GABA_A 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. Macdonald^{2,3}

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_A receptor subunit subtypes and GABA_A receptor pharmacological and biophysical properties. We used reverse transcription PCR and whole-cell-recording techniques to determine whether GABA_A 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 $\alpha 5$, $\beta 3$, $\gamma 3$, and π subtype mRNAs were detected. NT2 GABA_A receptor currents had a maximal amplitude of 52 pA and an EC₅₀ of 4.0 μ M, 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_A receptor subtype mRNAs were detected. Maximal peak whole-cell currents were ~50-fold larger than NT2 cell currents, and the

GABA EC₅₀ was higher (39.7 μ M). In 13 week NT2-N cells, diazepam, zolpidem, loreclezole, and lanthanum had only small effects on GABA_A receptor currents, and the zinc IC₅₀ 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 EC₅₀, and zinc IC₅₀ but that currents were robustly enhanced by diazepam, zolpidem, and loreclezole. During differentiation of NT2 cells to NT2-N cells, GABA_A receptors underwent changes in subunit expression and pharmacology that were similar to many of the developmental changes in GABA_A receptors that occur in CNS neurons.

Key words: GABA; GABA_A 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_A 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 ($\alpha 1$ – $\alpha 6$, $\beta 1$ – $\beta 4$, and $\gamma 1$ – $\gamma 4$) 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 ($\alpha 2$, $\alpha 4$, $\beta 1$, and $\gamma 1$), 5 ($\alpha 1$, $\alpha 6$, $\beta 2$, and $\gamma 2$), 15 ($\alpha 5$, $\beta 3$, and $\gamma 3$) (McLean et al., 1995; Rabow et al., 1995), and X ($\alpha 3$, ϵ , and $\beta 4$) (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-

Received Sept. 21, 1998; revised May 13, 1999; accepted June 2, 1999.

This research was supported by National Institutes of Health Grant RO1 NS33300 to R.L.M. We thank Dr. Ewen Kirkness for providing the π subunit cDNA.

Correspondence should be addressed to Dr. Robert L. Macdonald, Neuroscience Laboratory Building, 1103 East Huron Street, Ann Arbor, MI 48104-1687.

Dr. Neelands's present address: Vollum Institute, Oregon Health Sciences University, Portland, OR 97201.

Copyright © 1999 Society for Neuroscience 0270-6474/99/197057-09\$05.00/0

ical and pharmacological properties that were similar to those occurring during neuronal development.

MATERIALS AND METHODS

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 2×10^6 cells/75 cm² flask and differentiated by treatment with 1 μ M 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 μ M FUDR + 10 μ M uridine and 1 μ M 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 mOsm, with a pH of 7.4. Patch-clamp electrodes of 5–10 M Ω were filled with pipette solution containing (in mM): 153.33 KCl, 1 MgCl₂, 10 HEPES, 5 EGTA, and 2 ATP, 300–310 mOsm, 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 μ m 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. Stock solutions of loreclezole were dissolved in dimethylsulfoxide (DMSO) and diluted with sterile water (final DMSO concentration was less than 1:1000). Individual drugs were diluted in recording medium to their final concentration. Loreclezole was obtained from Janssen Biochimica (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 it as 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:

$$I = \frac{I_{\max}}{1 + 10^{(L \cdot \log_{10} C - L \cdot \log_{10} C_{50}) \cdot \text{Hill slope}}},$$

where I was the GABAR current at a given GABA concentration, and $I_{\max}$ was the maximal GABAR current. Maximal current and concen-

tration–response curves were obtained after pooling data from all cells tested for GABA and for all drugs. Concentration–response curves were also obtained from individual cells. The curve-fitting algorithm minimized the sum of the squares of the actual distance of points from the curve. Convergence 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 $\pm$ 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_{Cl} 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 Biotecx (Houston, TX). One milligram of total RNA was treated with DNase in a total volume of 10 ml composed of 1 ml of 10 $\times$ 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 then added to the mixture that was then incubated at 25°C for 15 min. Reverse transcription used the product of the above reaction mixed with the following: 1 ml of 5 $\times$ first-strand buffer, 2 ml of 0.1 M 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 procedure as 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 heat inactivated 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 mM primer mixture (3' primer and 5' primer), 2 ml of the reverse transcription product, 10 ml of 10 $\times$ 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 VI 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

