GABA<sub>A</sub> Receptors Expressed in Undifferentiated Human Teratocarcinoma NT2 Cells Differ from Those Expressed by Differentiated NT2-N Cells

Torben R. Neelands,¹ Jie Zhang,¹ and Robert L. Macdonald²,³

¹Graduate Program in the Neurosciences and Departments of ²Neurology and ³Physiology, University of Michigan Health Sciences Center, Ann Arbor, Michigan 48104-1687

During CNS development, changes occur in expression of GABA<sub>A</sub> receptor subunit subtypes and GABA<sub>A</sub> receptor pharmacological and biophysical properties. We used reverse transcription PCR and whole-cell-recording techniques to determine whether GABA<sub>A</sub> receptor expression and function also changed during retinoic acid–induced differentiation of human Ntera 2 (NT2) teratocarcinoma cells into neuron-like cells (NT2-N cells). In undifferentiated NT2 cells only α5, β3, γ3, and π subtype mRNAs were detected. NT2 GABA<sub>A</sub> receptor currents had a maximal amplitude of 52 pA and an EC<sub>50</sub> of 4.0 μM, were relatively insensitive to enhancement by zolpidem and diazepam, and were enhanced by loreclezole and inhibited by lanthanum, zinc, and furosemide. In contrast, in NT2-N cells after 13 weeks of retinoic acid treatment, all GABA<sub>A</sub> receptor subtype mRNAs were detected. Maximal peak whole-cell currents were ~50-fold larger than NT2 cell currents, and the GABA EC<sub>50</sub> was higher (39.7 μM). In 13 week NT2-N cells, diazepam, zolpidem, loreclezole, and lanthanum had only small effects on GABA<sub>A</sub> receptor currents, and the zinc IC<sub>50</sub> for current inhibition was significantly higher than that for NT2 cells. In a previous study, we showed that NT2-N cells after 5 weeks of retinoic acid treatment had moderate peak currents, GABA EC<sub>50</sub>, and zinc IC<sub>50</sub> but that currents were robustly enhanced by diazepam, zolpidem, and loreclezole. During differentiation of NT2 cells to NT2-N cells, GABA<sub>A</sub> receptors underwent changes in subunit expression and pharmacology that were similar to many of the developmental changes in GABA<sub>A</sub> receptors that occur in CNS neurons.

Key words: GABA; GABA<sub>A</sub> receptor; electrophysiology; patch clamp; RT-PCR; Ntera2; loreclezole; benzodiazepine; zinc; differentiation; NT2 cells; NT2-N cells

The Ntera 2/cl.D1 subclone (NT2) of neuronal precursor cells was isolated from the TERA2 cell line for its ability to differentiate into postmitotic neuron-like cells (NT2-N cells) when treated with retinoic acid (Pleasure et al., 1992). Therefore, NT2 cells provide a potentially interesting cell line for studies of the regulation of neuronal development (Andrews, 1998). We reported previously that after 5 weeks of retinoic acid treatment, differentiated NT2-N cells expressed a limited number of GABA<sub>A</sub> receptor (GABAR) subtype mRNAs and that the pharmacological and biophysical properties of GABAR currents were consistent with those of GABARs assembled from these subunit mRNAs (Neelands et al., 1998). In the CNS, expression of GABAR subunits is developmentally and regionally regulated and may be associated with changes in GABAR function and pharmacology (Laurie et al., 1992; Poulter et al., 1992; Fritschy et al., 1994; Oh et al., 1995). Little is known, however, about the developmental regulation of expression and assembly of CNS GABARs.

GABARs are composed of five subunits that together form a chloride ion channel that mediates fast IPSPs in mature CNS. Six different subunit families (α, β, γ, δ, π, and ε) and multiple subtypes (α1–6, β1–4, and γ1–4) have been identified (Macdonald and Olsen, 1994; Davies et al., 1997; Hedblom and Kirkness, 1997). Each subtype has a unique regional expression in the brain, and individual neurons often express multiple subtypes. The assembly (Angelotti et al., 1993; Saxena and Macdonald, 1994; Burgard et al., 1996; Neelands et al., 1999) and stoichiometry (Chang et al., 1996; Tretter et al., 1997) of GABARs are regulated so that all the potential combinations of subunits do not form functional channels. Studies of recombinant GABARs have shown that the subunit composition of the receptor isoform can influence their pharmacological and biophysical properties (Pritchett et al., 1989a; Angelotti and Macdonald, 1993; Macdonald and Olsen, 1994). The regional and developmental regulation of GABAR subunit subtypes regulates the GABAR isoforms expressed and, therefore, the function of the receptors.

