Journal of Neuroscience Research 76:801–811 (2004)

Role of γ-Aminobutyric Acid in Early Neuronal Development: Studies With an Embryonic Neuroectodermal Stem Cell Clone

M. Jelitai,[1]* M. Anděrová,[2] K. Markó,[1] K. Kékesi,[3] P. Koncz,[1] E. Syková,[2] and E. Madarász[1]

[1]Department of Neural Cell Biology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary

[2]Department of Neuroscience, Institute of Experimental Medicine, ASCR and Second Medical Faculty, Charles University, Prague, Czech Republic

[3]Hungarian Academy of Sciences Neurobiology Research Group of Eötvös Loránd University, Budapest, Hungary

γ-Aminobutyric acid (GABA) has been known to function as an autocrine/paracrine signal molecule in addition to its well-known inhibitory neurotransmitter function. Studies on the developing brain and on primary brain cell cultures provided evidence for a variety of GABA functions in periods preceding the formation of synapses. The exact role of GABA in the early neural development, however, is still not well understood. In this study, one-cell-derived NE-4C neuroectodermal stem cells were induced to form neurons and astrocytes in vitro, and the role of GABA was investigated in defined phases of neurogenesis. Noninduced NE-4C cells contained GABA, expressed GABA(A)R α subunits, and carried functional GABA(A) ion channels. A moderate cytoplasmic GABA content was detected during the entire period of differentiation. By the time of the formation of differentiated neurons, neuron-like cells with both high and low GABA content were clearly distinguishable. HPLC analysis indicated that NE-4C cells released GABA into their fluid environment during all stages of neuronal development. By using the patch-clamp technique, GABA-evoked currents were recorded during the entire proliferation/differentiation period, whereas a GABA-evoked increase in intracellular Ca^{2+} was detected only during the maturation of postmitotic neuronal precursors. Bicuculline blocked both the ion currents and the $[Ca^{2+}]_i$ increase in response to GABA. Neuron formation was facilitated by GABA through GABA(A) ion channels during postmitotic differentiation, but not earlier during the phases of cell fate commitment. Although the data clearly demonstrate an early responsiveness to GABA, understanding the significance of GABA influence in early neural cell fate decisions will require further investigation.

Key words: neural stem cell; neuronal development; nonsynaptic GABA; GABA-evoked current; calcium response

Studies performed on a wide range of preparations, both in vivo and in vitro, suggest that the classical neurotransmitters γ-aminobutyric acid (GABA) and glutamate are operative well before the formation of synapses. GABA serves as a development-regulatory signal in the genesis of neural tissues (Meier et al., 1991; Barker et al., 1998; Nguyen et al., 2001; Ben-Ari, 2002; Owens and Kriegstein, 2002). Components of the GABA signalling system, such as GABA-synthesizing enzyme, GAD, and GABA in the cytosol, appear very early in development (Barker et al., 1998). The earliest point when transcripts of the glutamic acid decarboxylase 1 (GAD1) gene, coding for GAD67 in adults, were detected was the ninth day after conception in nonneural regions of mouse embryos (Maddox and Condie, 2001). The embryonic forms of GAD67 (67 kD), two alternatively spliced truncated forms (44 kD and 25 kD), are expressed in mouse brain as early as the 10.5–12.5 embryonic days (E10.5–12.5; Szabó et al., 1994; Katarova et al., 2000).

Contract grant sponsor: Hungarian National Science Foundation (OTKA); Contract grant number: T 034692; Contract grant sponsor: Central Technological Development Program; Contract grant number: OMFB-00203/2002; Contract grant number: AV0Z5039906; Contract grant sponsor: European Commission Centre of Excellence Program; Contract grant number: ICA1-CT-200070028; Contract grant sponsor: Grant Agency of the Czech Republic; Contract grant number: GAÉR 305/02/1528; Contract grant number: GAÉR 305/03/1172; Contract grant number: MŠMT LN00A065; Contract grant number: J13/98:111300004.

*Correspondence to: Márta Jelitai, Institute of Experimental Medicine of the Hungarian Academy of Sciences, 1083 Budapest, Szigony u. 43, Hungary. E-mail: jelitai@koki.hu

Received 19 November 2003; Revised 26 January 2004; Accepted 29 January 2004

Published online 12 April 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20106

The presence of immunocytochemically detectable GABA has been demonstrated from the very beginning of neocortical development in rodents. GABA-containing neurons were detected in subpopulations of Cajal-Retzius cells, in cells of the intermediate zone (Lavdas et al., 1999), and in the developing striatum at E14 (Fiszman et al., 1993). Functional GABA(A) receptors and endogenous GABA(A) receptor activation were shown in the proliferative zone (VZ) of the murine telencephalon at E13–14 and from E16 in rat (Lo Turco and Kriegstein, 1991; Lo Turco et al., 1995; Haydar et al., 2000; Maric et al., 2001).

In the germinative areas of the developing CNS and in the developmental stages preceding the formation of synaptic contacts, GABA acts as an autocrine, paracrine signal mediator. The mechanisms underlying the regulatory function(s) of nonsynaptic GABA have been thoroughly studied but are not well understood. In defined phases of neuronal maturation, GABA causes depolarization (Cherubini et al., 1991) due to a relatively high concentration of intracellular Cl^- (Owens et al., 1996; Clayton et al., 1998). The depolarization, via the opening of GABA-gated Cl^- channels, increases $[Ca^{2+}]_i$ (Yuste and Katz, 1991), which in turn may elicit multiple cellular responses. It has been shown that GABA-induced depolarization regulates the proliferation of neural progenitors (LoTurco et al., 1995; Haydar et al., 2000), the migration (Behar et al., 1998) and differentiation of postmitotic neuronal precursors (Barbin et al., 1993; Ganguly et al., 2001), the elongation of neurites (Maric et al., 2001), and the activity-dependent formation and stabilization of synapses (Ben-Ari, 2002). Some data indicate that GABA can influence the development of neural progenitor cells via brain-derived neurotrophic factor (BDNF) expression (Berninger and Poo, 1995; Marty et al., 1996; Obrietan et al., 2002). The mechanisms of GABA action in the early stages of neuronal cell fate determination, however, are not well understood.

Neural progenitor cells from the early embryonic stages are not available for in vitro cell biological and molecular studies. Primary cultures established from embryonic brains contain a heterogeneous population of neural progenitors at various stages of differentiation. For in vitro studies of cell fate commitment and early neuronal differentiation, single-cell-derived clones of neuroectodermal progenitor cells can provide appropriate models.

The aim of the present study was to investigate the GABA content, the presence of GABA receptors, and the role of GABA in the course of in-vitro-induced neuronal differentiation of NE-4C embryonic neural stem cells. The NE-4C cell line (Schlett and Madarász, 1997), derived from E9 mouse embryos lacking functional p53, gives rise to neurons by the sixth and astrocytes by the eighth to tenth days of induction by all-*trans* retinoic acid (RA). Oligodendrocyte-specific characteristics could not be revealed in the 2-week periods of investigations, but the development of oligodendrocytes has not been specifically addressed. Detailed studies on the morphological maturation and changes in expression of intermediate filaments and neuron-specific markers, together with analyses on the development of bioelectrical properties, revealed a highly reproducible scheme of in vitro neurogenesis and led to an appropriate model for in vitro studies of neuron formation (Schlett et al., 2000; Herberth et al., 2002; Jelitai et al., 2002; Tárnok et al., 2002).

