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Conditionally-Immortalized Astrocytic Cell Line Expresses GAD and Secretes GABA Under Tetracycline Regulation

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We have engineered conditionally-immortalized mouse astrocytes to express β-galactosidase or GAD_{65} in a tetracycline-controlled fashion. The engineered cell lines, BASlinβgal and BASlin65, divide at 33°C but cease division at 39°C. We carried out morphological and biochemical analyses to further understand GABA production and release, and to determine the suitability of these cells for transplantation. Using the BASlinβgal cell line, we showed a dramatic regulation of β-galactosidase expression by tetracycline. The BASlin65 cell line showed functional GAD_{65} enzymatic activity and GABA production, both of which were suppressed by growth in the presence of tetracycline. When cultured in the absence of tetracycline, BASlin65 cells have a total GABA content equal to or greater than other GABA-ergic cell lines. Immunofluorescence microscopy revealed that GAD_{65} had a distinct perinuclear localization and punctate staining pattern. GABA, on the other hand, showed diffuse staining throughout the cytoplasm. BASlin65 cells not only synthesize GABA, they also release it into the extracellular environment. Their ability to produce and release significant amounts of GABA in a tetracycline-regulated manner makes BASlin65 cells a useful cellular model for the study of GABA production and release. Furthermore, their non-tumorigenicity makes them excellent candidates for transplantation into specific regions of the brain to provide a localized and regulatable source of GABA to the local neuronal circuitry. J. Neurosci. Res. 60: 302–310, 2000. © 2000 Wiley-Liss, Inc.

Key words: GAD; GABA; astrocytes; transplantation

We report the development of a murine astroglial cell line that synthesizes and releases GABA. These cells will be useful as a model system to study GABA production and release. In addition, recent research suggests the possible utility of ex vivo and in vivo gene transfer for the delivery of neurochemicals (Tuszynski et al., 1990; Cunningham et al., 1991; Salle et al., 1993). In the future, transplantation of these engineered GABA-producing cells into experimental animals will provide an opportunity to evaluate the behavioral effects of GABA replacement on specific neural circuits of the basal ganglia and the hippocampal formation, particularly in relation to the pathogenesis of Huntington's disease, Parkinson's disease, and epilepsy.

In the nervous system, GABA is synthesized from glutamate by the enzymatic action of glutamate decarboxylase (GAD). Both isoforms of this enzyme, GAD_{65} and GAD_{67}, depend on the co-factor pyridoxal phosphate (PLP) for full enzymatic activity, but they differ in their molecular weights, their affinities for PLP, and their postulated cellular function (Erlander and Tobin, 1991). GAD_{65} is membrane-associated, responds to added PLP with a large increase in enzymatic activity, and has a reported role in synaptic release of GABA (Erlander et al., 1991; Kaufman et al., 1991). In contrast, GAD_{67} is distributed throughout the cytoplasm, shows low levels of enzymatic stimulation with added PLP, and likely plays a role in cellular metabolism (Erlander et al., 1991; Kaufman et al., 1991).

Previous work in this and other laboratories has yielded stable clones of genetically modified cell lines engineered to express GAD and therefore to produce GABA (Segovia et al., 1992; Ruppert et al., 1993). Although these cell lines have been useful for studying the molecular mechanisms underlying GABA production and storage, their tumorigenicity after transplantation and their lack of regulated expression limit their use in transplantation studies. To overcome these limitations, we have engineered conditionally-immortalized astrocytic progenitor cells to produce GAD under the control of a tetracycline-regulatable promoter. These cells can be used to understand the production, storage and release of GABA from astrocytes, and in the future can be used as

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cellular implants of non-tumorigenic cells that produce GABA in a controllable manner.

