 4$ subunit.

$\alpha 4$ subunit expression is also increased following withdrawal from THP, *in vivo*, when a relative BZ insensitivity is observed (Smith et al., 1998). Chronic steroid exposure and/or withdrawal have also been shown to increase $\alpha 4$ expression in other areas such as the midbrain periaqueductal grey (Lovick et al., 2005) and cerebellum (Follesa et al., 2000).

Interestingly, increases in $\alpha 4$ expression in hippocampus and cortex are observed after prolonged exposure or withdrawal from many other known GABA-modulatory drugs, including BZs (Holt et al., 1996; Holt et al., 1997; Arnot et al., 2001; Follesa et al., 2001) and ethanol (Mahmoudi et al., 1997; Devaud et al., 1997; Sanna et al., 2003; Liang et al., 2007), suggesting that enhancing inhibition may be the common mediator which triggers $\alpha 4$ expression. For both THP and ethanol, at least one population of these receptors was of the $\alpha 4\beta 2$ subtype (Cagetti et al., 2003; Smith et al., 1998; Liang et al., 2007), based on the distinctive pharmacology of this receptor isoform (Wafford et al., 1996).

There is an extensive literature examining the mechanisms which underlie classic forms of synaptic plasticity produced by excitatory amino acids and other depolarizing conditions, which trigger voltage-dependent Ca^{++} influx leading to a cascade of intracellular events (Luscher et al., 2000). However, it is unclear how positive GABA-modulators act to trigger subunit plasticity in GABAR subunit composition. Thus, we considered two potential initial triggers for this event: i.) ligand-receptor interactions or ii.) changes in current through the GABAR. These possibilities were investigated using a pharmacological approach with selective GABA ligands to increase or decrease GABA-gated current or by altering the Cl^- driving force without direct interaction with the GABAR. To this end, we used NGF-differentiated IMR-32 cells, which express GABAR and have been characterized pharmacologically (Sapp and Yeh, 2000), and which we have previously shown (Zhou and Smith, 2007) are an optimal system for THP-induced upregulation of the GABAR $\alpha 4$ subunit.

In addition, we chose a neuroblastoma cell line because it represents a homogeneous population of cells, unlike primary neuronal cell cultures.

Methods

Cell Culture

Frozen IMR-32 human neuroblastoma cells were obtained from the American Tissue Culture Collection (ATCC CCL-127, Manassas, VA) and maintained under culture conditions according to Anderson et al (Anderson et al., 1993). Cells were maintained in modified Eagle's Minimum Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM, containing 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% FBS, 100 I.U./ml penicillin and 100 µg/ml Streptomycin (ATCC, Manassas, VA) and incubated in 5% CO₂ at 37°C. Cells were subcultured with 0.25% Trypsin/0.53 mM EDTA solution at a 1:6/1:7 subcultivation ratio every 6–7 days. After growing for 3–4 days, cells were differentiated into a neuronal phenotype, with a serum-free medium: Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12, 1:1 (DMEM/F-12) containing 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, 1.2 g/L sodium bicarbonate, 0.2% BSA, 100 I.U./ml penicillin, 100 µg/ml streptomycin and 20 ng/ml human NGF-β, as we have described (Zhou and Smith, 2007). Cells were used between 60 and 80 generations.

Steroid and Drug Administration

All steroids and drugs were administered once per 24 h for three days. In all cases, vehicle-treated cells were used as the control (medium or < 0.1% DMSO). For the K⁺ depolarization experiment, isoosmotic 180 mM KCl was added to the medium to achieve 10 mM KCl for periods of 0.5, 1, 1.5, 2, 3, 4, 24 or 48 h. All drugs were from Sigma (St. Louis, MO), except for THP (Steraloids, Inc., Newport, RI).

GABA analysis

The ambient GABA concentration present in cultures of differentiated IMR-32 cells was determined using mass spectroscopy techniques. Initially, spent media was collected from two dishes after a 1 h period. Then, the combined samples (6 ml cell culture medium) or the GABA standard (200 pmoles) were passed through a Sep-Pak C18 cartridge (Waters Corp., Milford, MA) preconditioned with solvent A (90:9:1 H₂O:CH₃CN:HCO₂H) and solvent B (90:9:1 CH₃CN:H₂O:HCO₂H). The flow-through was collected and concentrated to dryness in a speedvac rotary evaporator. The sample was reconstituted in solvent A for mass spectrometry analysis.

In the presence of an overabundant peak (mass/charge, m/z) of 104.20 which precluded detection of the GABA peak at 104.07, the sample was methyl esterified to provide a 14-Da mass shift (Goodlet et al., 2001). Briefly, control GABA and the experimental sample were treated as described above. After drying, both samples were reacted for 1 hr. at room temp. with 75 µl of methanolic HCl, freshly prepared by the stepwise addition of 60 µl acetyl chloride to 300 µl anhydrous methanol. Following the incubation, samples were concentrated and reconstituted in solvent A.

Samples were infused with a syringe at a flow rate of 3 µl/min. All spectra were obtained on a Finnigan LCQ Deca XP Ion Trap (ThermoElectron Corp., San Jose, CA). A full scan was acquired at m/z (mass/charge) 50–150 setting, and the parent ion m/z 118 was selected at an isolation width of 1 m/z and fragmented at 35% collision energy. In order to quantify the GABA concentrations of the sample, the daughter ion 101.07 was isolated, and the area under the curve integrated and compared with the 200 pmole standard.

Western Blot Analysis

$\alpha 4$ levels were estimated in cell membranes using Western blot procedures previously described (Smith et al., 1998). In all cases, two or three protein concentrations were tested per sample to ensure density readings in the linear range. Cell membranes electrophoresed onto 8–10% NuPage Bis-Tris gels were transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). The $\alpha 4$ subunit was detected with a rabbit antibody (Smith et al., 1998) against a peptide of rat $\alpha 4$ as a 67 kDa band. Band density was visualized by ECL (enhanced chemoluminescence, Pierce Supersignal WestFemto Substrate, Pierce Chemical Co., Rockford, IL), and the optical density of the immunoreactive bands was analyzed using a Microtek scanner and One-DScan Gel Analysis Software. Results were normalized to the GAPDH optical density from the same samples (36 kDa), and the results were expressed as a ratio of the control value (Smith et al., 1998).