MATERIALS AND METHODS

Maintenance and Neural Differentiation of NE-4C Cells

The NE-4C cell line (Schlett and Madarász, 1997) was derived from E9 mouse embryo and was maintained in minimum essential medium (MEM; Sigma-Aldrich Hungary) supplemented with 5% fetal calf serum (FCS; Gibco-BRL Life Technologies, Paisley, United Kingdom), 4 mM glutamine (Sigma-Aldrich), and 40 μg/ml gentamycin (Chinoin Hungary) at 37°C with 5% CO_2. Subconfluent cultures were regularly split by trypsinization with 0.05% trypsin (Sigma-Aldrich) in phosphate-buffered saline (PBS) and transferred into poly-L-lysine (PLL; Sigma-Aldrich)-coated dishes at a cell density of 10^4 cells/cm^2. For neural induction, NE-4C cells were plated at a density of 2.5×10^4 cells/cm^2 and were incubated with all-*trans* RA acid (10^{-7} M) for 48 hr after plating.

Estimation of Cell Number

Cell numbers were estimated according to the method of Mosmann (1983). The cultures were incubated with 3-(4,5 dimethylthiasole-2-il)-2,5-diphenyltetrazolium [MTT (Sigma); at a final concentration of 0.25 mg/ml] at 37°C for 1 hr. The cells and the reaction products were dissolved in acidic (0.08 M HCl) isopropyl alcohol, and the optical densities (OD) were determined by using an SLT-210 ELISA reader at dual wavelengths of 570 and 630 nm. Viability assays were carried out on six to eight identically treated sister cultures, and means and standard deviations were calculated.

Immunocytochemistry

For immunocytochemistry, cells were grown on poly-L-lysine-coated glass coverslips. Cells were fixed with a fixative containing 4% paraformaldehyde (TAAB Laboratories) and 0.1% glutaraldehyde (Sigma) for 10 min at room temperature (RT), then permeabilized with 0.1% Triton X-100 (Sigma) for 5 min. After 1 hr of blocking with 10% FCS, cultures were incubated with primary antibodies overnight at 4°C. Primary antibodies were diluted as follows: polyclonal anti-GABA (Sigma) 1/1,000, anti-IIIβ-tubulin (Exbio, Prague, Czech Republic) 1/1,000, anti-NeuN 1/2,000 (Chemicon, Temecula, CA), anti-GABA(A)R α subunit 1/1,000 (Chemicon). Biotin-conjugated anti-mouse or anti-rabbit Igs (Sigma) were used at a dilution of 1/1,000. Fluorescent-labeled anti-mouse and anti-rabbit antibodies (IgG-FITC) and streptavidin-TRITC (Sigma) were diluted 1/500 and applied for 1 hr at RT. Streptavidin-peroxidase was diluted 1/1,000 and was added for 1 hr, followed by incubation with 3,3′-diaminobenzidine (0.55 mg/ml) in the presence of 0.3% H_2O_2 for 20 min.

Estimation of the Average GABA Content in the Cultures by In Situ ELISA

The relative amount of GABA was determined on fixed microcultures by using in situ ELISA, as described by Doherty et

al. (1984), with slight modifications (Madarasz, 1987). For in situ ELISA, cells grown and induced on 96-well plates were fixed with a fixative containing 4% paraformaldehyde (TAAB Laboratories) and 0.1% glutaraldehyde (Sigma) in PBS for 10 min at RT, then permeabilized with 0.1% Triton X-100 (Sigma) for 5 min. After blocking of nonspecific binding sites with 10% FCS (in PBS) for 1 hr, rabbit anti-GABA (Sigma) diluted 1/1,000 was applied overnight at 4°C. After a 1-hr incubation with peroxidase-labeled anti-rabbit IgG (Sigma), the cultures were washed once with 25 mM citrate buffer (pH 5.0), and the peroxidase reaction was developed with o-phenylenediamine (0.8 mg/ml in citrate buffer; Reanal) in the presence of 0.03% H_2O_2. The reaction was stopped after 30 min with an equal volume of 4.5 M H_2SO_4. The light absorption was measured at wavelengths of 492 and 405 nm with an SLT 210 ELISA reader. The optical densities of GABA-ELISA were obtained from four to six identically treated sister cultures and were normalized by dividing the MTT OD. The normalized GABA content was determined 0, 2, 4, 6, 8, and 10 days after RA induction in four independent series of inductions.

Measurement of Intracellular Free Calcium ($[Ca^{2+}]_i$) Levels in Single Cells

NE-4C cells grown on PLL-coated glass coverslips in 60-mm dishes were washed with a physiological solution containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose (pH 7.2) and loaded with 5 μM Fura-2/AM (Molecular Probes, Eugene, OR) for 45 min at RT. At the end of the incubation, the excess Fura-2/AM was removed by washing, and the cultures were further incubated for 30 min to complete the hydrolysis of the Fura ester. Single-cell fluorescence evoked by excitation at 340 nm or 380 nm was measured at 510 nm with a PTI Ca-imaging system. Thirty milimolar KCl, 100 μM GABA, or 50 μM bicuculline (all from Sigma) was perfused at a 1 ml/1iter min flow rate for 30 sec and washed afterward for at least 3 min. Data were evaluated by using the IMPTI software provided by PTI.

Patch-Clamp Recordings

GABA-evoked currents were recorded by using the patch-clamp technique in the whole-cell configuration (Hamill et al., 1981). Recording pipettes with a tip resistance of 4–6 MΩ were made from borosilicate capillaries (Ruckl & Sons, Otvovice, Czech Republic) with a Brown-Flaming micropipette puller (P-97; Sutter Instruments Company, Novato, CA). Electrodes were filled with a solution containing (in mM) KCl 130, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 10. The pH was adjusted with KOH to 7.2. To visualize the recorded cells, the intracellular solution contained Alexa-Fluor hydrazid 594 (Molecular Probes). The extracellular solution contained (in mM) NaCl 117, KCl 3, $CaCl_2$ 1.5, $MgCl_2$ 1.3, Na_2HPO_4 1.25, $NaHCO_3$ 35, D-glucose 10, osmolality 300 mmol/kg. The solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2 to maintain a final pH of 7.4 (Chvátal and Kettenmann, 1991). The following substances were added to the extracellular solution in the concentrations indicated in the text: GABA (Sigma), bicuculline (Sigma), and muscimol (Sigma). The cells were approached with the patch electrode by using an Infrapatch system (Luigs & Neumann, Ratingen, Germany). PLL-coated coverslips with attached cells were placed in a chamber mounted on the stage of a fluorescence microscope (Axioskop FX; Carl Zeiss, Jena, Germany) and were continuously perfused with oxygenated extracellular solution at the rate of 3 ml/min. All experiments were carried out at 30°C. The cells and the recording electrode were imaged with a digital microscope camera (AxioCam; Carl Zeiss). Current signals were amplified with an EPC-9 amplifier (Heka Elektronik, Lambrecht/Pfalz, Germany), low-pass filtered at 3 kHz, and sampled at 5 kHz by an interface connected to an AT-compatible computer system, which also served as a stimulus generator. Data acquisition, storage, and analysis were performed with TIDA (Heka Elektronik).

RESULTS

The GABA content and the distribution of GABA-containing cells in cultures of NE-4C cells were monitored throughout a period of in-vitro-induced neural differentiation from a noncommitted neuroectodermal progenitor stage to the formation of morphologically developed neurons. This in vitro model, previously characterized by Schlett et al. (1997), Herberth et al. (2002), and Jelitai et al. (2002), allows us to correlate the data with distinct stages of neuronal determination.

GABA Is Present in All Neural Progenitors in the Phase of Early Neuronal Commitment

In noninduced NE-4C progenitor cells, a moderate and uniform GABA content was detected (Fig. 1). To exclude false positivity, WEHI-164 mouse macrophage cells were stained in parallel for GABA (Fig. 1a). WEHI cells were fed with latex beads (2.16 μm) and cocultured with noninduced NE-4C cells. The macrophages were easily distinguished from NE-4C cells by their bead content. Positive GABA staining was restricted to cells without beads, indicating that neural progenitor cells contained GABA above a general background level.