The majority of GABAR subunit subtype genes are found in four clusters on human chromosomes 4 (α2, α4, β1, and γ1), 5 (α1, α6, β2, and γ2), 15 (α5, β3, and γ3) (McLean et al., 1995; Rabow et al., 1995), and X (α3, ε, and β4) (Wilke et al., 1997). The π subunit is also located on chromosome 5 but is not tightly linked with the GABAR gene cluster (E. Kirkness, personal communication), and the δ subunit has been mapped to human chromosome 1 (Sommer et al., 1990). In general, GABAR subunits whose genes are located on chromosomes 4 and 15 are expressed early in the developing rat brain, and those on chromosome 5 are expressed in adults (Laurie et al., 1992). In this study we determined whether retinoic acid–induced differentiation of NT2 cells into neuron-like NT2-N cells produced changes in GABAR subunit mRNA expression and in GABAR biophys-
Cell culture. NT2 cells were grown and maintained in DMEM high glucose (HG) with 10% fetal bovine serum and penicillin and streptomycin added as described previously (Andrews, 1984). NT2 cells were plated directly onto 35 mm dishes for electrophysiological recordings or were plated at 2 × 10^5 cells/75 cm² flask and differentiated by treatment with 1 μM retinoic acid for 4 weeks. After retinoic acid treatment, cells were washed with versene and then treated with trypsin to dislodge the cells. Cells were resuspended after being triturated and replated at a 1:10 dilution with DMEM HG and maintained in 5% CO₂. The following day the medium was removed and saved as conditioned medium to feed replates II and III. Cells were again treated with trypsin and then spun for 5 min at 1000 rpm. The pellet was resuspended in 1 ml of medium containing mitotic inhibitors (10 μM FUDR + 10 μM uridine and 1 μM cytosine arabinoside) and replated (replate II). The same treatment was performed again after 1 week in culture (replate III) to obtain nearly 100% pure neuron-like cells. These cells were plated onto 10 cm dishes and kept in DMEM HG and maintained in 5% CO₂ for an additional 8 weeks. During the 13th week after the start of retinoic acid treatment, the 10 cm dishes of NT2-N cells were either treated with Ultraspec to isolate total RNA or cut into sections for electrophysiological recording.

Solutions and drug application. Cells were removed from the 5% CO₂ incubator, and the feeding medium was replaced with recording medium containing (in mM): 142 NaCl, 1 CaCl₂, 6 MgCl₂, 8.09 KCl, 10 glucose, and 10 HEPES, 315–325 mMosl, with a pH of 7.4. Patch-clamp electrodes of 5–10 MΩ were filled with pipette solution containing (in mM): 153.35 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, and 2 ATP, 300–310 mMosm, with a pH of 7.3. This combination of solutions results in nearly equivalent intracellular and extracellular chloride ion concentrations, hence an E_Cl near 0 mV, and produced an inward current when cells were voltage-clamped at negative potentials.

Compounds were applied to cells using a modified U-tube “multipuffer” application system (Greenfield and Macdonald, 1996). The U-tube system enabled us to position a micropipette with a 40–50 μm tip next to the cell for the duration of the recording and apply multiple concentrations of individual drugs to each cell. Stock solutions of GABA, diazepam, zinc, and zolpidem were made by dissolving each in sterile water. Dimethylsulfoxide (DMSO) and diluted with sterile water. Stock solutions of loreclezole were dissolved in dimethylsulfoxide (DMSO) and diluted with sterile water. DMSO stock solutions of loreclezole were dissolved in DMSO and diluted with sterile water. Loreclezole was obtained from Janssen Biochemicals (Berse, Belgium). All other compounds were from Sigma (St. Louis, MO).

Electrophysiology. Whole-cell voltage-clamp and single-channel recordings using the patch-clamp technique were obtained as described previously (Hamill et al., 1981). Patch-clamp electrodes were pulled from Labcraft microhematocrit capillary tubes (Curtin Matheson Scientific, Houston, TX) using a P-87 Flaming-Brown micropipette puller (Sutter Instrument Company, San Rafael, CA).

Whole-cell recordings were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Signals were digitized on-line at 200 Hz using a TL-1 Labmaster analog-to-digital converter, recorded, and subsequently analyzed off-line using Axotape 2.0 software (Axon Instruments). Single-channel recordings were obtained from “outside-out” patches formed using standard techniques with an Axopatch 200A amplifier.