Electrophysiology

GABA-activated current was recorded at room temperature (20–25°C) in a bath solution containing (in mM): NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, HEPES 10, and D-glucose 24, pH 7.4, 320 mOsm/kg H₂O following wash-out of steroid/drugs, as we have described (Smith et al., 1998). The pipette solution contained (in mM): *N*-methyl-D-glutamine chloride 120, Cs₄BAPTA 5, Mg ATP 5. The ATP regeneration system, Tris phosphocreatinine (20 mM) and creatine kinase (50 U/ml), was added to minimize GABA rundown. The input resistance of recording pipettes was 5 to 10 M Ω .

GABA-gated Cl[−] currents were recorded in these cells using whole-cell patch clamp techniques at a holding potential of −50 mV with an EPC7 amplifier (HEKA elektronik, Germany). GABA-evoked currents were filtered at 3 kHz and digitally sampled at a 10 kHz sampling frequency using the pClamp 8.2 software package (Axon Instruments, Foster City, CA). Drug delivery was accomplished via a solenoid-activated rapid superfusion system positioned within 50 μ m of the cell that released drug for 20 ms to achieve drug exposure times of at least 400 ms at 1–3 min intervals to prevent GABA rundown (Smith et al., 1998). To construct GABA concentration-response curves, the value of the peak current for each concentration of GABA was normalized to the maximal current obtained in the same cell. Peak GABA-gated current was calculated as GABA potentiation (or inhibition) for all drug concentrations as $(I_{\text{GABA drug}} - I_{\text{GABA control}}) / (I_{\text{GABA control}})$. Drugs included lorazepam (LZM), flumazenil, RO15-4513 and bicuculline (Research Biochemicals, Natick, MA).

The tight-seal cell-attached current-clamp recording method was used to investigate the membrane potential change after GABA application (Perkins, 2006). To this end, recordings were carried out with >1 G Ω seals at a 0 pA holding current (150 mM NaCl intra-pipette solution). For the bumetanide experiment, drugs were applied with a theta tube (Sutter Instruments, Novato, CA) controlled by a piezoelectric transducer (LSS-3100, Burleigh Instr, Fishers, NY) to achieve rapid onset and offset of response (Smith and Gong, 2005).

Statistical Analysis

Differences between groups were assessed using the Student's *t* test (two groups) or standard ANOVA and Tukey (>2 groups) *post hoc* procedures. Significance was determined when *p* < 0.05.

Results

Detection and quantification of GABA in the differentiated IMR-32 culture

We used mass spectroscopy techniques to detect and quantify levels of GABA present in cultures of NGF-differentiated IMR-32 cells. Media was collected from two cultures after 1 h,

combined and tested for the presence of GABA. The predicted peak at 104.07 was visualized (Fig. 1-I) for both the experimental sample and the 200 pmole GABA standard, but was not present in the blank (untreated media). However, this peak was contaminated with a secondary peak of similar wavelength (104.20) in the experimental sample which prevented conclusive identification of GABA. In order to increase the limit of detection in the presence of the high background produced by contaminating molecules in the media, methyl esterification was implemented to detect a parent peak of 118 (Fig. 1-II). Integration of the area under the curve yielded values of 14,000 for the 200 pmole standard, and 1,423 for the sample. The samples were further fragmented using collision-induced dissociation to a unique peak at 101.07, present for both the sample and GABA standard (Fig. 1-III), thus confirming the presence of GABA in the sample. Neither of the peaks at 118 and 101.07 were present in the blank. Therefore, the approximate amount of GABA in the medium per culture is 10 pmoles.

48 h THP administration produces insensitivity to BZ modulation associated with upregulation of the $\alpha 4$ GABAR subunit

Our previous study (Zhou and Smith, 2007) demonstrated that neuronal differentiation by serum deprivation plus NGF is the optimal condition for steroid-induced increases in expression of the BZ-insensitive $\alpha 4$ GABAR subunit in IMR-32 cells. Therefore, we used this paradigm to examine the GABA pharmacology of differentiated IMR-32 cells following 48 h treatment of differentiated IMR-32 cells with 100 nM THP or vehicle. Initially, we verified that GABA-gated current recorded from IMR-32 cells treated for 48 h with THP retained a significant level of response to acutely applied THP. Indeed, 100 nM THP increased GABA (EC_{20})-gated current by a mean 115 ± 12 pA in cells treated with THP for 48 h (Fig. 2A). In addition, 48 h treatment with THP did not alter the GABA-gated current. Our data demonstrate that GABA activated a concentration-dependent and saturable current (Fig. 2B) under both conditions, with an EC_{50} of 44.6 ± 3.8 μ M or 48.0 ± 2.9 μ M for vehicle and THP-treated cells, respectively (Fig. 2B). Similarly, neither the Hill coefficient (1.0 ± 0.1 , control and 1.2 ± 0.1 , THP-treated), nor the peak current amplitude induced by 10 μ M GABA (EC_{20}) was altered by 48 h THP treatment ($p > 0.05$, Fig. 2C).

Cycloheximide decreases $\alpha 4$ expression after 48 h THP exposure

In order to test whether active protein synthesis was necessary for the increase in $\alpha 4$ expression we have observed following THP treatment (Zhou and Smith, 2007), we co-administered 4 μ M cycloheximide for varying periods of time (2, 4, 6, 16 and 24 h) for the final portion of a 48 h THP exposure and quantified $\alpha 4$ expression levels using Western blot analysis of crude membrane fractions. In fact, protein synthesis was necessary for maintaining maximal levels of $\alpha 4$ expression for the entire 48 h duration of THP exposure except for the final 4 h. As shown previously (Zhou and Smith, 2007), THP produced significant 2.5-fold increases in $\alpha 4$ expression (Fig. 2D). Cycloheximide administered for the final 6 h produced almost a 50% decrease in THP-induced $\alpha 4$ expression (Fig. 2D, $p < 0.05$), while a 24 h exposure to cycloheximide reduced $\alpha 4$ expression to control levels. This suggests a receptor turnover ≥ 4 h, which is consistent with previous reports (Borden et al., 1984).