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 $\pm$ 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 $\pm$ SE.

shift in the GABA EC_{50} between the NT2 cells ($\text{EC}_{50} = 4.0$ μM ; $n_H = 1.1$) and the 13 week NT2-N cells ($\text{EC}_{50} = 39.7$ μM ; $n_H = 1.3$). Included in the two plots are the data we reported previously for the 5 week NT2-N cells ($I_{\text{max}} = 472 \pm 71$ pA; $\text{EC}_{50} = 21.8$ 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 recti-

Figure 2. Whole-cell current–voltage relationships for NT2 cells and 5 and 13 week NT2-N cells. Peak GABAR, represented as mean $\pm$ SEM, was plotted against the holding potential (HP). *Inset*, Amplitude ratios were calculated by dividing the peak current at $+75$ mV by the peak current at -75 mV (see Materials and Methods). Larger amplitude ratios indicate greater outward rectification. Data represent mean $\pm$ SEM.

fication 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 β_2 and β_3 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 $78.2 \pm 24.8\%$ in NT2 cells and by only $18 \pm 7\%$ 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 $133 \pm 34\%$ (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 α subunit 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 $20 \pm 12\%$ of control at 1 μM diazepam (Fig. 4A). Similarly, 1 μM diazepam minimally enhanced currents evoked by 1 μM GABA in NT2 cells by $25 \pm 8\%$ (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

Figure 3. Enhancement of GABAR currents by loreclezole. *A*, Representative current traces from NT2 cells (*left*) and 13 week NT2-N cells (*right*) showing enhancement of GABAR currents (1 and 10 μ M GABA, respectively) by coapplication of 3 μ M loreclezole (LOR). Horizontal bars, Drug applications. *B*, Loreclezole concentration-response relationship for GABAR current enhancement of NT2 cells ($n = 5$), 5 week NT2-N cells ($n = 5$), and 13 week NT2-N cells ($n = 4$). Data are mean $\pm$ SEM.

Figure 4. Enhancement of GABAR currents by diazepam. *A*, Concentration-response curve for the enhancement by diazepam of currents evoked by 10 μ M GABA from 13 week NT2-N cells ($n = 3$). Data are mean $\pm$ SEM. *B*, Comparison of the ability of a saturating concentration of diazepam (1 μ M) to enhance GABAR currents from NT2 cells ($n = 3$), 5 week NT2-N cells ($n = 5$), and 13 week NT2-N cells ($n = 3$). Data are mean enhancement $\pm$ SEM.

pA). Enhancement of GABAR currents by 1 μ M 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 ($232 \pm 34\%$).

Figure 5. Enhancement of GABAR currents by zolpidem. *A*, Concentration-response curve for the enhancement by zolpidem of currents evoked by 10 μ M GABA from 13 week NT2-N cells ($n = 3$). Data are mean $\pm$ SEM. *B*, Comparison of the ability of a high concentration of zolpidem (1 μ M) to enhance GABAR currents from NT2 cells ($n = 3$), 5 week NT2-N cells ($n = 5$), and 13 week NT2-N cells ($n = 3$). Data are mean enhancement $\pm$ SEM.