The pattern of GABA staining changed with advancing differentiation. The highest level of GABA content was found on the second day of induction (Fig. 2A), during a stage when the progenitors, still proliferating, clustered into compact aggregates (Tárnok et al., 2002) and many of them became committed to a neuronal fate (Herberth et al., 2002). During this period, all cells displayed nonstructured GABA immunoreactivity, as shown by the diffuse cytoplasmic staining (Fig. 1b2). After this stage, the total GABA content of the cultures (Fig. 2A) decreased. From the third to the fourth days of induction, the compact cell aggregates spread out, and substrate-attached, flat cells settled on the bottom. On the top, individual neuron-like cells with elongated processes appeared. In parallel with these morphological changes, the uniform pattern of GABA staining changed: Some cells with neuronal morphology lost GABA immunoreactivity, whereas others showed more intense GABA staining (Fig. 1c2,d2). In the majority of substrate-attached cells, on the other hand, a moderate GABA staining (Fig. 1c2,d2) was sustained. By the sixth day of neural induction, when about half of the cells displayed neuronal morphology and

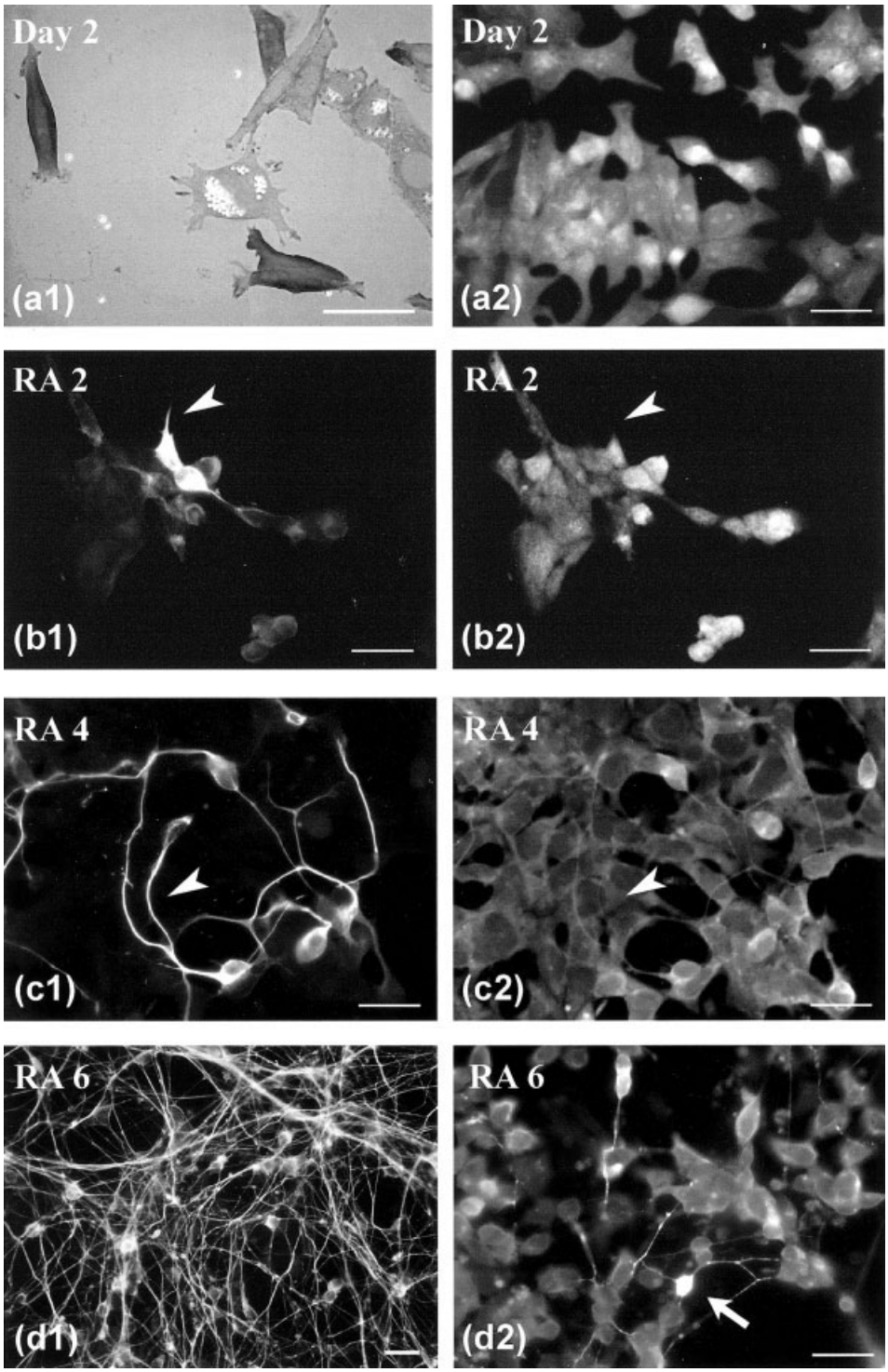

Fig. 1. Demonstration of GABA (**a1,a2,b2,c2,d2**) and neuron-specific IIIβ-tubulin (**b1,c1,d1**) in noninduced NE-4C cells (a) and in cells on the second (b), fourth (c), and sixth (d) days of RA induction. In comparison with GABA-negative latex bead-labelled WEHI cells as NE-4C cells displayed a moderate GABA immunoreactivity (dark cells in a1) and a uniform staining pattern (fluorescent cells in a2). b–d: Double staining for GABA (b2,c2,d2; Texas red) and IIIβ-tubulin (b1,c1,d1; FITC) on the second (b), fourth (c), and sixth (d) days of neuronal induction. The first N-tubulin-positive cells appeared on the second day of induction (arrowhead in b), although the distribution of GABA did not change. By the fourth day of induction (c), neuron-like cells with long, N-tubulin-positive processes had developed (c1). At this time, all cells in the cultures displayed GABA immunoreactivity (c2). By the sixth day of induction, a dense network of IIIβ-tubulin-positive neurons had formed (d1), but only a few neuron-like cells showed strong GABA staining (arrow in d2). Scale bars = 20 μm.

contained neuron-specific IIIβ-tubulin, the GABA content, normalized to the total number of cells in the culture, was lower than that in noninduced cultures (Fig. 2A). Meanwhile, the number of GABA-containing neurons increased, and the GABA staining became stronger, suggesting that some differentiated neurons could accumulate GABA. In some neurons, strong GABA staining was detected in the neurites and in the growth cones. The proportion of GABA-accumulating neurons, however, was relatively low in comparison with the total number of IIIβ-tubulin-positive cells (Fig. 1d). Double staining for GABA and IIIβ-tubulin showed that only a fraction (less