BZ responses of IMR-32 cells after 48 h THP treatment

Because 48 h THP treatment increases expression of the GABAR $\alpha 4$ subunit, which is insensitive to BZs (Wafford et al., 1996), we predicted that responses of GABA-gated current recorded after 48 h THP exposure would be unresponsive to modulation by the BZ agonist LZM. Indeed, after 48 h THP administration to NGF-differentiated IMR-32 cells, LZM was ineffective in potentiating GABA-gated current (Fig. 3A,B). In contrast, under control conditions, LZM potentiated GABA-gated current ($p < 0.05$) from 18.4 ± 1.1 to $100.2 \pm 4.9\%$ in a concentration-dependent manner (0.01 μ M – 100 μ M), with maximal potentiation observed

at 10 μ M LZM. These data suggest that $\alpha 4$ -containing GABARs predominate on the cell membrane after 48 h THP exposure.

48 h THP exposure alters response to BZ ligands

The $\alpha 4$ subunit can co-express with either $\gamma 2$ or δ subunits (Sur et al., 1999), which both produce receptors insensitive to BZ-modulation. However, $\alpha 4\beta\gamma 2$ GABARs respond to BZ inverse agonists and antagonists, such as RO15-4513 and flumazenil, respectively, in an atypical BZ agonist-like fashion (Wafford et al., 1996). Therefore, we tested responses of GABA(EC_{20})-gated current to these compounds after 48 h THP treatment, when expression of the $\alpha 4$ subunit is increased.

After 48 h exposure of differentiated IMR-32 cells to 100 nM THP, flumazenil significantly enhanced GABA-gated current from $17.7 \pm 3.1\%$ to $55.7 \pm 9.5\%$ across a $0.01 \mu\text{M} - 100 \mu\text{M}$ concentration range ($p < 0.05$, Fig. 3C). Similarly, RO15-4513 potentiated GABA-gated current from 25.2 ± 2.7 to 157.5 ± 17.6 across the same concentration range after 48 h THP treatment ($p < 0.05$, Fig. 3D). In contrast, when tested under control conditions, neither drug significantly altered GABA-gated current, consistent with the known lack of effect of these compounds on non- $\alpha 4$ GABAR (Wafford et al., 1996). These data suggest that there is an increase in functional $\alpha 4\beta\gamma 2$ GABAR expression in IMR-32 cells after 48 h THP exposure.

48 h THP exposure decreases $\alpha 1$ expression, but does not change δ expression

Work from the present lab (Shen et al., 2005) and others (Devaud et al., 1997) suggests that there is a reciprocal relationship between $\alpha 4$ and $\alpha 1$ expression. Therefore, we also investigated potential changes in $\alpha 1$ expression following 48 h THP exposure. As predicted by our *in vivo* findings, $\alpha 1$ expression declined by a mean $56 \pm 10\%$ after 48 h THP treatment relative to control levels ($p < 0.01$, Fig. 4A, B). In contrast, expression of the δ subunit remained unchanged by the 48 h THP treatment compared to control (Fig. 4A, C).

GABA current is inward in differentiated IMR-32 cells

In most cell cultures, such as primary cortical cells and differentiated cell lines (including P19 cells and NT2-N cells), GABAR stimulation leads to a depolarization, rather than the hyperpolarization associated with mature GABAR responses (Khirug et al., 2005). Therefore, we investigated the membrane potential change after GABA application in NGF differentiated IMR-32 cells. To this end, we used the tight-seal cell-attached patch clamp technique, where steady-state and dynamic changes in transmembrane potential can be recorded using current clamp mode (Perkins, 2006). At a holding current of 0 pA, a depolarizing potential change (15 ± 1.3 mV) was recorded after 10 μ M GABA application, with the resting membrane potential recorded at $-50 \sim -55$ mV (Fig. 5A), suggesting that GABA generates an inward current in these cells.

48 h picrotoxin treatment prevents the THP-induced increase in $\alpha 4$ expression

In order to investigate whether increased current flow resulting from the GABA-modulatory effect of THP played a role in THP-induced $\alpha 4$ upregulation, we tested whether use of a GABAR non-competitive antagonist such as picrotoxin could prevent THP-induced increases in $\alpha 4$ expression in differentiated IMR-32 cells. Western blot results showed that concomitant administration of 50 μ M picrotoxin with THP for 48 h prevented the 3-fold increase in $\alpha 4$ expression normally produced by 48 h THP (Fig. 5, C,D). As we have shown previously (Zhou and Smith, 2007), the $\alpha 4$ presents as a single band in these Western blots, possibly due to a fully glycosylated state of the receptor.

Reduction of GABA-gated current decreases expression of the $\alpha 4$ GABAR subunit

In order to further investigate whether compounds which block GABA-gated current alter $\alpha 4$ expression, we tested whether sub-maximal concentrations of penicillin G (PG), the GABAR channel blocker (Twyman et al., 1992), or gabazine (SR95531 hydrobromide), the competitive GABAR antagonist ($\leq 10 \mu\text{M}$), could affect $\alpha 4$ expression in differentiated IMR-32 cells. In fact, 48 h administration of 1 mM PG or 200 nM gabazine both significantly ($p < 0.05$) decreased $\alpha 4$ expression (Fig. 6).