Zolpidem

The imidazopyridine zolpidem is another GABAR benzodiazepine site agonist that has a higher affinity for GABARs containing an $\alpha 1$ subtype (BZ 1 receptors). GABARs containing $\alpha 5$ 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 μ M in Figure 5*A* GABA in 13 week NT2-N cells in a concentration-dependent manner (Fig. 5*A*). Zolpidem (3 μ M) maximally enhanced GABAR currents by $23 \pm 6\%$. Average normalized data were fit with a logistic equation with an EC_{50} of 455 nM ($n = 3$) (Fig. 5*A*). Coapplication of 1 μ M zolpidem with 1 μ M GABA to NT2 cells did not produce a significant effect ($-2 \pm 11\%$) (Fig. 5*B*). As for diazepam, full concentration-response curves were not generated because the high zolpidem concentration (1 μ M) did not alter GABAR currents. The ability of 1 μ M 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 ($98 \pm 14\%$) (Neelands et al., 1998).

Lanthanum

The trivalent cation lanthanum (La^{3+}) either potentiated ($\alpha 1$ subtype) or inhibited ($\alpha 5$ and $\alpha 6$ 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 but inhibited currents evoked from NT2 cells (Fig. 6*A*). 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.

Figure 6. Effects of lanthanum on GABAR currents. *A*, Current traces from NT2 cells (*left*) and 13 week NT2-N cells (*right*) showing concentration-dependent effects of 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 cells ($n = 2-3$), 5 week NT2-N cells ($n = 11$), and 13 week NT2-N cells ($n = 3$) on peak GABAR current amplitudes. Data are mean $\pm$ SEM.

6B). Average normalized data were plotted and fit with a four-parameter logistic equation (see Materials and Methods). Lanthanum inhibited NT2 cell GABAR currents ($\text{IC}_{50} = 137 \mu\text{M}$; $n_H = -0.7$; $n = 2-3$) and enhanced 13 week neuron NT2-N cell GABAR currents ($\text{EC}_{50} = 96.5 \mu\text{M}$; $n = 3$). GABAR currents from the NT2 cells were inhibited $79 \pm 4\%$ by 1 mM lanthanum in contrast to currents evoked from the 13 week NT2-N cells that were enhanced by $9 \pm 4\%$ by 1 mM lanthanum. Lanthanum had been shown previously to have no significant effect on GABAR currents from 5 week NT2-N cells (Fig. 6B, open diamonds, dashed line).

Zinc

The divalent cation zinc has been shown to be an antagonist of GABAR currents. Zinc reduced whole-cell GABAR currents from both NT2 cells and 13 week NT2-N cells in a concentration-dependent manner (Fig. 7A). Inhibitory concentration–response curves were obtained by increasing the concentration of zinc (100 nM to 1 mM) coapplied with a high concentration of GABA for NT2 cells (10 μM) 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 zinc inhibition of GABAR currents was less for NT2 cells ($\text{IC}_{50} = 5.4 \mu\text{M}$; $n_H = -0.8$; $n = 2-4$) than for 13 week NT2-N cells ($\text{IC}_{50} = 64.7 \mu\text{M}$; $n_H = -0.4$; $n = 3$). 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 were blocked only 85% by 1 mM zinc. Zinc has been shown previously to inhibit GABAR currents from 5 week NT2-N cells with a moderate IC_{50} of 14.7 μM ($n_H = -0.9$) and a maximal inhibition by 1 mM zinc of 95% (Fig. 7B, open diamonds, dashed line).

Figure 7. Inhibition of GABAR currents by zinc. *A*, Current traces from NT2 cells (*left*) and 13 week NT2-N cells (*right*) showing concentration-dependent inhibition of GABAR currents (10 or 30 μM GABA, respectively) by coapplication of either 3 or 30 μM zinc. The current trace 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 zinc on NT2 cells ($n = 2-4$), 5 week NT2-N cells ($n = 5$), and 13 week NT2-N cells ($n = 3$) on peak GABAR current amplitudes. Data are mean $\pm$ SEM.