Astrocytes have several potential advantages for grafting studies (Kesslak et al., 1986; Smith et al., 1986; Smith and Silver, 1988; Cunningham et al., 1991; Franklin et al., 1991; Jiao et al., 1993; La Gamma et al., 1993; Mucke et al., 1993; Takshima et al., 1994; Bradbury et al., 1995). Unlike cell types such as myoblasts and fibroblasts, astrocytes are components of the brain itself, and they successfully survive transplantation (Fisher et al., 1991; Jiao et al., 1993; La Gamma et al., 1993; Mucke et al., 1993). The machinery for the release and uptake of compounds to and from the extracellular environment already exists in astrocytes, making them suitable to release neurotransmitters. Finally, astrocytes facilitate recovery from injury of the nervous system by taking up excess Ca^{2+} and by providing molecules that stimulate neuronal growth and differentiation (Kesslak et al., 1986; Smith et al., 1986; Franklin et al., 1991; Takshima et al., 1994).

Many previously used cell lines, derived from spontaneous tumors or from tumors induced by mutagenic agents, form malignant tumors after transplantation (Tumilowicz et al., 1970; Schubert et al., 1974). To avoid the problem of tumorigenicity after cell transplantation, we used a conditionally immortalized astrocytic cell line (Bongarzone et al., 1996). We employed a temperature-sensitive version of the SV40 large T antigen (*ts*A58) to develop cell lines that divide at the permissive temperature (33°C), allowing rapid clonal expansion (Jat and Sharp, 1989). At the non-permissive temperature (39°C), however, cells stop dividing and undergo cytodifferentiation (Jat and Sharp, 1989; Whittemore and White, 1993). Such conditionally immortalized cells, transplanted into the brains of developing rats, can form mature structures without any apparent tumors (Bredesen et al., 1990; Whittemore et al., 1991).

Giordano et al. (1993, 1996) have previously reported GAD expression in an immortalized striatal cell line, with GABA production occurring in a constitutive fashion. Although useful for studying the processes of GABA production and secretion, such constitutively expressing cells can be problematic in brain transplantation studies. With cells that constantly secrete GABA, it is difficult to separate the effects of the GABA release from the effects of the implant itself. Moreover, tonic widespread activation of GABA receptors may actually induce absence seizures (Hosford et al., 1992). To avoid potential problems of uncontrolled GABA release, we engineered cells to express GAD, and therefore synthesize and release GABA, under tetracycline regulation.

Gossen and Bujard (1992) have developed a tetracycline-responsive promoter system that allows the tight regulation of gene expression. In this system, gene expression depends on a minimal human cytomegalovirus (HCMV) immediate-early promoter fused to seven copies of the Tn-10 tetracycline operator. Cells constitutively express the tetracycline-controlled transactivator (tTA), a fusion protein that consists of the tetracycline repressor combined with the transcriptional transactivation domain of VP16 from herpes simplex virus. In the presence of tetracycline, tTA does not bind to the *tet* operator, and transcription effectively ceases. After removal of tetracycline, however, tTA binds to the *tet* operator, and robust expression occurs.

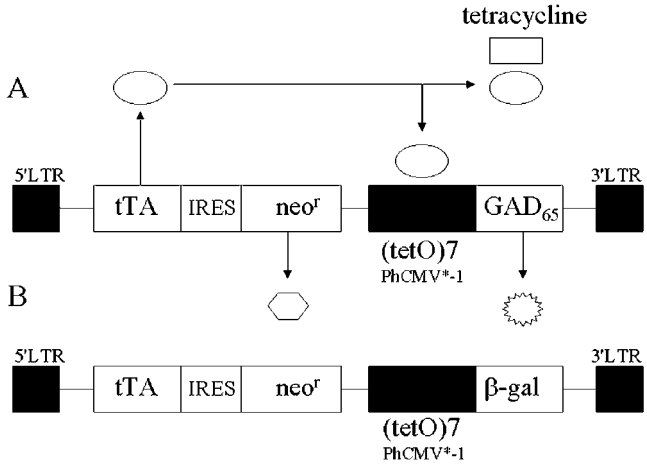

Fig. 1. Tetracycline-regulated expression of GAD_{65} and β-galactosidase. The LIN(2.3RG65) construct (**A**) and the LIN(βgal) construct (**B**) produce GAD_{65} and β-galactosidase under the control of tetracycline. The LinX vector contains a long terminal repeat (LTR) of Moloney murine sarcoma virus. The LTR drives the expression of both tTA (oval) and, using an internal ribosome entry site (IRES), neomycin phosphotransferase (hexagon) genes. In the absence of tetracycline, tTA binds the tetracycline operator sequences present in the hybrid promoter to stimulate transcription from the minimal HCMV promoter (PhCMV*-1). Transcription yields the 2.3-kb mRNA for GAD_{65} (star) or the 3.4-kb mRNA for β-galactosidase in LIN(2.3RG65) or LIN(βgal). Tetracycline (1 μg/ml) (rectangle) binds the tTA to prevent stimulation of PhCMV*-1, thus controllably inhibiting GAD_{65} or β-galactosidase production.