We further predicted that 48 h exposure to a negative modulator of the GABAR would also decrease $\alpha 4$ expression in differentiated IMR-32 cells. For this purpose, we chose the BZ inverse agonist dimethoxy-4-ethyl- β -carboline-3-methoxylate (DMCM), which decreases GABA-gated current by 40% – 60% at a $1 \mu\text{M}$ concentration (Neelands et al., 1998). Indeed, 48 h administration of $1 \mu\text{M}$ DMCM decreased $\alpha 4$ expression by 40%, compared to control values ($p < 0.05$, Fig. 7,A,B). Consistent with a low level of $\alpha 4$ expression, LZM potentiated GABA-gated current by $102.7 \pm 3.3\%$ in 48 h DMCM treated cells ($p < 0.05$), an effect not significantly different from control ($100.2 \pm 4.9\%$, $p > 0.05$, Fig. 7,C,D). This suggests that non- $\alpha 4$ containing receptors predominate on the cell membrane after 48 h DMCM treatment. Taken together, these data support the proposal that $\alpha 4$ expression is positively correlated with the change in GABA-gated current across a 48 h period.

Bumetanide administration decreases expression of the $\alpha 4$ GABAR subunit

In order to determine if the change in GABA-gated Cl^- current was the trigger for $\alpha 4$ upregulation, we tested whether 48 h THP treatment would increase $\alpha 4$ expression when the magnitude of the Cl^- current was decreased. To this end, we used $10 \mu\text{M}$ bumetanide, which selectively blocks the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter NKCC1 (Isenring et al., 1998; Payne 1997), to decrease the Cl^- driving force during 48 h THP application. Initially we verified that $10 \mu\text{M}$ bumetanide decreased the membrane potential change induced by $10 \mu\text{M}$ GABA application (by a mean $37 \pm 5.3\%$ after 10 min) recorded with tight-seal cell attached techniques from differentiated IMR-32 cells at a holding current of 0 pA ($p < 0.05$, Fig. 8,A,B). In addition, 48 h bumetanide treatment resulted in a significantly reduced response to application of $10 \mu\text{M}$ GABA ($72.6 \pm 11\%$, $p < 0.05$, Fig. 8,C,D). This effect increased with repeated applications of GABA, such that the response to the 4th pulse of GABA was reduced to almost undetectable levels, an additional decrease of $80.3 \pm 10\%$ ($p < 0.001$ versus control) after 48 h bumetanide treatment. This decrease in membrane potential change was likely a result of depletion of intracellular Cl^- via GABA activation of the receptor. Concomitant blockade of the Cl^- transporter with bumetanide would prevent the replenishment of intracellular Cl^- that would normally occur and result in a progressively smaller current due to a diminishing driving force (reduced Cl^- gradient).

Co-administration of $10 \mu\text{M}$ bumetanide with THP for 48 h successfully prevented THP-induced upregulation of $\alpha 4$ expression (Fig 8,E,F). In fact, 48 h administration of bumetanide alone significantly decreased $\alpha 4$ expression by 40% ($p < 0.05$, Fig. 8,E,F). These data suggest that $\alpha 4$ expression is related to changes in current through the GABAR.

High K^+ does not alter GABAR $\alpha 4$ expression

Because enhancing GABA-gated depolarizing current increased $\alpha 4$ expression, we tested whether direct depolarization mediated by high K^+ would result in increased expression of the $\alpha 4$ subunit. To this end, we administered 10 mM K^+ , a concentration which has been shown to effectively result in neuronal depolarization (DeFazio et al., 2000). We initially confirmed membrane depolarization by directly recording the shift in the holding potential with tight seal cell-attached recording techniques (Fig 5B), where application of 10 mM KCl produced a mean $32 \pm 5 \text{ mV}$ shift in the holding potential.

When tested across a range of incubation durations (0.5 – 4 h, 24 h and 48 h) depolarization using 10 mM K⁺ failed to increase $\alpha 4$ expression beyond levels seen with 4 mM K⁺, the concentration in the control culture medium. In fact, values for averaged optical densities were almost identical between high K⁺ and control groups (Table 1).

Flumazenil treatment does not change expression of the $\alpha 4$ GABAR subunit

Because our results indicate that $\alpha 4$ expression was correlated with the magnitude of the change in GABA-gated current, we predicted that exposure to the BZ antagonist flumazenil would not alter $\alpha 4$ expression. Under conditions of low $\alpha 4$ expression, as seen in the control IMR-32 cells, flumazenil binds to the BZ site on the GABAR, but has no effect on GABA-gated current (see Fig. 2). Indeed, 48 h administration of 10 μ M flumazenil did not change $\alpha 4$ expression ($p > 0.05$, Fig. 9,A,B). Thus, these results suggest that alterations in GABA-gated current are required for changes in $\alpha 4$ expression.

48 h treatment with pentobarbital increases $\alpha 4$ expression

In order to test whether positive GABA modulators other than THP increase expression of the $\alpha 4$ subunit, we administered 10 μ M pentobarbital (Pb) to differentiated IMR-32 cells for 48 h. Indeed, Pb treatment also produced significant 2.5-fold increases in $\alpha 4$ expression ($p < 0.05$), similar to THP (Fig. 9C, D). These results suggest that Pb is also effective in triggering plasticity of the $\alpha 4$ GABAR subunit.

Discussion

The results from the present study demonstrate that sustained changes in GABA-gated current regulate expression of the GABAR $\alpha 4$ subunit in a reciprocal relationship with $\alpha 1$ expression. Increased $\alpha 4$ expression produced by 48 h steroid treatment was prevented by picrotoxin, as well as by a reduction in the Cl⁻ driving force through the receptor, suggesting that changes in current trigger the subunit change. Protein synthesis was shown to be necessary for maintaining peak levels of $\alpha 4$ expression, except for the final 4 – 6 h. This suggests a relatively short receptor half-life, consistent with previous reports of GABAR (Borden et al., 1984). These findings may be important in understanding the initial trigger for changes in this highly regulatable subunit produced as a compensatory response to alterations in the GABAergic system.