Furosemide

The anthranilic acid derivative furosemide inhibited recombinant GABAR currents with apparent IC_{50} values in the micromolar range when an $\alpha 4$ or $\alpha 6$ subtype was expressed and in the millimolar range when other α subunits are present (Wafford et al., 1996). Furosemide inhibited GABAR currents evoked from both NT2 cells and 13 week NT2-N cells (Fig. 8A). Data were normalized to control currents, and the average data were fit with a logistic equation with apparent IC_{50} values of 873 μM ($n_H = -0.9$; $n = 3$) for NT2 cells and 1.5 mM ($n_H = -0.6$; $n = 4$) for 13 week NT2-N cells (Fig. 8B). In individual fits of the four 13 week NT2-N cells in which a full furosemide concentration response was obtained, there were two distinct populations of IC_{50} values ($\sim 600 \mu\text{M}$ and 1.5 mM). Maximal inhibition of control currents from NT2 cells was $53.8 \pm 5.7\%$ at 1 mM furosemide and for 13 week NT2-N cells was $64.4 \pm 4.0\%$ 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 IC_{50} ($\text{IC}_{50} = 1.4 \text{ mM}$; $n_H = -1.3$) and a maximal inhibition at 3 mM furosemide of $78.2 \pm 1.2\%$ (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 $\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, and π subunit subtypes

Figure 8. Inhibition of GABAR currents by furosemide. *A*, Current traces from NT2 cells (left) and 13 week NT2-N cells (right) showing concentration-dependent inhibition of GABAR currents (10 or 30 μ M GABA, respectively) by coapplication of 1 mM furosemide (FUR). Horizontal bars, Drug applications. *B*, Concentration–response curves for the effects of furosemide on NT2 cells ($n = 3$), 5 week NT2-N cells ($n = 3$), and 13 week NT2-N cells ($n = 3$) on peak GABAR current amplitudes. Data are mean $\pm$ SEM.

(Table 2) (Neelands et al., 1998). In repeated experiments with RNA isolated from NT2 cells, major bands were found for the $\alpha 5$, $\beta 3$, and π subtype, and a faint band was found for the $\gamma 3$ subtype (Fig. 9). No bands were found for $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\beta 1$, $\beta 2$, $\gamma 1$, and $\gamma 2$ subtype mRNAs (Table 2). The mRNA expression of GABAR subunits was qualitatively similar for both 10 and 13 week NT2-N cells; major bands were found for $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 2$, $\beta 3$, $\gamma 1$, $\gamma 3$, and π subtype mRNAs, whereas faint bands were found for $\alpha 1$, $\alpha 4$, $\alpha 6$, $\beta 1$, and $\gamma 2$ subtype mRNAs (Table 2). The emergence of the previously unexpressed $\alpha 4$, $\beta 1$, and $\gamma 1$ GABAR subtype mRNAs after at least 10 weeks of retinoic acid treatment was illustrated in Figure 10 (RT-PCR of 10 week RNA shown) along with $\beta 3$ and $\gamma 3$ 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).

DISCUSSION

GABAR subunit subtype expression changed during differentiation of NT2 cells

Untreated NT2 cells strongly expressed $\alpha 5$, $\beta 3$, and π subtype mRNAs and weakly expressed $\gamma 3$ subtype mRNA. The $\alpha 5$, $\beta 3$, and $\gamma 3$ subtype 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 $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 3$, $\gamma 3$, and π subunit mRNAs (Neelands et al., 1998). In addition to the subtype mRNAs whose genes are located on chromosome 15 ($\alpha 5$, $\beta 3$, and $\gamma 3$ subtypes), some mRNAs were present in the 5 week NT2-N cells from genes located on human chromosomes 4 ($\alpha 2$ subtype) and X ($\alpha 3$ subtype). Conspicuously absent, however, were subtype mRNAs whose genes are in the chromosome 5 gene cluster ($\alpha 1$, $\alpha 6$,