Data analysis. The magnitude of the enhancement or inhibition of GABAR current by a drug was measured by dividing the peak amplitude of GABAR currents elicited in the presence of a given concentration of the drug (with GABA) by the peak amplitude of control current elicited by GABA alone and multiplying the fraction by 100 to express the percent of control. Thus the control response was 100%. Peak GABAR currents at various drug concentrations were fitted to a sigmoidal function using a four-parameter logistic equation (sigmoidal concentration–response) with a variable slope. The equation used to fit the concentration–response relationship was:

\[ I = \frac{I_{\text{max}}}{1 + 10^{-E_{\text{EC50}} - Log\text{drug}/Hill\,\text{slope}}} \]

where \( I \) was the GABAR current at a given GABA concentration, and \( I_{\text{max}} \) was the maximal GABAR current. Maximal current and concentration–response curves were obtained after pooling data from all cells tested for GABAR and for all drugs. Concentration–response curves were also obtained from individual cells. The curve-fitting algorithm minimized the sum of the actual distance of points from the curve. Convergence of the algorithm was reached when two consecutive iterations changed the sum of the squares by <0.01%. The curve fit was performed on an IBM PC compatible personal computer using Prism 2.0 (Graph Pad, San Diego, CA). Data were presented as mean ± SEM. To quantify whole-cell current rectification, peak amplitudes of responses to GABA were measured at holding potentials of −75 and +75 mV. An amplitude ratio (+75 mV/−75 mV) was calculated, and rectification was determined with respect to a linear ratio of 1.0 using the predicted E_CV of 0 mV. An amplitude ratio >1.0 indicated outward rectification.

Reverse transcription PCR. Total RNA was isolated from NT2 cells and NT2-N cells using the Ultraspec method by Biotec (Houston, TX). One milligram of total RNA was treated with DNase in a total volume of 10 ml composed of 1 ml of 10X DNase I buffer, 1 ml of DNase I (1 U/ml), and 7 ml of DEPC water. This mixture was incubated for 15 min at 25°C. Addition of 1 ml of 25 mM EDTA was followed by a 10 min incubation at 70°C to heat inactivate the DNase I. The reaction mixture was then chilled immediately on ice. One milliliter of a random primer (0.6 mg/ml) was added to the mixture that was then incubated at 70°C for 15 min. Reverse transcription used the product of the above reaction mixed with the following: 1 ml of 5X first-strand buffer, 2 ml of 0.1 mM DTT, 1 ml of 10 mM dNTP mix, and 1 ml of RNAse inhibitor (10 U/ml). The mixture was prewarmed for 2 min at 42°C before addition of 1 ml of Superscript II (10 U/ml) and then was incubated for 45 min at 42°C. Using the same mixture, we described above, with the exception that 1 ml of DEPC water was substituted for Superscript II, acted as a negative control. The reverse transcription product was heattreated for 15 min at 70°C before PCR. PCR was performed for each subunit in 100 ml of the following mixture: 1 ml of a 20 μM primer mixture (3′ primer and 5′ primer), 2 ml of the reverse transcription product, 10 ml of 10X buffer, 16 ml of 25 mM MgCl₂, 1 ml of 25 mM dNTP mixture, 0.5 ml of Amplitaq (5 U/ml), and 72.5 ml of DEPC-treated water. Positive controls were performed using cDNA for each subunit subtype as the PCR template. PCR was performed as follows: a 2 min period at 94°C to denature the mixture; 35 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min; and a 7 min extension period at 72°C. PCR products (5 ml for positive controls; 15 ml for both test and negative controls) were mixed with dye and run on a 1.5% agarose gel at 120 V for 40 min and then stained with ethidium bromide and photographed. Reaction reagents were purchased as follows: Amplitaq from Perkin-Elmer (Norwalk, CT); random primer, Superscript II, RNAse inhibitor, and DNase I from Life Technologies (Gaithersburg, MD); and the dNTP mix and DNA molecular weight marker V1 from Boehringer Mannheim (Indianapolis, IN).

RESULTS

Whole-cell GABAR currents in NT2 cells and 13 week NT2-N cells

NT2 cells and 13 week NT2-N cells were voltage clamped at −75 mV, and increasing concentrations of GABA were applied for 6–10 sec (Fig. 1A). Whole-cell GABAR currents recorded from both NT2 cells and 13 week NT2-N cells had concentration-dependent activation rates (Fig. 1A). At the higher GABA concentrations, the currents decreased in the continued presence of GABA, consistent with desensitization. The rate of apparent desensitization increased in a GABA concentration-dependent manner. The GABAR currents were similar to those seen in recordings from 5 week NT2-N cells, human neurons, and rat dentate granule cells (Sah, 1995; Kapur and Macdonald, 1996; Neelands et al., 1998). Peak current amplitudes evoked by a range of GABA concentrations (100 nM to 1 mM) were pooled and fitted to a sigmoidal logistic function (Fig. 1B). The maximal current was substantially larger for 13 week NT2-N cells (2894 ± 504 pA; n = 4) than for NT2 cells (52 ± 11 pA; n = 5). Peak currents evoked at each GABA concentration were normalized to the maximal current obtained from each cell. The averaged data were plotted and fitted to a sigmoidal logistic function (Fig. 1C). Plotting the data as a percent maximum illustrated the 10-fold


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evoked at 2

dividing the peak current evoked at

titude ratio was calculated for each stage of differentiation by

resulted in slight outward rectification for 13 week NT2-N cells

mV. Plots of the average peak currents at each holding potential

tained for NT2 cells and 13 week NT2-N cells by measuring peak

currents increased during differentiation with retinoic acid.