For the present study, we employed a human neuroblastoma cell line, IMR-32 (Sapp and Yeh, 2000), which we have shown (Zhou and Smith 2007), following neuronal differentiation, is an effective model to investigate upregulation of the GABAR $\alpha 4$ subunit following 48 h THP treatment. It also provides a homogeneous population of cells to investigate GABAR expression, unlike traditional neuronal cell culture. Furthermore, results from the present study suggest that GABA-gated current recorded from differentiated IMR-32 cells is inward, as it is in two of the areas with high levels of $\alpha 4$ expression, cortex and dentate gyrus (Gulledge and Stuart, 2003; Staley and Mody, 1992), where GABA can produce a shunting inhibition. Thus, it may be an especially relevant model system for study of the regulation of $\alpha 4$ expression.

Our earlier *in vivo* findings (Smith et al., 1998, Gulinello et al., 2001) also demonstrated that 48 h THP treatment increases $\alpha 4$ expression in CA1 hippocampal pyramidal cells. Steroid-induced increases in $\alpha 4$ expression produced an insensitivity of CA1 pyramidal neurons to the GABA-modulatory effects of a BZ because it was prevented when $\alpha 4$ expression was suppressed using selective antisense suppression (Smith et al., 1998).

$\alpha 4\beta 2\gamma 2$ GABAR pharmacology

This study replicates the *in vivo* findings using an *in vitro* model, where increased $\alpha 4$ expression was accompanied by pharmacological responses characteristic of $\alpha 4\beta 2\gamma 2$ GABAR (Wafford et al., 1996), i.e., the GABA-gated current was relatively insensitive to BZ agonists such as LZM, but responsive to the BZ antagonist flumazenil and inverse agonist RO15-4513 with increases in GABA-gated current. In contrast, GABA-gated current recorded from control neurons prior to steroid treatment responded to LZM with robust potentiation, as previously shown for CA1 hippocampal pyramidal neurons (Smith et al., 1998), but was unresponsive to either flumazenil or RO15-4513, suggesting low ambient $\alpha 4$ expression.

Similar changes in BZ pharmacology have been reported in earlier studies after chronic intermittent ethanol treatment (Cagetti et al., 2003; Liang et al., 2007), when $\alpha 4\beta 2\gamma 2$ GABAR are also increased. Interestingly, flumazenil treatment is also effective behaviorally after alcohol withdrawal, when it has anxiolytic actions (Moy et al., 1997), an effect mediated via the amygdala (Knapp et al., 2007).

GABA quantification

The results from the present study suggest that GABA is released from the differentiated neuronal cultures. The estimated GABA concentration we measured in a culture of about 1×10^6 neurons is about 10 pmoles. Given that estimates of synaptic volume are around 0.1 femtoliter (Bhalla, 2004) and estimating 10–100 synapses per neuron, this would suggest a synaptic concentration of around 1–10 μ M, which represents an EC_{15-20} for GABA-gated current in our differentiated neuronal cultures. This may be an underestimate of the volume, however, because transporter-activated GABA release (Wu et al., 2007) may also contribute to the pool of available GABA. This measurement was especially important in showing that GABA modulators alter expression of the $\alpha 4$ GABAR subunit. In fact, the 100 nM concentration of THP used here is ineffective as a GABA-mimetic agent (Zhou and Smith, 2007), and would act solely as a GABA modulator. Similarly, the various BZ ligands investigated are also known to be ineffective in the absence of GABA.

Cl^- driving force

Because sustained increases in GABA-gated current produced by 48 h exposure to THP and other positive GABA modulators are effective at increasing $\alpha 4$ expression, we directly investigated the role of the current generated by GABAR activation as the initial trigger for changes in $\alpha 4$ expression. The most direct evidence for this is the fact that reducing the Cl^- gradient using bumetanide prevented $\alpha 4$ upregulation by THP. Bumetanide at a 10 μ M concentration is a selective blocker of the NKCC1 co-transporter (Isenring et al., 1998; Payne, 1997) which is predominantly expressed by cultured neurons (Khirug et al., 2005). Most importantly, bumetanide does not directly bind to the GABAR, thereby eliminating the possibility of an allosteric effect on receptor function. Picrotoxin also prevented steroid-induced $\alpha 4$ expression, but is thought to directly bind to a modulatory site on the receptor which may trigger allosteric effects (Ramakrishnan and Hess, 2005).

$\alpha 4$ expression and GABA-gated current

Results from the present study also determined that the change in $\alpha 4$ expression was correlated with the direction of the change in GABA-gated current across the 48 h exposure period (Fig. 9). That is, compounds, such as THP and Pb, which *increase* GABA-gated current *increased* $\alpha 4$ expression, while compounds which *decrease* GABA-gated current, such as the BZ inverse agonist DMCM, *decreased* $\alpha 4$ expression. Sub-maximal concentrations of GABAR blockers, such as gabazine and PG, also effectively reduced $\alpha 4$ expression, suggesting that interaction with a modulatory site is not required. In addition, these two GABAR blockers act via distinct

mechanisms, with gabazine a competitive antagonist of the GABA binding site, and PG, an open channel blocker (Twyman et al., 1992). The fact that blockade of the Cl^- channel decreased $\alpha 4$ expression also supports the idea that the current through the channel triggers expression of the $\alpha 4$ subunit.

Although increased expression of the $\alpha 4$ subunit decreased BZ responsiveness of the IMR-32 cells, decreased $\alpha 4$ expression produced by DMCM did not increase BZ responsiveness. This apparent discrepancy could be due to the fact that BZ responsiveness was at a maximal level or that receptors which substituted for the $\alpha 4$ were unresponsive to BZs, such as $\alpha 1\beta 2$, which have been shown to express under physiological conditions (Mortensen and Smart, 2006). Alternatively, the $\alpha 4$ expressed in control cells may not be part of a functional receptor, in which case, its decrease would not alter BZ responses.