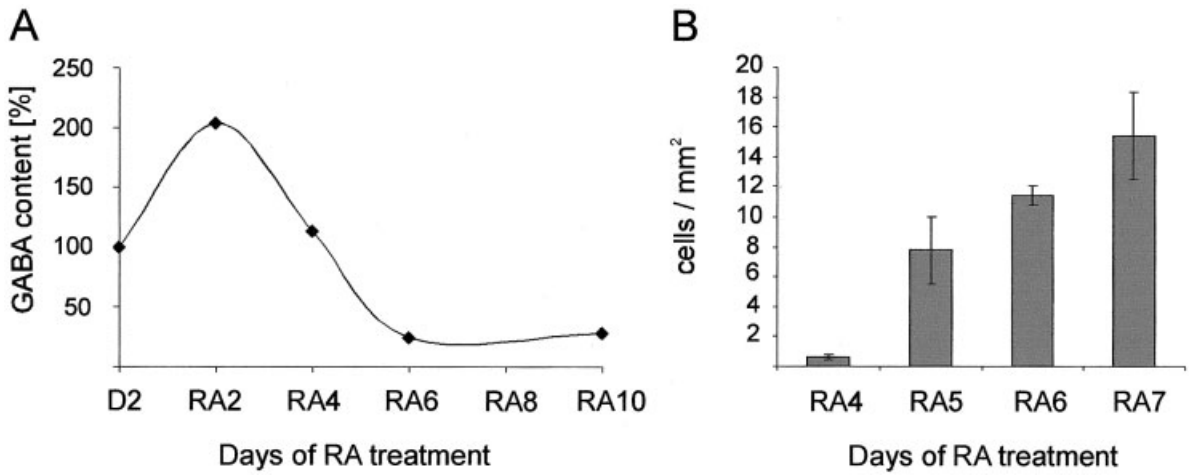

Fig. 2. **A:** The relative GABA content determined by in situ ELISA in NE-4C cultures at different stages of differentiation. D2 indicates noninduced cells. The GABA content was normalized to the cell density of identically treated sister cultures. Data from a representative series of experiments are presented as percentages of normalized GABA values obtained in noninduced cultures (100%; n = 4). **B:** GABA-accumulating, highly GABA-positive cells were counted in NE-4C cultures at different stages of differentiation. Data obtained from three independent induction series are presented as means and SDs (n = 3).

than 30%) of neurons displayed GABA immunoreactivity, whereas the majority of substrate-attached cells showed slight GABA staining.

In summary, each cell contained GABA at the beginning of RA-induced development. The overall GABA content of the cultures decreased with advancing neuronal maturation (Fig. 2A), but the frequency of individual cells with a very high GABA content increased (Fig. 2B). The majority of GABA-accumulating cells had neuronal morphology, but not all neurons contained GABA.

GABA Content in the Culture Medium During Neuronal Differentiation

The GABA content was determined in the culture media collected at different stages of induction and compared with the fresh feeding medium containing 5% FCS and approximately 1 μM GABA (Fig. 3). HPLC assays demonstrated that culture-conditioned media contained more GABA than the fresh medium, at all stages of differentiation. This observation indicated that NE-4C cells produced and released GABA into the fluid environment during the entire period of investigation. In the initial phase of neural induction, the GABA concentration increased significantly in the medium (Fig. 3), and the high extracellular GABA content decreased during the maturation period of morphologically differentiated neurons, i.e., in the second week of induction.

Although the data argued for a net GABA outflow, a low level of GABA uptake by NE-4C cells was also detected. The uptake which was measured at extracellular GABA concentrations found in conditioned media, increased slightly with the advancement of neuronal differentiation (data not shown).

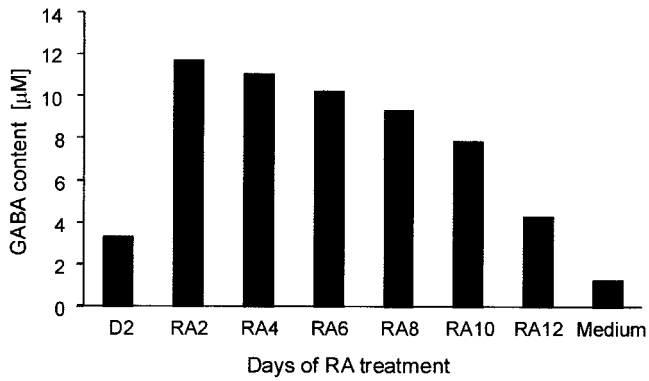

Fig. 3. GABA content in the media of NE-4C cells during neural induction. D2 indicates the noninduced culture, RA2, -4, -6, -8, -10, and -12 the days after the onset of induction by RA. HPLC assays were carried out on samples obtained from seven separate induction series, with the same results. HPLC data from a representative series are shown.

Presence of GABA(A) Receptors During In Vitro Neurogenesis

The α subunits of GABA(A) receptors were evenly distributed and showed a dotted staining in all noninduced progenitor cells (Fig. 4a2). They were observed both in the cytoplasm and on the cell membrane via confocal microscopy.

The strongest staining for GABA(A)R α subunits was found in the compact aggregates on the second day of induction (Fig. 4b), when all cells displayed strong, dotted staining in the cytoplasm. The intensity of the staining decreased gradually thereafter. With advancing neuronal maturation, the majority of cells retained

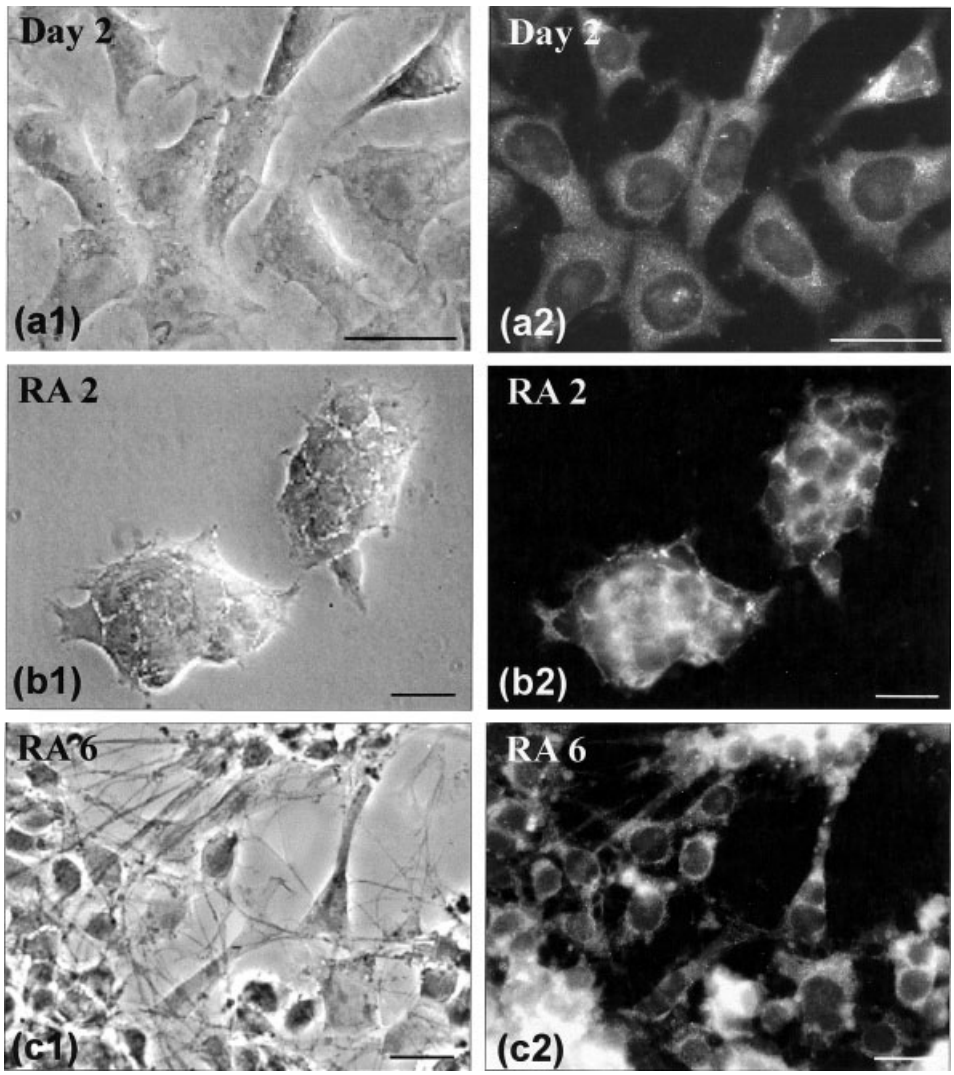

Fig. 4. Demonstration of GABA(A)R α subunits in noninduced cells (**a1,2**) and after 2 (**b1,2**) and 6 (**c1,2**) days of RA induction. The left column shows phase-contrast views, and the right column demonstrates immunocytochemical staining of the corresponding fields. Noninduced NE-4C cells displayed a uniform, dotted GABA(A)R α subunit immunoreactivity (a). The strongest staining was detected on the second day of induction (b). With advancing neuronal maturation, the majority of the cells maintained a moderate GABA(A)R α subunit staining (c), but strong GABA(A)R staining was not found in cells with neuronal morphology. Scale bars = 20 μm.

moderate GABA(A)R α subunit staining (Fig. 4c), but we failed to detect any strong GABA(A)R staining in cells with neuronal morphology, in contrast to the presence of some highly GABA-immunoreactive neurons.