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

	Stem cells	5 weeks of RA	13 weeks of RA
GABA			
Peak	52 $\pm$ 11 pA	472 $\pm$ 71 pA	2894 $\pm$ 504 pA
EC ₅₀	4.0 μ M	21.8 μ M	39.7 μ M
Hill slope	1.1	1.2	1.3
Diazepam			
max (1 μ M)	+25%	+232%	+20%
EC ₅₀		74.2 nM	27.9 nM
Zolpidem			
max (1 μ M)	–2.5%	+93%	+20%
EC ₅₀		527 nM	455 nM
Loreclezole			
max (3 μ M)	+78%	+140%	+7%
EC ₅₀	$\sim$ 1 μ M	1.2 μ M	$\sim$ 1 μ M
Zinc			
max	–96%	–95%	–83%
IC ₅₀	5.4 μ M	14.7 μ M	64.7 μ M
Furosemide			
max (3 mM)	–54%	–80%	–60%
IC ₅₀	873 μ M	1.4 mM	1.5 mM
Lanthanum			
max (1 mM)	–79.5%	No significant effect	+10%
EC ₅₀			96.5 μ M
IC ₅₀	137 μ M		

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 μ M) and zolpidem (1 μ M) were obtained in NT2 cells.

$\beta 2$, or $\gamma 2$ subtypes). RT-PCR of total RNA isolated from 13 week NT2-N cells amplified mRNA for all α , β , γ , and π subunit subtypes.

Cultures of NT2-N cells after 5 weeks of retinoic acid treatment were reported to be 99% pure (Pleasure et al., 1992) with minimal contamination by NT2 cells. The time point at which an individual NT2 cell begins differentiation during the 4 weeks of retinoic acid treatment, before replating, is unknown. Therefore, it is possible that persistence of some NT2 cells and the potential difference in “age” of NT2-N cells at the 5 and 13 week time points may contribute to the high level of expression of the subtypes found in “immature” cells ($\alpha 5$, $\beta 3$, and π) in RNA preparations from these cultures. The results of RT-PCR experiments alone could not determine whether all the GABAR subunits were expressed in individual cells, whether there was a subset of younger NT2-N cells contaminating the RNA, or whether during differentiation separate populations of NT2-N cells with different GABAR subunit expression patterns emerged.

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 $\sim$ 600 μ M and 2 mM when tested on four NT2-N cells from the same culture dish. In addition,

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

	Stem cells	5 weeks of RA	13 weeks of RA
$\alpha 1$	—	—	+
$\alpha 2$	—	++	++
$\alpha 3$	—	++	++
$\alpha 4$	—	—	+
$\alpha 5$	++	++	++
$\alpha 6$	—	—	+
$\beta 1$	—	+	+
$\beta 2$	—	—	++
$\beta 3$	++	++	++
$\gamma 1$	—	—	++
$\gamma 2$	—	—	+
$\gamma 3$	+	++	++
δ	?	?	?
π	++	++	?
ϵ	?	?	?

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 cells only had high levels of message for $\alpha 5$, $\beta 3$, and π and low levels of $\gamma 3$, which is consistent with the insensitivity to benzodiazepines. NT2-N cells at the 5 week time point had high levels of $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 3$, $\gamma 3$, and π and low levels of $\beta 1$, 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 $\alpha 1$ subtype should have increased the affinity of modulation by benzodiazepine site ligands. The slight decrease in the diazepam and zolpidem EC_{50} 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 $\alpha 1$ subtype into some of the 13 week NT2-N cell GABARs. In addition, formation of benzodiazepine-insensitive GABAR isoforms containing $\alpha 4$ and/or $\alpha 6$ subtypes in 13 week NT2-N cells may be responsible for the decreased diazepam and zolpidem enhancement of GABAR currents. The expression of $\alpha 4$ and $\alpha 6$ 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 IC_{50} for furosemide when either an $\alpha 4$ or $\alpha 6$ 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 $\beta 1$ subtype. When $\beta 1$ and $\beta 3$ subtypes were coexpressed in recombinant receptors, loreclezole sensitivity was similar to that in loreclezole-insensitive $\beta 1$ subtype-containing isoforms (Fisher and Macdonald, 1997). The increase in expression of $\gamma 1$ and $\gamma 2$ subtypes may have accounted for the increase in the IC_{50} for zinc inhibition of GABAR currents in 13 week NT2-N cells and the rightward shift in the GABA EC_{50} . The calculated GABA EC_{50} for recombinant GABARs, however, has