In contrast to these compounds that alter GABA-gated current, flumazenil is a BZ antagonist which binds to the GABAR, but under control conditions, produces no effect on GABA-gated current. As predicted by our hypothesis, 48 h flumazenil treatment did not alter $\alpha 4$ expression, suggesting that a change in current through the receptor is the necessary trigger for $\alpha 4$ expression.

K⁺ depolarization and $\alpha 4$ expression

Our data suggest that the current generated by GABAR in these differentiated IMR-32 cells is in the depolarizing direction, as shown for most cultured neurons and cell lines (Khirug et al., 2005). However, global depolarization alone using 10 mM K^+ was ineffective in increasing $\alpha 4$ expression in differentiated IMR-32 cells. K^+ was applied for a range of duration times, from 0.5 to 48 h, without significantly altering $\alpha 4$ expression. It is unlikely that this relatively low concentration of K^+ is toxic, because cell morphology and growth remained at normal levels, even following the 48 h incubation. This procedure has in fact, been reported in the slice, where it was the lowest concentration of K^+ to achieve depolarization without neurotoxicity (DeFazio et al., 2001).

The reason for the lack of effect of K^+ on $\alpha 4$ expression may be due to the fact that it was a continual, tonic depolarization rather than a phasic depolarization, as would be achieved synaptically. Alternatively, it may be that local depolarizing current in the vicinity (or through) the GABAR is required. This lack of effect of K^+ exposure on $\alpha 4$ expression is consistent with findings from other labs (Lyons et al., 2001), which reported that neither high K^+ , TTX nor blockade of glutamate receptors, induced uncoupling of GABA and BZ binding. Although the mechanism for GABA-BZ uncoupling is unknown, it is consistent with an increase in expression of the BZ-insensitive $\alpha 4$ subunit.

Sub-maximal changes in GABA-gated current

In fact, consistent with the lack of effect of K^+ on $\alpha 4$ expression, our results also suggest that only concentrations of drugs which produce sub-maximal changes in GABA-gated current are effective in altering $\alpha 4$ expression. Only sub-maximal concentrations of GABAR blockers were effective in this regard, while complete GABAR blockade with picrotoxin failed to decrease $\alpha 4$ expression. The sub-maximal blockade with 200 nM gabazine (Stell and Mody, 2002) would be expected to selectively block synaptic receptors, and suggests that this population may be the trigger for $\alpha 4$ plasticity. Our pharmacological results suggest that THP treatment increases expression of $\alpha 4\beta 2$ receptors, which can be localized synaptically (Hsu et al., 2003), while δ expression levels did not change. Although not tested in the present study, $\alpha 1$ expression would likely undergo a complementary upregulation to maintain constant levels of GABAR expression, as demonstrated here for the reciprocal relationship of $\alpha 4$ upregulation

with $\alpha 1$ downregulation. This reciprocal relationship has been reported previously (Devaud et al., 1997, Matthews et al., 1998, Shen et al., 2005).

Conversely, constant activation of the GABAergic system with a GABA agonist, muscimol, also failed to increase $\alpha 4$ expression (data not shown). Interestingly, while withdrawal from other GABA modulators can increase $\alpha 4$ expression (Holt et al., 1996; Mahmoudi et al., 1997), withdrawal from GABA itself does not alter GABAR subunit expression, but instead decreases GABAR density (Casasola et al., 2001). It may be that other systems, involving excitatory synaptic events, override changes in the GABAergic system when constant and complete blockade or activation is involved, similar to the effect of constant K^+ -induced depolarization.

Cl⁻ triggered events

The mechanism by which GABA-gated current could alter GABAR expression is not yet elucidated. It has long been known that Cl^- can act in a modulatory capacity, stabilizing the receptor in the low affinity state where it is more likely to be allosterically enhanced by modulators (Leed-Lundberg et al., 1980; Olsen and Snowman, 1982; Lo and Snyder et al., 1983; Bormann et al., 1987). Indeed, modulation of GABA binding is not evident when Cl^- is absent from the medium (Peroutka et al., 1980). In addition, recent evidence suggests that local areas of charge transfer may play a role in triggering ionotropic or metabotropic events. Voltage sensitive sites have been identified for hyperpolarization-activated cation channels (Ih) which are triggered by Cl^- and can be reduced by selective mutation of basic residues in the channel (Wahl-Schott et al., 2005). Recent results from our laboratory have also identified a basic residue which mediates Cl^- -dependent desensitization of $\alpha 4\beta 2\delta$ GABAR (Shen et al., 2007). In addition, a recent report has demonstrated the existence of voltage sensors which trigger phosphorylation events in a membrane protein devoid of an ion-conducting pore (Ramsey et al., 2006). Thus, increases in Cl^- current which trigger $\alpha 4$ expression may indeed be mediated by selective sites which result in allosteric effects leading to second messenger cascades, such as phosphorylation and internalization of non- $\alpha 4$ -containing GABAR (Kittler et al., 2005).

Regulation of $\alpha 4$ transcription

Recent reports suggest that depolarizing events such as seizure activity increase $\alpha 4$ expression. This activity-dependent increase in $\alpha 4$ expression may be due to the development of depolarizing GABA currents, frequently the result of increased afferent activity due to the collapse of the Cl^- gradient (DeFazio et al., 2000), rather than to direct depolarization, as suggested by our study and others (Lyons et al., 2001). One recent study showed that seizure-induced $\alpha 4$ expression via BDNF which increases early growth factor 3 (Egr3) that leads to activation of the $\alpha 4$ promoter (Roberts et al., 2006; Roberts et al., 2005). Recent studies also suggest that L-type calcium channel activation may be an important regulator of GABAR expression under some conditions (Wand and Greenfield, 2009). Other studies have demonstrated that heat shock protein can induce $\alpha 4$ expression (Pignataro et al., 2007), while earlier studies also suggested that this was the case because seizure activity reduced BZ responsiveness of dentate gyrus granule cells (Kapur, 2000), a possible indicator of $\alpha 4$ expression. Nevertheless, multiple mechanisms may be involved in $\alpha 4$ regulation due to the fact that the $\alpha 4$ promoter has multiple transcriptional initiation sites (Ma et al., 2004).