GABA Elicits Ionic Responses Through GABA(A) Receptors From the Very Beginning of Neurogenesis

In noninduced, homogeneous cultures, and at the beginning of induction, cells were chosen randomly for the patch-clamp recording of GABA-evoked currents. After the appearance of process-bearing cells, the responses were recorded mainly in cells showing neuron-like morphology.

In 65% of noninduced cells, 100 μM GABA elicited a small inward current (Table I, Fig. 5A). GABA-evoked responses were recorded by whole-cell patch-clamp technique, at a holding potential of −70 mV. In responsive cells, inward currents were detected with amplitudes varying around 100 pA and with unusual long kinetics (with an activation time of about 11 sec) in comparison with the average rise and decay time of the postsynaptic GABA(A) ion channels.

TABLE I. GABA-Responsive (100 μM) Cells Measured With Patch-Clamp Techniques in the Course of Neuronal Differentiation

	Days of treatment			
	D2	RA2	RA6	RA8
Percentage of GABA-responding cells	65	46	53	42
Number of cells recorded	17	28	19	24

During the entire course of development, about 50% of the investigated cells responded to GABA (Table I). The responses, however, changed markedly with neural

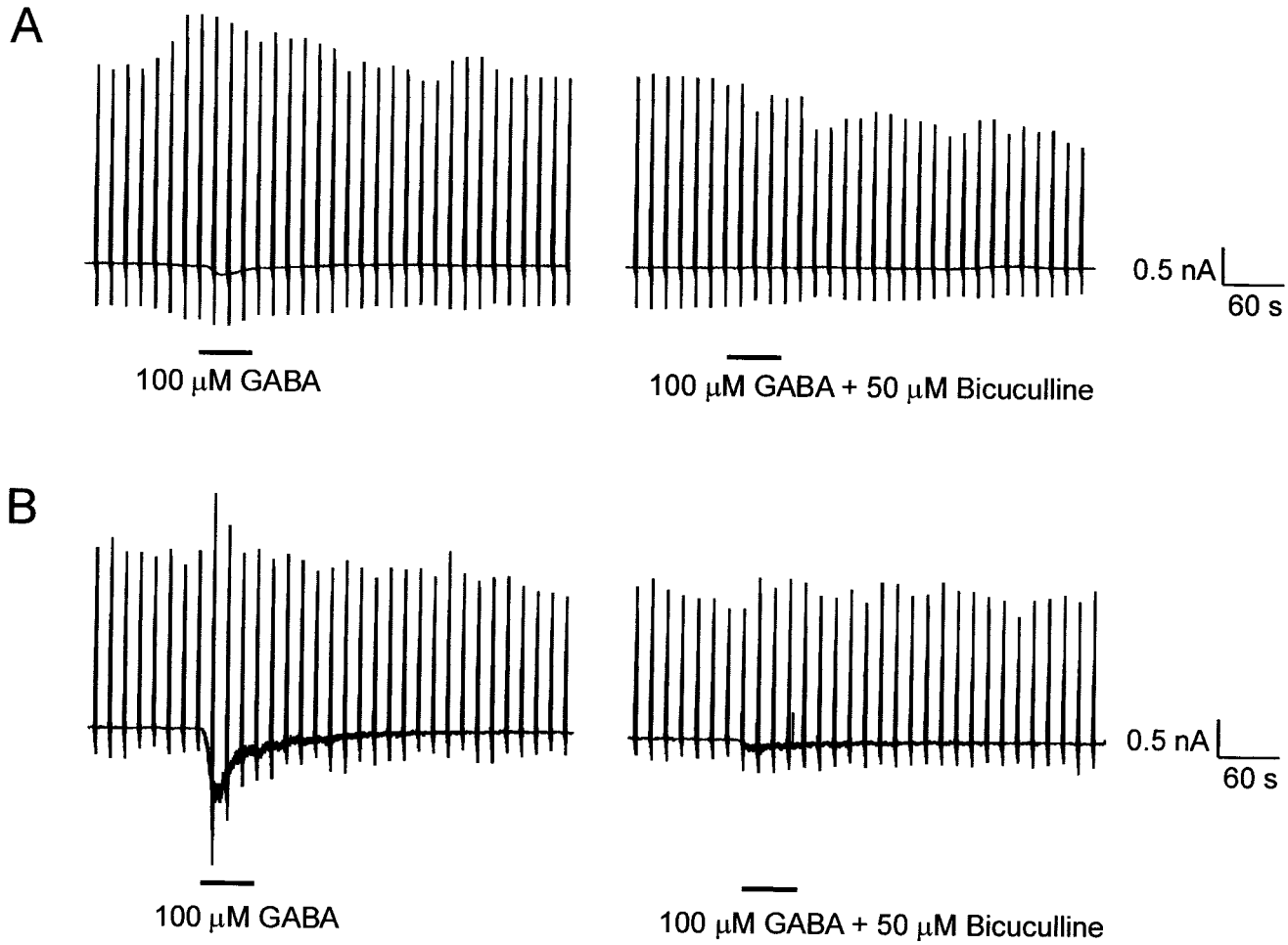

Fig. 5. GABA-evoked responses recorded by the whole-cell patch-clamp technique at a −70 mV holding potential. On the left side, representative GABA responses are shown from a noninduced progenitor cell (**A**) and from a NE-4C neuron on the sixth day of RA-induced differentiation (**B**). Bicuculline blocked the responses (right). The membrane currents were measured while briefly clamping the cell membrane potential to different values, by rectangular voltage steps (200 msec in duration) from the holding potential (−70 mV) to potentials of −140, −105, −35, 0, and +35 mV.

maturation. The amplitude of the currents significantly increased, but the long activation time persisted (Fig. 5B). Fifty micromolar muscimol, a GABA(A) receptor agonist, elicited responses similar to those with GABA, whereas bicuculline, a GABA(A) receptor antagonist, blocked the responses (Fig. 5). The data demonstrate that functional GABA(A) receptors are present in neural progenitors from the very beginning of neurogenesis.

GABA-Evoked Ca Responses in the Course of Neurogenesis

Under the assumption that GABA-evoked responses were mediated through changes in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ was measured in single cells at various stages of neural differentiation. In noninduced, homogeneous cultures, and at the beginning of induction, the cells were chosen randomly for measurement. After the appearance of process-bearing cells, Ca^{2+} responses were recorded mainly in cells showing neuron-like morphology. Ionomycin-evoked Ca elevation was used as method control at the end of each recording.