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 $\alpha 5$ but not $\alpha 2$ or $\alpha 3$ [$\alpha 2$, lanes 2–4 (numbered from the left); $\alpha 3$, lanes 5–7; $\alpha 5$, lanes 8–10; molecular weight standards, lane 1]. *B*, β and γ subunits showing major bands for $\beta 3$, only a faint band for $\gamma 3$, and no band for $\beta 1$ ($\beta 1$, lanes 2–4; $\beta 3$, lanes 5–7; $\gamma 3$, 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).

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 EC_{50} 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 $\alpha 5\beta 3\pi$ and $\alpha 5\beta 3\gamma 3\pi$ 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: $\alpha 5\beta 3$, $\alpha 5\beta 3\gamma 3$, $\alpha 5\beta 3\pi$, and $\alpha 5\beta 3\gamma 3\pi$ GABARs. The limited number of possible isoforms allowed determination of the isoform(s) that was likely to be

Figure 10. RT-PCR reaction amplification of GABAR subunits in 10–13 week NT2-N cells. Total RNA was isolated from NT2-N cells treated with retinoic acid for 10–13 weeks. RT-PCR amplified all the GABAR subunit mRNAs tested at these developmental stages. No changes in the subunit subtype expression pattern were detected over this time period (results from 10 week NT2-N cells are shown). 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. *Top*, Agarose gel of GABAR α and β subunits showing only faint bands for $\alpha 4$, $\beta 1$, and $\beta 3$ [$\alpha 4$, lanes 2–4 (numbered from the left); $\beta 1$, lanes 7–9; $\beta 3$, lanes 10–12; molecular weight standards, lanes 1, 5, 6, and 13]. *Bottom*, γ subunits showing bands for $\gamma 1$ and $\gamma 3$ ($\gamma 1$, lanes 2–4; $\gamma 3$, lanes 5–7; molecular weight standards, lanes 1 and 8).

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 $\alpha 5$ 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 $\beta 3$ subtype along with the $\alpha 5$ subunit in NT2 cell GABARs was likely because GABAR currents were enhanced by loreclezole (Wafford et al., 1994) and $\beta 3$ homomers were not activated by GABA (Wooltorton et al., 1997). Incorporation of both $\alpha 5$ and $\beta 3$ subtypes was likely because all GABARs formed from $\alpha 5$, $\beta 3$, $\gamma 2L$, and π subtype combinations contained both $\alpha 5$ and $\beta 3$ subtypes (Burgard et al., 1996; Neelands and Macdonald, 1998). The relative insensitivity of GABAR currents to enhancement by both diazepam and zolpidem suggested that although low levels of $\gamma 3$ subtype mRNA were detected, the $\gamma 3$ subtype did not assemble into the major GABAR isoform expressed by NT2 cells. Recombinant GABAR currents containing the π subunit were insensitive to benzodiazepine site ligands and had a low zinc IC_{50} but did not display as much outward rectification as NT2 cell currents (Neelands and

Macdonald, 1998). Therefore, the large degree of outward rectification of whole-cell currents, the relative insensitivity to benzodiazepine site ligands, and the low zinc IC_{50} suggest that the $\alpha 5\beta 3$ isoform was the predominant GABAR isoform expressed by NT2 cells. In addition, preliminary results showed the single-channel conductance of NT2 cell GABARs (~ 15 pS; data not shown) was more similar to that of the recombinant $\alpha 5\beta 3$ isoform (15.2 pS) than to that of the $\alpha 5\beta 3\pi$ (23.8 pS) or $\alpha 5\beta 3\gamma 3$ (26.9 pS) isoforms (Neelands and Macdonald, 1998). These differences suggested that although the π subunit was highly expressed, it was not incorporated into NT2 cell GABARs. These data do not rule out the possibility that other isoforms were also assembled and composed a small fraction of the functional receptors on the cell surface.