$\alpha 4$ expression and hyperexcitability states

In addition to imparting a distinctive pharmacological response, increases in $\alpha 4$ -containing GABAR may also have implications for the level of inhibitory tone, as $\alpha 4$ -GABAR exhibit a faster rate of deactivation than other GABAR isoforms tested (Smith and Gong 2005), which would result in a lower inhibitory charge transfer. Several studies have noted faster decaying mIPSCs (miniature inhibitory post-synaptic currents) produced by increased $\alpha 4$ expression

(Hsu et al., 2003; Liang et al., 2003; Liang et al., 2007; Liang et al., 2008). This faster decay is reversed when $\alpha 4$ expression is prevented by antisense administration (Hsu et al., 2003) or the use of $\alpha 4$ knock-out animals (Chandra et al., 2006). In fact, increases in $\alpha 4$ -GABAR lead to decreases in paired pulse inhibition in CA1 hippocampus (Hsu and Smith 2003) and increased seizure susceptibility (Smith et al., 1998), both of which can also be prevented with antisense-induced suppression of $\alpha 4$ expression. Thus, the trigger for $\alpha 4$ expression may be a pivotal event in the homeostatic response to prolonged levels of inhibition produced by GABA-modulatory drugs.

$\alpha 4\beta\delta$ GABAR

In addition to combining with $\gamma 2$, $\alpha 4$ coexpresses with δ (Sur et al., 1999), supplying a tonic inhibitory current (Stell et al., 2003) that may counteract the hyperexcitability state. Cyclic changes in $\alpha 4\beta\delta$ expression have been observed across the ovarian cycle (Maguire et al., 2005; Lovick et al., 2005) and at puberty (Shen et al., 2007) when THP levels decline. However, in the present study, δ subunit expression was unchanged, suggesting that the increase in $\alpha 4$ reflects an increase in the $\alpha 4\beta\gamma 2$ isoform. This possibility is further strengthened by the resultant pharmacological profile (Wafford et al., 1996), as well as by the GABA concentration-response characteristics (Wafford et al., 1996). In contrast to most GABAR isoforms, $\alpha 4\beta\delta$ GABAR have a high sensitivity to GABA (Brown et al., 2002), which is clearly distinguishable from $\alpha 4\beta\gamma 2$.

Clinical relevance

Interestingly, hormonally-regulated increases in GABAR $\alpha 4$ expression (Smith et al., 1998; Gulinello et al., 2001; Lovick and Griffiths 2005; Shen et al., 2007), are correlated with increased anxiety. Thus, this altered GABAergic state may represent a useful model of premenstrual dysphoric disorder, where fluctuating levels of THP accompany adverse mood changes, including anxiety, and a relative BZ insensitivity, which could represent increased $\alpha 4$ expression (Sundstrom et al., 1997).

The results from the present study suggest that GABA-modulatory compounds, such as THP, increase GABAR $\alpha 4$ subunit expression *in vitro* as a result of increasing the current gated by the receptor. These results may help to elucidate the cellular mechanisms underlying plasticity of this normally underexpressed GABA subunit, which is associated with changes in CNS excitability and mood.

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Fig. 1. Mass spectroscopy analysis of GABA content

Following neuronal differentiation of IMR-32 cells, media was collected from two cultures, combined and analyzed (C), compared with a 200 pmole standard (B) and blank (A). I, Initial identification of the 104.07 peak (arrows) produced by GABA. II, Methyl esterification yields a peak at 118.07 (arrows) for both GABA (B) and the experimental sample (C). III, Collision-induced dissociation following methyl esterification yields a common peak at 101.07 (arrows) for both GABA (B) and the experimental sample (C).

Fig. 2. 48 h THP administration does not change the concentration-response relationship or the amplitudes of GABA-gated current recorded from differentiated IMR-32 cells

A, Differentiated IMR-32 cells incubated with 100 nM THP for 48 h were tested for sensitivity to THP modulation. Responses to 10 μ M GABA before (upper trace) and after (lower trace) co-application with 100 nM THP. Representative of responses obtained from 6 cells. **B**, Concentration-response curves of GABA-gated current. Peak current amplitudes are plotted as a function of GABA concentration. Data derived from each cell were normalized to the maximal peak current amplitude. Concentration-response curves were generated by data derived from 22 cells (control, Con) and 10 cells (48 h THP). **C** There is no significant difference in the peak amplitudes of GABA(10 μ M)-induced currents between these two groups ($p > 0.05$). **D**, Effects of cycloheximide on α 4 expression after 48 h exposure of differentiated IMR-32 cells to 100 nM THP. Cycloheximide (4 μ M) was administered for the final 2, 4, 6, 16 or 24 h of the 48 h THP incubation period. * $p < 0.05$ vs control; ** $p < 0.05$ vs THP alone.

Fig. 3. 48 h THP administration alters benzodiazepine pharmacology

A, GABA (10 μ M) current traces before and during application of LLM (0.1 – 100 μ M). Representative of 18–24 cells/group. Con, control. **B**, This graph depicts no significant enhancement of GABA-gated current by LLM at any dose after 48 h THP administration to differentiated IMR-32 cells (48 h THP) ($n = 24$, $p > 0.05$). In contrast, LLM significantly potentiated GABA-gated current by 20–100% in sham control cells (Control) ($n = 18$, $*p < 0.05$ vs GABA alone and vs responses after 48 h THP treatment). **C**, Modulation of GABA-gated currents by flumazenil, the BZ antagonist, which increases GABA-gated current after THP treatment. ($*p < 0.05$ vs. GABA alone and vs. control, $n = 10$ cells per group). **D**, Effects of RO15-4513 on GABA-gated currents, which also increases GABA-gated current after THP treatment ($*p < 0.05$ vs. GABA alone and vs. control, $n = 10$ –14 cells per group).