In contrast to the GABA-evoked currents, a GABA-evoked increase in $[Ca^{2+}]_i$ was not detected in the early phases of RA-induced differentiation (from day 0 to day 6). Moreover, the Ca responses were always of less amplitude (Fig. 6A) and appeared in later phases of development than the Ca responses elicited by 30 mM KCl (Herberth et al., 2002), which were detected already on day 4. The first GABA-evoked responses appeared on day 6 of induction, when large numbers of neurons were already present and networks of neuronal processes were forming. Between the sixth and the eighth days of induction, about 30% of cells with neuronal morphology re-

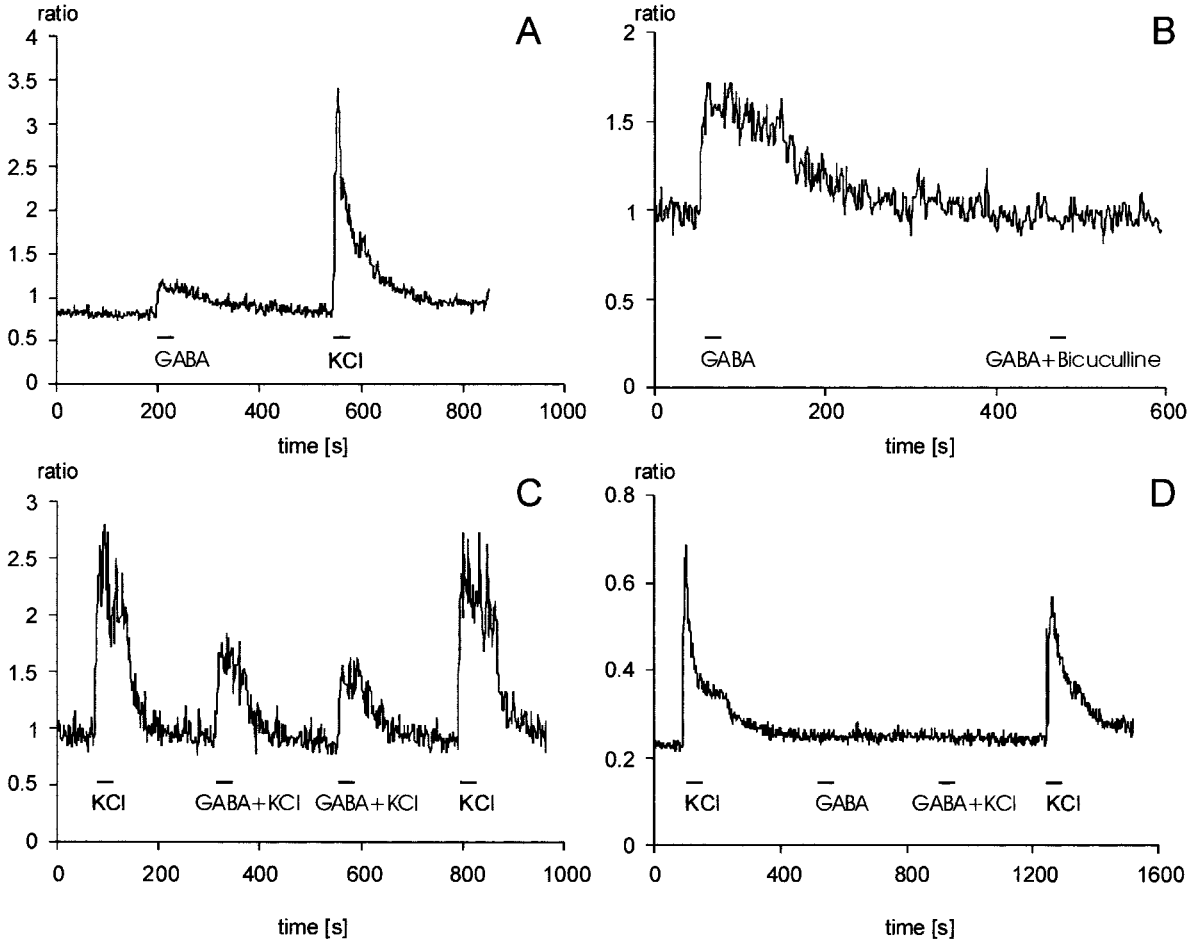

Fig. 6. $[Ca^{2+}]_i$ responses of NE-4C neurons on the seventh (**A**), eighth (**B,C**), and fourteenth (**D**) days of RA induction. Ratio of the fluorescence intensity F1/F2 emitted at 340 nm and 380 nm, respectively, is shown from representative assays. The small (in comparison with 30 mM KCl) but positive Ca response to 100 μM GABA (A) was totally abolished by adding 50 μM bicuculline (B). In a few cells (which did not respond to GABA), 100 μM GABA reduced (C; record was taken on the eighth day of RA induction) or totally abolished (D; record from the fourteenth day of induction) the KCl-evoked Ca elevation in later phases of neuronal maturation.

TABLE II. Number of GABA-Responsive (100 μM) Cells With Intracellular Calcium Increasing in the Course of Neuronal Differentiation

	Days of treatment					
	D2	RA2	RA4	RA6	RA7	RA8
Percentage of GABA-responding cells	0	0	0	31	28	32
Number of cells measured	82	84	94	58	86	81

sponded to GABA by the elevation of $[Ca^{2+}]_i$ (Table II). Bicuculline completely blocked the GABA-evoked Ca^{2+} responses (Fig. 6B). From the eighth day onward, exposure to GABA decreased or totally abolished the KCl-evoked Ca response (Fig. 6C,D) in a few cells, which did not respond to GABA by itself.

GABA Promotes the Maturation of Postmitotic Neuronal Precursors

The early presence of GABA and GABA-gated ion channels together with the enhanced GABA content in the culture media suggested that extracellular GABA played a role in the neuronal differentiation of NE-4C cells. To address this question, the cells were maintained for various periods in the presence or absence of 100 μM bicuculline. The number of neurons was determined on the seventh day of induction by counting those cells that displayed the neuron-specific markers IIIβ-tubulin or NeuN (Fig. 7). The number of neurons was significantly lower when the cells were grown in the presence of bicuculline either during the whole induction period (from the first to the seventh days) or between the fourth and the seventh days of induction. In contrast, there were no changes in neuron formation when bicuculline was added only for the first 3 days of induction, i.e., in the

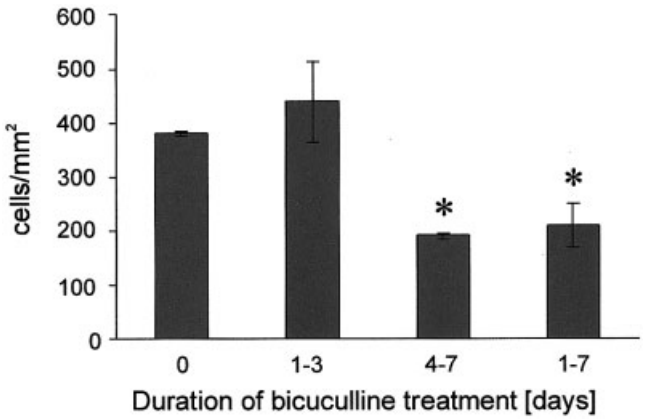

Fig. 7. Effects of bicuculline on the neuronal differentiation of NE-4C cells. Bicuculline (100 μM) was present either for the first 7 days (1–7) or for the first 3 days (1–3) or was added between the fourth and seventh days (4–7) of induction. Control cultures (0) were grown in the absence of bicuculline. The number of neurons was estimated by counting the Neu-N-immunoreactive cells on day 7. Data were collected from four separate induction series and are presented as means and SDs (two-sample *t*-test, $^{\star}P < .05$).

period of proliferation and initial neuronal cell fate decision (Schlett and Madarász, 1997). The data showed that GABA through the GABA(A) receptors could facilitate the formation of neurons in the postmitotic period of maturation.