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 $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 3$, and $\gamma 3$ 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; Poulter et al., 1992). In contrast, the level of expression for $\alpha 1$, $\alpha 6$, $\beta 2$, and $\gamma 2$ 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

- Andrews PW (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. *Dev Biol* 103:285–293.
- Andrews PW (1998) Teratocarcinomas and human embryology: pluripotent human EC cell lines. Review article [84 refs]. *APMIS* 106:158–167.
- Angelotti TP, Macdonald RL (1993) Assembly of GABA_A receptor subunits: $\alpha 1 \beta 1$ and $\alpha 1 \beta 1 \gamma 2S$ subunits produce unique ion channels with dissimilar single-channel properties. *J Neurosci* 13:1429–1440.
- Angelotti TP, Uhler MD, Macdonald RL (1993) Assembly of GABA_A receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J Neurosci* 13:1418–1428.
- Bloch W (1991) A biochemical perspective of the polymerase chain reaction. *Biochemistry* 30:2735–2747.
- Burgard EC, Tietz EI, Neelands TR, Macdonald RL (1996) Properties of recombinant gamma-aminobutyric acid A receptor isoforms containing the $\alpha 5$ subunit subtype. *Mol Pharmacol* 50:119–127.
- Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant GABA_A receptor. *J Neurosci* 16:5415–5424.
- Davies PA, Hanna MC, Hales TG, Kirkness EF (1997) Insensitivity to anesthetic agents conferred by a class of GABA_A receptor subunit. *Nature* 385:820–823.
- Fisher JL, Macdonald RL (1997) Functional properties of recombinant GABA(A) receptors composed of single or multiple β subunit subtypes. *Neuropharmacology* 36:1601–1610.
- Fritschy JM, Paysan J, Enna A, Mohler H (1994) Switch in the expression of rat GABA_A-receptor subtypes during postnatal development: an immunohistochemical study. *J Neurosci* 14:5302–5324.
- Greenfield LJJ, Macdonald RL (1996) Whole-cell and single-channel $\alpha 1 \beta 1 \gamma 2S$ GABA_A receptor currents elicited by a “multipuffer” drug application device. *Pflügers Arch* 432:1080–1090.

- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391:85–100.
- Hedblom E, Kirkness EF (1997) A novel class of GABA_A receptor subunit in tissues of the reproductive system. *J Biol Chem* 272:15346–15350.
- Kapur J, Macdonald RL (1996) Pharmacological properties of gamma-aminobutyric acid_A receptors from acutely dissociated rat dentate granule cells. *Mol Pharmacol* 50:458–466.
- Laurie DJ, Wisden W, Seeburg PH (1992) The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* 12:4151–4172.
- Macdonald RL, Olsen RW (1994) GABA_A receptor channels. *Annu Rev Neurosci* 17:569–602.
- McLean PJ, Farb DH, Russek SJ (1995) Mapping of the $\alpha 4$ subunit gene (GABRA4) to human chromosome 4 defines an $\alpha 2-\alpha 4-\beta 1$ -gamma 1 gene cluster: further evidence that modern GABA_A receptor gene clusters are derived from an ancestral cluster. *Genomics* 26:580–586.
- Neelands TR, Macdonald RL (1998) Incorporation of the π -subunit into GABA_A receptors. *Soc Neurosci Abstr* 24:103.
- Neelands TR, Greenfield LJJ, Zhang J, Turner RS, Macdonald RL (1998) GABA_A receptor pharmacology and subtype mRNA expression in human neuronal NT2-N cells. *J Neurosci* 18:4993–5007.
- Neelands TR, Fisher J, Bianchi M, Macdonald RL (1999) Spontaneous and γ -aminobutyric acid (GABA)-activated GABA_A receptor channels formed by ϵ subunit-containing isoforms. *Mol Pharmacol* 55:168–178.
- Oh KS, Lee CJ, Gibbs JW, Coulter DA (1995) Postnatal development of GABA_A receptor function in somatosensory thalamus and cortex: whole-cell voltage-clamp recordings in acutely isolated rat neurons. *J Neurosci* 15:1341–1351.
- Pleasure SJ, Page C, Lee VM (1992) Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. *J Neurosci* 12:1802–1815.
- Poulter MO, Barker JL, O'Carroll AM, Lolait SJ, Mahan LC (1992) Differential and transient expression of GABA_A receptor α -subunit mRNAs in the developing rat CNS. *J Neurosci* 12:2888–2900.
- Pritchett DB, Seeburg PH (1990) Gamma-aminobutyric acid_A receptor $\alpha 5$ -subunit creates novel type II benzodiazepine receptor pharmacology. *J Neurochem* 54:1802–1804.
- Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989a) Importance of a novel GABA_A receptor subunit for benzodiazepine pharmacology. *Nature* 338:582–585.
- Pritchett DB, Luddens H, Seeburg PH (1989b) Type I and type II GABA_A-benzodiazepine receptors produced in transfected cells. *Science* 245:1389–1392.
- Rabow LE, Russek SJ, Farb DH (1995) From ion currents to genomic analysis: recent advances in GABA_A receptor research [review; 675 refs]. *Synapse* 21:189–274.
- Sah DW (1995) Human fetal central neurons in culture: voltage- and ligand-gated currents. *J Neurophysiol* 74:1889–1899.
- Saxena NC, Macdonald RL (1994) Assembly of GABA_A receptor subunits: role of the delta subunit. *J Neurosci* 14:7077–7086.
- Saxena NC, Neelands TR, Macdonald RL (1997) Contrasting actions of lanthanum on different recombinant gamma-aminobutyric acid_A receptor isoforms expressed in L929 fibroblasts. *Mol Pharmacol* 51:328–335.
- Sommer B, Poustka A, Spurr NK, Seeburg PH (1990) The murine GABA_A receptor delta-subunit gene: structure and assignment to human chromosome 1. *DNA Cell Biol* 9:561–568.
- Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant GABA_A receptor subtype. *J Neurosci* 17:2728–2737.
- Wafford KA, Bain CJ, Quirk K, McKernan RM, Wingrove PB, Whiting PJ, Kemp JA (1994) A novel allosteric modulatory site on the GABA_A receptor β subunit. *Neuron* 12:775–782.
- Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS, Whiting PJ (1996) Functional characterization of human gamma-aminobutyric acid_A receptors containing the $\alpha 4$ subunit. *Mol Pharmacol* 50:670–678.
- Wilke K, Gaul R, Klauck SM, Poustka A (1997) A gene in human chromosome band Xq28 (GABRE) defines a putative new subunit class of the GABA_A neurotransmitter receptor. *Genomics* 45:1–10.
- Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992) The distribution of 13 GABA_A receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J Neurosci* 12:1040–1062.
- Wooltorton JR, Moss SJ, Smart TG (1997) Pharmacological and physiological characterization of murine homomeric $\beta 3$ GABA(A) receptors. *Eur J Neurosci* 9:2225–2235.
- Zhang JH, Sato M, Tohyama M (1991) Region-specific expression of the mRNAs encoding β subunits ($\beta 1$, $\beta 2$, and $\beta 3$) of GABA_A receptor in the rat brain. *J Comp Neurol* 303:637–657.