Using this system, we have genetically engineered conditionally immortalized astrocytes that are non-tumorigenic and exhibit tightly regulated expression of GAD and production of GABA. We believe these cells will prove useful for studies addressing the role of GABA in normal brain circuitry as well as in neurological disorders such as Parkinson's disease, Huntington's disease, and epilepsy.

MATERIALS AND METHODS

Construction of GAD_{65} and β-Galactosidase cDNAs

We obtained a 2.3-kb EcoR1 fragment containing the 1,755-bp coding region of the rat GAD_{65} cDNA from pL(2.4RG65)SN (Ruppert et al., 1993). We then produced a GAD_{65} construct, LIN(2.3RG65), by blunt-end ligation into the Cla1 site of the LinX vector (a gift of Dr. F. Gage; Hosimaru et al., 1996) (Fig. 1A). We produced a similar construct with β-galactosidase, LIN(βgal), cloning a 3.4-kb EcoR1 fragment of the *E. coli* β-galactosidase cDNA from pUHG16-3 (a gift of Dr. H. Bujard; Gossen and Bujard, 1992) into the Cla1 site of the LinX vector by blunt-end ligation (Fig. 1B).

The retroviral LinX vector contains a long terminal repeat (LTR) of Moloney murine sarcoma virus. The LTR drives the expression of both tTA and, using an internal ribosome entry site (IRES), neomycin phosphotransferase genes. In the absence of tetracycline, tTA binds the tetracycline operator sequences present in the hybrid promoter to stimulate transcription from the minimal HCMV promoter (PhCMV*-1). Transcription yields the 2.3-kb mRNA for GAD_{65} or the 3.4-kb mRNA for β-galactosidase in LIN(2.3RG65) or LIN(βgal). Tetracycline (1 μg/ml) binds the tTA to prevent stimulation of PhCMV*-1, thus controllably inhibiting GAD_{65} or β-galactosidase production.

After blunt-end ligation of $rGAD_{65}$ or β-galactosidase cDNA into the Cla1 site of the LinX vector, DH5α competent cells were transformed and grown overnight on ampicillin selection plates. After this selection, the DNA was isolated, using a miniprep procedure, and digested with restriction enzymes (BamHI, BSP1061, and EcoRI) to determine orientation. DNA was purified using QIAGEN maxipreps and stored at −80°C.

Cell Culture and Transfection

The astrocytic cell line BAS8.1 was immortalized from 0–3 days postnatal BALB/*c* mouse primary glial cortical cultures using the temperature-sensitive SV40 large T antigen (Bongarzone et al., 1996). To engineer the BAS8.1 cell line, these primary cultures were infected with virus from the ψ2 packaging cell line, that had been transfected with the recombinant pZ-IPSV*ts*A58 retrovirus containing the genes for SV40 large T-antigen and puromycin resistance. BAS8.1 cells were maintained at 33°C in 5% CO_2 in DMEM/F12, 10% FCS, 3 μg/ml puromycin, and penicillin/streptomycin. Cells were passaged twice a week using trypsin-EDTA. Characterization of this astrocyte cell line showed they were positive for glial fibrillary acidic protein (GFAP), and negative for the oligodendroglial marker myelin basic protein (MBP) and the neuronal marker neuron specific enolase (NSE).