GABAR currents (Neelands et al., 1998).

Horizontal bars

are shown.

Figure 1. GABA concentration–response characteristics for GABARs

expressed in NT2 cells and 5 and 13 week neurons. A, Representative

traces of GABAR currents from NT2 cells (left) and 13 week NT2-N cells

(right) in response to application of increasing concentrations of GABA

are shown. Horizontal bars, GABA application. B, Peak currents as a

function of GABA concentration were fit with a four-parameter logistic

equation. Data are presented as mean ± SE (V_h = -75 mV). Peak

currents increased during differentiation with retinoic acid. C, Normalized

concentration–response curves show an increase in the GABA EC_{50}

during differentiation by retinoic acid. Data are mean ± SE.

shift in the GABA EC_{50} between the NT2 cells (EC_{50} = 4.0 μM; n\_h = 1.1) and the 13 week NT2-N cells (EC_{50} = 39.7 μM; n\_h = 1.3). Included in the two plots are the data we reported previously for the 5 week NT2-N cells (I_{max} = 472 ± 71 pA; EC_{50} = 21.8 μM; n\_h = 1.2) that fall between the other two stages of differentiation (Neelands et al., 1998).

Whole-cell GABAR current–voltage (I–V) relations were obtained for NT2 cells and 13 week NT2-N cells by measuring peak currents evoked by GABA at holding potentials from −75 to +75 mV. Plots of the average peak currents at each holding potential resulted in slight outward rectification for 13 week NT2-N cells and large outward rectification for NT2 cells (Fig. 2). An amplitude ratio was calculated for each stage of differentiation by dividing the peak current evoked at +75 mV by the peak current evoked at −75 mV. The amplitude ratios were 3.75 ± 0.25 for NT2 cells (n = 3) and 1.37 ± 0.26 for 13 week NT2-N cells (n = 5). Amplitude ratios >1 indicated that there was outward rectification for cells from both stages of differentiation (Fig. 2, inset) (see Materials and Methods). In contrast, 5 week NT2-N cells showed no evidence of inward or outward rectification of peak current (Fig. 2, dashed line) as is illustrated by an amplitude ratio of 1.06 ± 0.12 (inset).

Pharmacology of GABAR currents in NT2 cells and 13 week NT2-N cells

Loreclezole

The novel anticonvulsant drug loreclezole has been shown to interact with a site on the β2 and β3 subtypes to enhance GABAR currents (Wafford et al., 1994). Loreclezole enhanced whole-cell GABAR currents recorded from NT2 cells and 13 week NT2-N cells in a concentration-dependent manner (Fig. 3A). Average normalized data (excluding the data point for 30 μM loreclezole) were fit with logistic equations with EC_{50} values of 1.0 μM for NT2 cells (n = 5) and 1.1 μM for 13 week NT2-N cells (n = 3) (Fig. 3B). Loreclezole (3 μM) maximally enhanced GABAR currents by 78.2 ± 24.8% in NT2 cells and by only 18 ± 7% in 13 week NT2-N cells. We reported previously that loreclezole enhanced GABAR currents from 5 week NT2-N cells in a concentration-dependent manner (EC_{50} of 1.2 μM) and 3 μM loreclezole increased currents by 133 ± 34% (Neelands et al., 1998).

Diazepam

The benzodiazepine diazepam only enhanced currents from recombinant GABARs containing a γ subunit (Pritchett et al., 1989a), and the relative diazepam EC_{50} varied with the a subtype expressed (Pritchett et al., 1989b). Diazepam enhanced whole-cell GABAR currents in 13 week NT2-N cells in a concentration-dependent manner (Fig. 4A). Concentration–response curves were obtained in three cells by coapplication of diazepam with 10 μM GABA and fitted with an EC_{50} of 27.9 nM. Diazepam maximally enhanced GABAR currents by 20 ± 12% of control at 1 μM diazepam (Fig. 4A). Similarly, 1 μM diazepam minimally enhanced currents evoked by 1 μM GABA in NT2 cells by 25 ± 8% (Fig. 4B). Full concentration–response curves for diazepam were not generated for NT2 cells because of the small maximal effect in combination with the small peak currents (~20
Enhancement of GABAR currents by 1 mM diazepam was significantly less in both the NT2 cells and 13 week NT2-N cells compared with the enhancement reported for GABAR currents in 5 week NT2-N cells (232 ± 34%).