DISSCUSSION

In the present study, the role of GABA was investigated in a well-characterized in vitro model, the RA-induced neurogenesis of a one-cell-derived neuroectodermal stem cell clone, NE-4C (Schlett and Madarász 1997; Schlett et al., 1997, 2000; Herberth et al., 2002; Jelitai et al., 2002; Tárnok et al., 2002). NE-4C neural stem cells derived from the anterior brain vesicles of 9-day-old mouse embryos represent early neuroectodermal progenitors. These cells proliferate continuously and provide large populations of homogeneous cells. Induction with RA results in heterogeneity: Cells committed to neuronal and later to astroglial fate appear in succession and among cells maintaining a nondifferentiated neuroectodermal phenotype. During the relatively short investigation period, oligodendrocytes were not formed, and terminally specified neuronal subtypes could not develop. Despite these later limitations, the model provides a tool to study some early steps of pan-neuronal phenotype determination. The availability of an unlimited amount of homogeneous neural progenitors as a starting material is an advantage that cannot be provided with primary brain cell preparations. Moreover, the direct monitoring of the loss of homogeneity and the distribution of transient phenotypes during the successive steps in cell fate commitments help in understanding the complex in vivo changes during the course of neural tissue genesis. Although an in-vitro-induced differentiation by a model cell population should not be regarded as an equivalent of any tissue-type development, some conclusions concerning the main steps and the desired conditions can be drawn. Validation of some of these conclusions is in progress in our laboratory through histochemical studies on early mouse embryos and cell biological investigations on neurospheres.

The data demonstrate that important components of the GABA signalling system, namely, GABA itself and functional GABA(A) receptors, are present in neural progenitors from the very beginning of neuronal determination. Despite the presence of these components in noninduced, noncommitted progenitors, GABA did not influence the rate of neuron formation when it was present in the proliferating phase (Schlett and Madarász, 1997) of early neuronal determination. Induced NE-4C cells cease proliferating after the third day of induction, and bicuculline was effective only later, the GABA(A)R blockage should not decrease the rate of neuron formation by acting on proliferating progenitors. In stages of postmitotic differentiation, however, the bicuculline blockade of GABA action impaired neuron formation. Consequently, GABA should facilitate the differentiation of already committed neuronal precursors. In this effective period, GABA evoked inward currents and elevated the intracellular Ca level, and both were abolished by the addition of bicuculline. Even if bicuculline might have a role other than that of a GABA(A) receptor antagonist (Mestdagh and Wulfert, 1999), this finding indicates that some effects of GABA on postmitotic neuronal differentiation are mediated through intracellular Ca signalling.

The GABA-evoked elevation of intracellular Ca level appeared at a relatively late phase of neuron formation, when process-bearing neuronal precursors already displayed neuron-specific marker proteins, among them the NeuN nuclear protein (Mullen et al., 1992) characteristic of mature neurons. In contrast, KCl could elicit an elevation of Ca in NE-4C cells in much earlier phases of neuronal differentiation (Herberth et al., 2002). We have to admit, however, that the sensitivity of the Ca imaging technique employed did not allow for the monitoring of local Ca transients in fine, developing processes and in growth cones. Therefore, the different responsiveness to KCl and GABA might reflect some differences not only in their effectiveness in affecting the intracellular K^+ and Cl^- concentrations but also in the distribution of K^+-permeable and GABA-gated ion channels between the soma and the fine processes.

In the majority of cells with neuronal morphology, KCl could initiate a Ca increase, whereas GABA responses were recorded only in approximately 30% of the neurons; the proportion of responding cells did not increase with advancing neuronal maturation. Among the nonresponding cells, a few neurons were found that decreased their KCl-evoked Ca responses in the presence of GABA. The response inhibition might indicate a more advanced phase of maturation, when GABA might evoke a hyperpolarization in developing neurons instead of a depolarization.

Whereas GABA-evoked Ca responses were detected in relatively late phases of neuronal determination, intracellular GABA content, GABA(A) receptor α subunits, GABA-evoked ion currents, and GABA release to the extracellular medium were demonstrated in all stages of NE-4C differentiation, including the noncommitted stem cell stage. Patch-clamp results showed that GABA could evoke some slight ionic fluxes in noninduced progenitor cells at the stage when they were still proliferating. The extremely slow activation time of the currents raises the question of whether such currents were direct or indirect responses to GABA. Whatever the mechanism, the data clearly demonstrate a remarkable responsiveness of proliferating neural stem cells to GABA.

Some recent results (Chen et al., 2000; Demarque et al., 2002) demonstrated that extrasynaptic GABA(A) ion channels work with slower kinetics than clustered postsynaptic GABA(A) receptors. Our immunocytochemical analysis could not reveal any clustering of GABA(A) receptor α subunits on neuronal somata or the processes of maturing NE-4C neurons, even on the eighth day of induction, when the production of some presynaptic proteins, such as synaptophysin and synapsin-1, were demonstrated (Jelitai et al., 2002). The overall density of GABA(A) receptor α subunits was lower on neuronal somata and processes than in the membranes of noninduced NE-4C cells. Without electron microscopic evidence, we do not know whether the absence of receptor accumulation was due to the lack of GABAergic synapses or to the stage of maturation when postsynapses have not yet been formed.

During the initial phase of neuronal induction, the intracellular GABA content increased in NE-4C cells, reaching its maximum when progenitors were still proliferating regardless of whether they were committed to a neuronal fate (Herberth et al., 2002). With advancing neuronal differentiation, nonneuronal, substrate-attached cells showed only faint GABA staining, whereas some neurons became highly GABA immunoreactive. The appearance of populations of GABA-accumulating and -nonaccumulating neurons from uniformly GABA-containing progenitors suggests that the initial GABA content of early progenitors has no direct relation with the later decision on transmitter accumulation.

The calcium-independent release of transmitters may also be attributed to the unconventional features of the communication between immature neural cells (Demarque et al., 2002). The mechanism of transmitter release and the source of the GABA found in noninduced cells, or in those at the very earliest stages of neural induction, are far from clear. As our HPLC and GABA uptake data show, the cells do not accumulate GABA from the fresh medium. On the contrary, the net outflow of GABA from the cells suggests some inherent GABA production by NE-4C cells. Our earlier studies on a sister clone, NE-7C2 (Varju et al., 2002), indicated that noninduced neuroectodermal stem cells did not express the enzyme-active embryonic (GAD44) form (Szabó et al., 1994) of glutamate decarboxylase GAD67, and the adult GAD forms (GAD67 and GAD65) could not be detected at the protein level in these cells. Western blot analyses also failed to demonstrate any GAD proteins in NE-4C cells (data not shown). In this respect, NE-4C cells seem to resemble a developmental stage preceding the expression of GAD enzymes (Katarova et al., 2000). There are some alternative metabolic processes, however, that can generate GABA as well (Watanabe et al., 2002). The low level and even the cytoplasmic distribution of GABA suggest a role for such mechanisms as the source of "nonneuronal" or "preneuronal" GABA (Waagepetersen et al., 1999). Further studies will be needed to clarify the source of GABA in noncommitted NE-4C progenitor cells and also to clarify whether the described GABA distribution is valid for the early in vivo neural progenitors as well.

We have shown (Jelitai et al., 1998), in accordance with in vivo data (LoTurco et al., 1995; Haydar et al., 2000), that GABA can regulate the rate of proliferation of primary neural precursors depending on their stage of differentiation. In the early proliferating phase of development, however, the inhibition of GABA signalling through GABA(A) receptors or treatment with GABA (nonpublished data) failed to influence the production of neurons by one-cell-derived NE-4C cells. An understanding of the importance and role of GABA responsiveness in this very early period of neuronal commitment requires further examination. Detailed electrophysiological analyses (Jelitai et al., 2003) are in progress on the NE-4C model, in order to obtain data on the possible role(s) of early, "preneuronal" GABA in the neuronal cell fate decision.

REFERENCES

Barbin G, Pollard H, Gaiarsa JL, Ben-Ari Y. 1993. Involvement of GABAA receptors in the outgrowth of cultured hippocampal neurons. Neurosci Lett 152:150–154.

Barker JL, Behar TN, Li YX, Liu QY, Ma W, Maric D, Maric I, Schaffner AE, Serafini R, Smith SV, Somogyi R, Vautrin JY, Wen XL, Xian H. 1998. GABAergic cells and signals in CNS development. Perspect Dev Neurobiol 5:305–322.