Fig. 4. Effect of 48 h THP administration on GABAR $\alpha 1$ and δ subunit expression in IMR-32 cells
A, Western blot analysis of $\alpha 1$ and δ subunit levels in membrane fractions of differentiated IMR-32 cells following 48 h exposure to 100 nM THP. 48 h THP decreased $\alpha 1$ expression, but did not alter expression of the δ subunit. **B,C**, Averaged optical densities, normalized to GAPDH level and expressed relative to control ($n = 9$, * $p < 0.05$ vs control).

Fig. 5. 48 h treatment with the GABAR channel blocker picrotoxin prevents THP-induced increases in $\alpha 4$ expression

A, GABA-mediated depolarization of the cell. A depolarizing potential change (~ 15 mV) was produced by 10 μ M GABA application, recorded using tight seal cell-attached patch techniques in current clamp mode (holding current of 0 pA; Resting membrane potential ≈ -50 to -55 mV). Representative of results from 5 cells. Con, control. **B**, Representative trace depicting membrane depolarization induced by 10 mM KCl recorded using tight seal cell-attached techniques ($n=5$). **C**, Representative Western blot showing that 48 h picrotoxin (Pic) prevents the effect of THP on $\alpha 4$ expression. **D**, Averaged optical densities, normalized to GAPDH level and expressed relative to control (Con). (* $p < 0.05$ vs all other groups, $n = 7$).

A **B**

Fig. 6. 48 h treatment with GABA blockers decreases expression of the $\alpha 4$ subunit of the GABAR Submaximal concentrations of the GABAR channel blocker penicillin G (PG, 1 mM) or the competitive GABA antagonist gabazine (200 nM) were tested. **A**, Representative Western blots. Con, control. **B**, Averaged optical densities, normalized to GAPDH and expressed relative to control. (* $p < 0.05$ vs control, $n = 7$).

Fig. 7. 48 h treatment with DMCM, a BZ inverse agonist, decreases expression of the $\alpha 4$ GABAR subunit

A, Representative Western blot. **B**, Averaged optical densities, normalized to GAPDH level and expressed relative to control (Con). (* $p < 0.05$ vs control, $n = 6$). **C**, GABA (10 μ M) current traces before and during application of LZM (10 μ M). Representative of 10–18 cells/group. **D**, LZM significantly potentiated GABA-gated current by ~100% in both groups (* $p < 0.05$ vs. GABA alone, $n = 10$).

Fig. 8. 48 h bumetanide administration prevents THP-induced increases in α 4 expression

A, B. Acute application of bumetanide (10 μ M Bum) for 10 min suppressed GABA(10 μ M)-mediated membrane depolarization. **A**, GABA(10 μ M)-mediated membrane potential change, recorded by tight seal cell-attached current clamp techniques with a holding current of 0 pA. Traces illustrate the cell response before and after bumetanide application, respectively. Con, control. **B**, Statistical analysis of the data is represented as a ratio relative to control (* p < 0.05, n = 4). **C, D**, 48 h bumetanide treatment reduces the GABA-mediated response. **C**, Representative responses to the 1st and 4th GABA pulse after 48 h bumetanide (10 μ M) treatment, **D**, Averaged data (* p < 0.05 versus control, ** p < 0.05 versus 1st GABA pulse, n = 4). **E**, Representative Western blot showing that 48 h bumetanide treatment prevents THP-induced increases in α 4 expression. **F**, Averaged optical densities, normalized to the GAPDH level and expressed relative to control. (* p < 0.05 vs other groups, ** p < 0.05 vs control, n = 6).

Fig. 9. 48 h treatment of IMR-32 cells with pentobarbital or flumazenil. Effects on $\alpha 4$ expression
A, Representative Western blot. Con, control. Flu, flumazenil (10 μ M), a BZ antagonist which binds to the GABA receptor without gating current does not alter $\alpha 4$ expression. **B**, Averaged optical densities, normalized to GAPDH level and expressed relative to control ($p > 0.05$, $n = 11$). **C,D**, In contrast, 48 h exposure of differentiated IMR-32 cells to 10 μ M pentobarbital (Pb), a positive GABA modulator, increases $\alpha 4$ expression. **C**, Representative Western blot; **D**, Averaged optical densities, normalized to GAPDH level and expressed relative to control. (* $p < 0.05$ vs control, $n = 9$).

Fig. 10. Summary figure. $\alpha 4$ expression is correlated with the change in GABA-gated current
Compounds which bind to the GABAR and increase GABA-gated current, including the positive GABA-modulators THP, Pb, BZ and ethanol, increase $\alpha 4$ expression. In contrast, sustained 48 h decreases in the GABA-gated current produced by the NKCC1 blocker bumetanide, the BZ inverse agonist DMCM, the GABA competitive antagonist gabazine and the open channel blocker PG reduce $\alpha 4$ expression. However, 48 h administration of the BZ antagonist flumazenil had no effect on $\alpha 4$ expression. EtOH: ethanol. *Mahmoudi et al., 1997. **Holt et al., 1996.

Table 1**Effect of high K⁺ on $\alpha 4$ expression**

Treatment of differentiated IMR-32 cells with 10 mM KCl in the media across a range of duration times (left panel) and the effect of GABAR $\alpha 4$ subunit expression (right panel) assessed by Western blot techniques. (n=3/group, performed in triplicate.)

Group		n	O.D. (relative to control) (Mean $\pm$ SEM)
Control	4 mM KCl	15	1 $\pm$ 0.1
0.5 h		3	1 $\pm$ 0.3
1 h		11	1 $\pm$ 0.1
1.5 h		3	1 $\pm$ 0.5
2 h	10 mM KCl	3	1.2 $\pm$ 0.2
3 h		3	1.1 $\pm$ 0.1
4 h		3	1 $\pm$ 0.01
24 h		3	1.1 $\pm$ 0.1
48 h		6	0.98 $\pm$ 0.35