Zolpidem

The imidazopyridine zolpidem is another GABAR benzodiazepine site agonist that has a higher affinity for GABARs containing an α1 subtype (BZ 1 receptors). GABARs containing α5 subtypes can be distinguished from other benzodiazepine-sensitive isoforms based on their insensitivity to zolpidem (BZ 2c receptors) (Pritchett and Seeburg, 1990). Zolpidem enhanced GABAR currents evoked by 10 μM GABA in 13 week NT2-N cells in a concentration-dependent manner (Fig. 5A). Zolpidem (3 μM) maximally enhanced GABAR currents by 23 ± 6%. Average normalized data were fit with a logistic equation with an EC50 of 455 nM (n = 3) (Fig. 5A). Coapplication of 1 mM zolpidem with 1 mM GABA to NT2 cells did not produce a significant effect (2 ± 11%) (Fig. 5B). As for diazepam, full concentration–response curves were not generated because the high zolpidem concentration (1 mM) did not alter GABAR currents. The ability of 1 μM zolpidem to enhance GABAR currents was significantly less in both the NT2 cells and 13 week NT2-N cells compared with that reported previously for 5 week NT2-N cells (98 ± 14%) (Neelands et al., 1998).

Lanthanum

The trivalent cation lanthanum (La3+) either potentiated (α1 subtype) or inhibited (α5 and α6 subtypes) GABAR currents depending on the α subtype composition of the receptor (Saxena et al., 1997; Neelands and Macdonald, 1998). Lanthanum increased peak whole-cell GABAR currents from 13 week NT2-N cells in a concentration-dependent manner (Fig. 6A). Concentration–response curves were generated for both NT2 cells and 13 week NT2-N cells by increasing the concentration of lanthanum (100 nM to 1 mM) coapplied with an EC equivalent concentration of GABA (Fig. 6B).
respectively) by coapplication of 1 mM lanthanum (La$^{3+}$) coapplied with 100 mM tetrodotoxin (TTX) to block Na$^+$ currents evoked from the 13 week NT2-N cells that were enhanced by 95% by 1 mM lanthanum. Lanthanum had a concentration-dependent inhibition of GABAR currents (10 or 30 μM GABA, respectively) by coapplication of either 3 or 30 μM zinc. The current traces from the NT2 cell was abnormally large but illustrated the inhibition by zinc better than the typical 20–40 pA currents. Horizontal bars, Drug applications. B, Concentration–response curves for the effects of lanthanum on NT2 cell GABAR currents (3 or 30 μM GABA, respectively) by coapplication of 1 mM lanthanum (La$^{3+}$). Horizontal bars, Drug applications. B, Concentration–response curves for the effects of lanthanum on NT2 cell GABAR currents. Lanthanum inhibited NT2 cell GABAR currents (IC$_{50}$ = 96.5 μM; $n_{H}$ = -0.7; $n$ = 2–3) and enhanced 13 week neuron NT2-N cell GABAR currents (EC$_{50}$ = 96.5 μM; $n_{H}$ = -0.7; $n$ = 2–3) and enhanced 13 week neuron NT2-N cell GABAR currents. The divalent cation zinc has been shown to be an antagonist of GABAR currents. Zinc reduced whole-cell GABAR currents from both NT2 cells (10 μM and 1.5 mM) and 13 week NT2-N cells (30 μM) (Fig. 7B). Average normalized data were plotted and fitted by a four-parameter logistic equation. The IC$_{50}$ for inhibition of GABAR currents was less for NT2 cells (IC$_{50}$ = 5.4 μM; $n_{H}$ = -0.8; $n$ = 2–4) than for 13 week NT2-N cells (IC$_{50}$ = 64.7 μM; $n_{H}$ = -0.4; $n$ = 3). NT2 cell GABAR currents were maximally inhibited by 100 μM zinc (96% of control); in contrast, currents evoked from the 13 week NT2-N cells that were enhanced by 95% by 1 mM lanthanum. 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Maximal inhibition of control currents from NT2 cells was 53.8 ± 5.7% at 1 mM furosemide and for 13 week NT2-N cells was 64.4 ± 4.0% at 3 mM furosemide (the solubility limit of furosemide in 0.1% DMSO). Furosemide had been shown previously to inhibit GABAR currents from 5 week NT2-N cells with a high IC50 (IC$_{50}$ = 1.4 mM; $n_{H}$ = -1.3) and a maximal inhibition at 3 mM furosemide of 78.2 ± 1.2% (Fig. 8B, open diamonds, dashed line).