Behar TN, Schaffner AE, Scott CA, O'Connell C, Barker JL. 1998. Differential response of cortical plate and ventricular zone cells to GABA as a migration stimulus. J Neurosci 18:6378–6387.

Ben-Ari Y. 2002. Excitatory actions of gaba during development: the nature of the nurture [review]. Nat Rev Neurosci 9:728–739.

Berninger B, Poo M. 1995. Fast actions of neurotrophic factors [review]. Curr Opin Neurobiol 3:324–330.

Chen L, Wang H, Vicini S, Olsen RW. 2000. The gamma-aminobutyric acid type A (GABAA) receptor-associated protein (GABARAP) promotes GABAA receptor clustering and modulates the channel kinetics. Proc Natl Acad Sci USA 97:11557–11562.

Cherubini E, Gaiarsa JL, Ben-Ari Y. 1991. GABA: an excitatory transmitter in early postnatal life. TINS 14:515–519.

Chvátal A, Kettenmann H. 1991. Effect of steroids on γ-aminobutyrate-induced currents in cultuted rat astrocytes. Pflugers Arch 419:263–266.

Clayton GH, Owens GC, Wolff JS, Smith RL. 1998. Ontogeny of cation-Cl^- cotransporter expression in rat neocortex. Brain Res Dev Brain Res 109:281–292.

Demarque M, Represa A, Becq H, Khalilov I, Ben-Ari Y, Aniksztejn L. 2002. Paracrine intercellular communication by a Ca^{2+}- and SNARE-independent release of GABA and glutamate prior to synapse formation. Neuron 36:1051–1061.

Doherty P, Dickson JG, Flanigan TP, Walsh FS. 1984. Quantitative evaluation of neurite outgrowth in cultures of human foetal brain and dorsal root ganglion cells using an enzyme-linked immunoadsorbent assay for human neurofilament protein. J Neurochem 42:1116–1122.

Fiszman ML, Behar T, Lange GD, Smith SV, Novotny EA, Barker JL. 1993. GABAergic cells and signals appear together in the early postmitotic period of telencephalic and striatal development. Brain Res Dev Brain Res 73:243–251.

Ganguly K, Schinder AF, Wong ST, Poo M. 2001. GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105:521–532.

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. Pflugers Arch 391:85–100.

Haydar TF, Wang F, Schwartz ML, Rakic P. 2000. Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci 20:5764–5774.

Herberth B, Pataki Á, Jelitai M, Schlett K, Deák F, Spät A, Madarász E. 2002. Changes of KCl sensitivity of proliferating neural progenitors during in vitro neurogenesis. J Neurosci Res 67:574–582.

Jelitai M, Herberth B, Varju P, Tóth B, Baranyi M, Madarász E. 1998. Non-synaptic effects of glutamic acid and GABA in cultures of developing neural cells. Neurobiology 6:471–472.

Jelitai M, Schlett K, Varju P, Eisel U, Madarász E. 2002. Regulated appearance of NMDA receptor subunits and channel functions during in vitro neuronal differentiation. J Neurobiol 51:54–65.

Jelitai M, Andìrová M, Marko K, Madarász E, Syková E. 2003. Electrophysiological characterization of neural stem cells after induction of differentiation by retinoic acid. Paper presented at the Sixth IBRO Worldcongress of Neuroscience.

Katarova Z, Sekerková G, Prodan S, Mugnaini E, Szabó G. 2000. Domain-restricted expression of two glutamic acid decarboxylase genes in midgestation mouse embryos. J Comp Neurol 424:607–627.

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG. 1999. The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci 19:7881–7888.

Lo Turco JJ, Kriegstein AR. 1991. Clusters of coupled neuroblasts in embryonic neocortex. Science 252:563–566.

LoTurco JJ, Owens DF, Heath MJS, Davis MBE, Kriegstein AR. 1995. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 15:1287–1298.

Madarász E. 1987. Effects of triidothyorine on the development of GFAP-immunoreactivity and CAT-activity in monolayer cultures of embryonal rat forebrain cells. Int J Dev Neurosci 5:255–262.

Maddox JM, Condie BG. 2001. Dynamic expression of glutamate decarboxilase gene in multiple non-neural tissues during mouse development. BMC Dev Biol Vol. 1.

Maric D, Liu QY, Maric I, Chaudry S, Chang YH, Smith SV, Sieghart W, Fritschy JM, Barker JL. 2001. GABA expression dominates neuronal lineage progression in the embryonic rat neocortex and facilitates neurite outgrowth via GABA(A) autoreceptor/Cl^- channels. J Neurosci 21:2343–2360.

Marty S, Berninger B, Carroll P, Thoenen H. 1996. GABAergic stimulation regulates the phenotype of hippocampal interneurons through the regulation of brain-derived neurotrophic factor. Neuron 16:565–570.

Meier E, Hertz L, Schousboe A. 1991. Neurotransmitters as developmental signals. Neurochem Int 19:1–15.

Mestdagh N, Wulfert E. 1999. Bicuculline increases Ca^{2+} transients in rat cerebellar granule cells through non-GABA(A) receptor associated mechanisms. Neurosci Lett 265:95–98.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Mullen RJ, Buck CR, Smith AM. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. Development 116:201–211.

Nguyen L, Rigo JM, Rocher V, Belachew S, Malgrange B, Rogister B, Leprince P, Moonen G. 2001. Neurotransmitters as early signals for central nervous system development. Cell Tissue Res 305:187–202.

Obrietan K, Gao XB, Van Den Pol AN. 2002. Excitatory actions of GABA increase BDNF expression via a MAPK-CREB-dependent mechanism—a positive feedback circuit in developing neurons. J Neurophysiol 88:1005–1015.

Owens DF, Kriegstein AR. 2002. Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715–727.

Owens DF, Boyce LH, Davis MB, Kriegstein AR. 1996. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci 16:6414–6423.

Schlett K, Madarász E. 1997. Retinoic acid induced neural differentiation in a neuroectodermal cell line immortalized by p53 deficiency. J Neurosci Res 47:405–415.

Schlett K, Herberth B, Madarász E. 1997. In vitro pattern formation during neurogenesis in neuroectodermal progenitor cells immortalized by p53-deficiency. Int J Dev Neurosci 15:795–804.

Schlett K, Czirók A, Tárnok K, Vicsek T, Madarász E. 2000. Dynamics of cell aggregation during in vitro neurogenesis by immortalized neuroectodermal progenitors. J Neurosci Res 60:184–194.

Szabó G, Katarova Z, Greenspan R. 1994. Distinct protein forms are produced from alternatively spliced bicistronic glutamic acid decarboxylase mRNAs during development. Mol Cell Biol 14:7535–7545.

Tárnok K, Pataki A, Kovács J, Schlett K, Madarász E. 2002. Stage-dependent effects of cell-to-cell connections on in vitro induced neurogenesis. Eur J Cell Biol 81:403–412.

Varju P, Katarova Z, Madarász E, Szabó G. 2002. Sequential induction of embryonic and adult forms of glutamic acid decarboxylase during in vitro-induced neurogenesis in cloned neuroectdermal cell-line, NE-7C2. J Neurochem 80:605–615.

Waagepetersen HS, Sonnewald U, Schousboe A. 1999. The GABA paradox: multiple role as metabolite, neurotransmitter, and neurodifferentiative agent. J Neurochem 73:1335–1342.

Watanabe M, Maemura K, Kanbara K, Tamayama T, Hayasaki H. 2002. GABA and GABA receptors in the central nervous system and other organs. Int Rev Cytol 213:1–47.

Yuste R, Katz LC. 1991. Control of postsynaptic Ca^{2+} influx in developing neocortex by excitatory and inhibitory neurotransmitters. Neuron 6:333–344.