The pharmacological properties of GABARs at these three stages of differentiation are summarized in Table 1.

**PCR analysis of GABAR subunit mRNAs in NT2 cells and 13 week NT2-N cells**

Total RNA was isolated from the same cultures of NT2 cells and 13 week NT2-N cells that were used for the electrophysiological studies. Reverse transcription (RT)-PCR was used to determine the presence or absence of GABAR subunit mRNAs using primers specific for human α1–6, β1–3, γ1–3, and π subunit subtypes.


Table 1. Summary of the pharmacology of NT2 cells and 5 and 13 week NT2-N cells

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<th>Stem cells</th>
<th>5 weeks of RA</th>
<th>13 weeks of RA</th>
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<tr>
<td><strong>GABA</strong></td>
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<td>Peak</td>
<td>52 ± 11 pA</td>
<td>472 ± 71 pA</td>
<td>2894 ± 504 pA</td>
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<td><strong>IC₅₀</strong></td>
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<td>4.0 µM</td>
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<td>Hill slope</td>
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<td><strong>Diazepam</strong></td>
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<td>Max (1 µM)</td>
<td>+25%</td>
<td>+232%</td>
<td>+20%</td>
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<td><strong>Zolpidem</strong></td>
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<td>Max (1 µM)</td>
<td>-2.5%</td>
<td>+93%</td>
<td>+20%</td>
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<td>EC₅₀</td>
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<td>455 nM</td>
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<td><strong>Loreclezole</strong></td>
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<tr>
<td>Max (3 µM)</td>
<td>+78%</td>
<td>+140%</td>
<td>+7%</td>
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<td>EC₅₀</td>
<td>-1 µM</td>
<td>1.2 µM</td>
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<td><strong>Zinc</strong></td>
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<tr>
<td>Max</td>
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<td>-83%</td>
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<td>Max (3 mM)</td>
<td>-54%</td>
<td>-80%</td>
<td>-60%</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>873 µM</td>
<td>1.4 mM</td>
<td>1.5 mM</td>
</tr>
<tr>
<td><strong>Lanthenum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max (1 mM)</td>
<td>-79.5%</td>
<td>No significant</td>
<td>+10%</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>137 µM</td>
<td></td>
<td>96.5 µM</td>
</tr>
</tbody>
</table>

Summary comparison of the pharmacological profile of NT2 cells and 5 and 13 week NT2-N cells. Maximal effect and EC₅₀/IC₅₀ are given for data in which complete concentration–response curves were acquired. Only single points for diazepam (1 µM) and zolpidem (1 µM) were obtained in NT2 cells.

DISCUSSION

**GABAR subunit subtype expression changed during differentiation of NT2 cells**

Untreated NT2 cells strongly expressed α₅, β₃, and π subtypes of mRNAs, and weakly expressed γ₂ subtype mRNA. The α₅, β₃, and γ₃ subtypes genes are clustered on human chromosome 15, whereas the π subunit gene is on chromosome 5, unassociated with any gene cluster. We reported previously that 5 week NT2-N cells expressed high levels of α₂, α₃, α₅, β₂, γ₁, and π subunit mRNAs (Neelands et al., 1998). In addition to the subtype mRNAs, among which were β₂, γ₁, and π subunit mRNAs, we also found faint bands for α₁, α₄, α₆, and γ₂ subtype mRNAs (Table 2). The emergence of the previously unexpressed α₂, β₂, and γ₂ GABAR subunit mRNAs after at least 10 weeks of retinoic acid treatment was illustrated in Figure 10 (RT-PCR of 10 week RNA shown) along with β₂ and γ₂ subtype mRNAs that remained throughout differentiation in these cultures. The change in expression of GABAR subunit mRNAs as a function of retinoic acid treatment was shown in Table 2 (including 5 week NT2-N cells that we reported previously).

**Changes in pharmacological properties associated with differentiation of NT2 cells**

The calculated half-maximal responses (EC₅₀ or IC₅₀) of the GABARs for allosteric modulators were evenly distributed around the mean for individual NT2 cells and 5 week NT2-N cells. In contrast, the effects of many of the allosteric modulators tested were more varied among individual 13 week NT2-N cells. This was clearly illustrated by furosemide that inhibited GABAR currents with two IC₅₀ values of ~600 µM and 2 µM when tested on four NT2-N cells from the same culture dish. In addition,
neurons; NT2 cells only had high levels of message NT2-N cells and the rightward shift in the GABA EC₅₀. The slight decrease in the diazepam and should have increased the affinity of modulation by benzodiazepines. NT2-N cells at the 5 week time point had high levels of a insensitive GABAR isoforms containing a subunit (Fisher and Macdonald, 1997). The increase in expression of the subunit mRNA; 11 week NT2-N cells could have been caused by increased ex- pression of subunit mRNA, low level expression of subunit mRNA; –, no detectable expression of subunit mRNA). NT2 cells only had high levels of message for α5, β3, and π and low levels of γ₂, which is consistent with the insensitivity to benzodiazepines. NT2-N cells at the 5 week time point had high levels of α₂, α₃, α₅, β₃, γ₃, and π and low levels of β₁, consistent with decreased zinc sensitivity and moderate benzodiazepine affinities. All subunit subtypes studied were found in the 13 week NT2-N cells and may be the reason for the variability in the pharmacological results.

Table 2. Summary of the GABAR subunit expression of NT2 cells and 5 and 13 week NT2-N cells

<table>
<thead>
<tr>
<th>Stem cells</th>
<th>5 weeks of RA</th>
<th>13 weeks of RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁</td>
<td>–</td>
<td>plus</td>
</tr>
<tr>
<td>α₂</td>
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</tr>
<tr>
<td>α₃</td>
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<tr>
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</tr>
<tr>
<td>α₅</td>
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<td>plus</td>
</tr>
<tr>
<td>α₆</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>β₁</td>
<td>–</td>
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<td>–</td>
<td>plus</td>
</tr>
<tr>
<td>β₃</td>
<td>plus</td>
<td>plus</td>
</tr>
<tr>
<td>γ₁</td>
<td>–</td>
<td>plus</td>
</tr>
<tr>
<td>γ₂</td>
<td>–</td>
<td>plus</td>
</tr>
<tr>
<td>γ₃</td>
<td>plus</td>
<td>plus</td>
</tr>
<tr>
<td>δ</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>π</td>
<td>plus</td>
<td>plus</td>
</tr>
<tr>
<td>ε</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

Comparison of the GABA receptor subunit subtypes identified by RT-PCR from mRNA isolated from NT2 cells and 5 and 13 week NT2-N cells (+, prominent expression of subunit mRNA; +, low level expression of subunit mRNA; –, no detectable expression of subunit mRNA). NT2-N cells only had high levels of message for α5, β3, and π and low levels of γ₂, which is consistent with the insensitivity to benzodiazepines. NT2-N cells at the 5 week time point had high levels of α₂, α₃, α₅, β₃, γ₃, and π and low levels of β₁, consistent with decreased zinc sensitivity and moderate benzodiazepine affinities. All subunit subtypes studied were found in the 13 week NT2-N cells and may be the reason for the variability in the pharmacological results.

there were large variations in the effects of diazepam on GABAR currents across the whole range of concentrations. Most of the pharmacological changes reported in 13 week NT2-N cells were likely caused by expression of all the GABAR subunit subtypes present in all RT-PCR reactions. The large number of GABAR subunits expressed at this stage of differentiation may also be responsible for the increased variability in responses of the 13 week cells to allosteric modulators. Expression of the α₁ subtype should have increased the affinity of modulation by benzodiazepine site ligands. The slight decrease in the diazepam and zolpidem EC₅₀ values for 13 week NT2-N cells compared with that for 5 week NT2-N cells may have been attributable therefore to incorporation of an α₁ subtype into the 13 week NT2-N cell GABARs. In addition, formation of benzodiazepine-insensitive GABAR isoforms containing α₄ and/or α₆ subtypes in 13 week NT2-N cells may be responsible for the decreased diazepam and zolpidem enhancement of GABAR currents. The expression of α₄ and α₆ subtypes may also have been responsible for the variability in furosemide inhibition of GABAR currents seen among individual 13 week NT2-N cells. Recombinant GABAR isoforms had a lower IC₅₀ for furosemide when either an α₄ or α₆ subtype was expressed (160 and 6 μM, respectively) compared with when other α subtypes were expressed (~1.5 mM). The much smaller effect of loreclezole on currents recorded from 13 week NT2-N cells could have been caused by increased expression of the β₁ subtype. When β₁ and β₃ subtypes were coexpressed in recombinant receptors, loreclezole sensitivity was similar to that in loreclezole-insensitive β₁ subtype–containing isoforms (Fisher and Macdonald, 1997). The increase in expression of γ₁ and γ₂ subtypes may have accounted for the increase in the IC₅₀ for zinc inhibition of GABAR currents in 13 week NT2-N cells and the rightward shift in the GABA EC₅₀. The calculated GABA EC₅₀ for recombinant GABARs, however, has been shown to depend on the α and β subunit subtypes and whether or not a γ, δ, ε, or π subunit was incorporated. Therefore, it was not possible to determine which subunit(s) produced the rightward shift in the GABA EC₅₀ of 13 week NT2-N cells compared with NT2 cells; however, the change occurred as the number of GABAR subunit subtype mRNAs increased and as the cellular morphology became more neuronal.

What GABAR isoform(s) was assembled in NT2 cells?

The subtype composition of NT2 cell GABARs could not be determined unambiguously by characterizing their pharmacological properties or by determining mRNA expression alone. However, the homogeneity of the pharmacological properties suggested a single isoform, and the mRNA expression pattern placed considerable constraints on the GABAR isoforms that could be expressed. Recombinant receptors expressed in mammalian cell lines typically require at least an α and a β subunit to form functional GABARs (Angelotti et al., 1993; Neelands et al., 1998). We have demonstrated that the π subunit can incorporate into recombinant GABARs composed of the α₅β₃π and α₅β₃γ₃π subunit combinations (Neelands and Macdonald, 1998). With the restrictions imposed by the limited number of subtype mRNAs expressed by NT2 cells, only four potential GABAR isoforms could likely be formed: α₅β₃, α₅β₃γ₃, α₅β₃π, and α₅β₃γ₃π. The limited number of possible isoforms allowed determination of the isoform(s) that was likely to be

Figure 9. RT-PCR reaction amplification of GABAR subunits in NT2 neuronal precursor cells. Lanes are designated as follows: M, marker; C, human cDNA for each subunit subtype used as a positive control; +, NT2-N RNA with Superscript II; and –, NT2-N RNA without Superscript II used as a negative control. A, Agarose gel of GABAR α subunits showing the presence of α5 but not α2 or 3 (2, lanes 2–4 (numbered from the left), α₃, lanes 5–7; α₅, lanes 8–10; molecular weight standards, lane 1). B, β and γ subunits showing major bands for β₃, only a faint band for γ₁, and no band for β₁ (β₁, lanes 2–4; β₃, lanes 5–7; γ₃, lanes 8–10; molecular weight standards, lane 1). C, Agarose gel showing a major band for the π subunit (π, lanes 2–4; molecular weight standards, lane 1). In some lanes bands of lower molecular mass than that predicted for the product of interest were stained that are nonspecific PCR amplification products (Bloch, 1991).
responsible for the electrophysiological properties of NT2 cell GABARs by comparing these results with those from recombinant GABARs. Identification of specific isoforms in most native neurons would be too complex because of the large number of possible GABAR isoforms.

Incorporation of the α5 subtype into NT2 cell GABARs was supported by inhibition of currents by lanthanum and insensitivity to zolpidem (Pritchett and Seeburg, 1990; Neelands and Macdonald, 1998). Functional expression of the β3 subtype along with the α5 subunit in NT2 cell GABARs was likely because GABAR currents were enhanced by loreclezole (Wafford et al., 1994) and β3 homomers were not activated by GABA (Wooltorton et al., 1997). Incorporation of both α5 and β3 subtypes was likely because all GABARs formed from α5, β3, γ2L, and π subtype combinations contained both α5 and β3 subtypes (Burgard et al., 1996; Neelands and Macdonald, 1998). The relative insensitivity of GABAR currents to enhancement by both diazepam and γ1, γ2, and γ3 (γ1, lanes 2–4; γ3, lanes 5–7; molecular weight standards, lanes 1 and 8).

Changes in GABAR subtype expression with differentiation of NT2 cells

The changes in the expression of GABAR subunit subtypes as a function of retinoic acid treatment were similar to the changes in subunit expression in the developing rat brain. Expression of α2, α3, α5, β3, and γ3 subtype mRNAs, whose genes are located within the chromosome 4, 15, and X clusters, was highest in perinatal rat brain and decreased in the adult brain (Zhang et al., 1991; Laurie et al., 1992; Pouler et al., 1992). In contrast, the level of expression for α1, α6, β2, and γ2 subtypes, whose genes are located in the chromosome 15 gene cluster, was minimally expressed early in development and reached maximal expression in the adult brain (Zhang et al., 1991; Laurie et al., 1992; Wisden et al., 1992). The changes in the expression of GABAR subunits during the differentiation of NT2 cells were similar to developmental changes in the rat brain. Although the NT2 cells undoubtedly did not follow the developmental pathway of any specific human neuron when grown in isolated cultures, the study of NT2 cells may provide useful information about the mechanisms underlying developmental regulation of GABAR subunits.

REFERENCES


Angelotti TP, Macdonald RL (1993) Assembly of GABA_A receptor subunits: α 1 β 1 and α 1 β 1 gamma 2S subunits produce unique ion channels with dissimilar single-channel properties. J Neurosci 13:1429–1440.