Figure 1 shows the constructs used to generate the stable cell lines, called BASlin65 and BASlinβgal, that produced GAD_{65} or β-galactosidase, in a tetracycline-controllable fashion (see Results). BAS8.1 cells were electroporated (275 mV, 50 mA) in the presence of 10 μg of LIN(2.3RG65) or LIN(βgal). Transfected astrocytes were selected two days after electroporation with the neomycin analogue, G418 (500 μg/ml, Sigma). Individual colonies, transferred to separate flasks using glass cloning cylinders, were expanded and maintained in the presence of 250 μg/ml G418, and then analyzed for the expression of GAD_{65} or β-galactosidase. Whereas BASlin65 and BASlinβgal stable cell lines were usually grown at 33°C, some experiments were also done at 39°C. At 39°C, the cells effectively cease mitosis, and survive for at least 3 weeks. Regulatable expression of GAD_{65} and β-galactosidase in engineered BAS8.1 cells was achieved by addition or removal of 1 μg/ml tetracycline to the growth media.

Cell growth was monitored by harvesting passage 15 cells grown at 33°C and then plating equal number of cells (2×10^5) into replicate 4 cm dishes. At time zero, dishes were placed into incubators at 33°C or 39°C. After 48, 72, or 96 hr, cells were harvested and cell numbers were estimated by quantitating DNA using Hoescht dye (Larbarca et al., 1980).

GAD Assay and β-Galactosidase Assay

GAD and β-galactosidase assays were performed on BASlin65 and BASlinβgal colonies. We chose one GAD-producing line and one β-galactosidase-producing line for further characterization.

GAD activity was measured in triplicate on cell homogenates in the presence or absence of 0.05 mM pyridoxal phosphate (PLP), the obligate cofactor for GAD_{65} (Kaufman et al., 1991). We assayed GAD activity by a $^{14}CO_2$-trapping procedure using a filter wet with pH basic tissue solubilizer TS-2 (RPI) (Erlander et al., 1991). Filters were then counted to determine GAD activity as nmol CO_2 released/mg protein/hr. Protein concentrations, used to normalize results, were determined using the BioRad microassay (Bradford, 1976). β-galactosidase activity was measured by a colorimetric assay using *o*-nitrophenyl-β-D-galactopyranoside (ONPG).

Western Blot Analysis

Extracts of BASlin65 and other cells corresponding to 15 μg of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel. The fractionated proteins were transferred to a 0.45-μm nitrocellulose filter and probed with GAD6, a monoclonal antibody specific for GAD_{65} (Gottlieb et al., 1986). Analysis for GAD_{65} protein was by ECL (Amersham) detection method, using prestained molecular weight markers (BioRad).

Immunofluorescence

Cells were grown on poly-D-lysine-coated glass slides until 80% confluent. Cells were rinsed with phosphate-buffered saline (PBS, diluted from 10X PBS, GIBCO-BRL, 90.0 g/L NaCl, 2.10 g/L KH_2PO_4, 7.26 g/L $Na_2HPO_4 \cdot \cdot 7H_2O$, pH7.2) and then fixed in 4% formaldehyde for 30 min at room temperature. After two quick rinses with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min. After 3 additional rinses in PBS, cells were incubated overnight at 4°C in primary antibody plus 0.5% sheep serum blocking solution and 0.01% Tween-20 in PBS. Antibodies and their concentrations for the various experiments were as follows: GAD6 (a mouse monoclonal IgG raised to GAD_{65}), 1:15; anti-GABA rabbit polyclonal IgG (Sigma), 1:1000; anti-SV40 mouse IgG (Pharmingen), 1:200. Bound primary antibodies were detected by anti-mouse IgG or anti-rabbit IgG secondary antibodies, labeled with FITC or rhodamine, at 1:200, for 30 min at room temperature.

HPLC

Cells were washed with PBS and processed to assay either total cellular GABA content or GABA release. GABA content was determined by adding 0.4 M perchloric acid in PBS for 10 min before harvesting the cells by scraping. Lysates were then sonicated and filtered. GABA release was determined by incubating the cells in basal medium (144 mM NaCl, 1 mM $MgCl_2$, 4 mM KCl, 1.8 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.1) for 1 hr, after which time medium was collected and filtered. GABA was detected in triplicate by reverse-phase HPLC and fluorescence detection of *o*-phthaldehyde-derivatized adducts as described previously (Lindroth and Mopper, 1979).

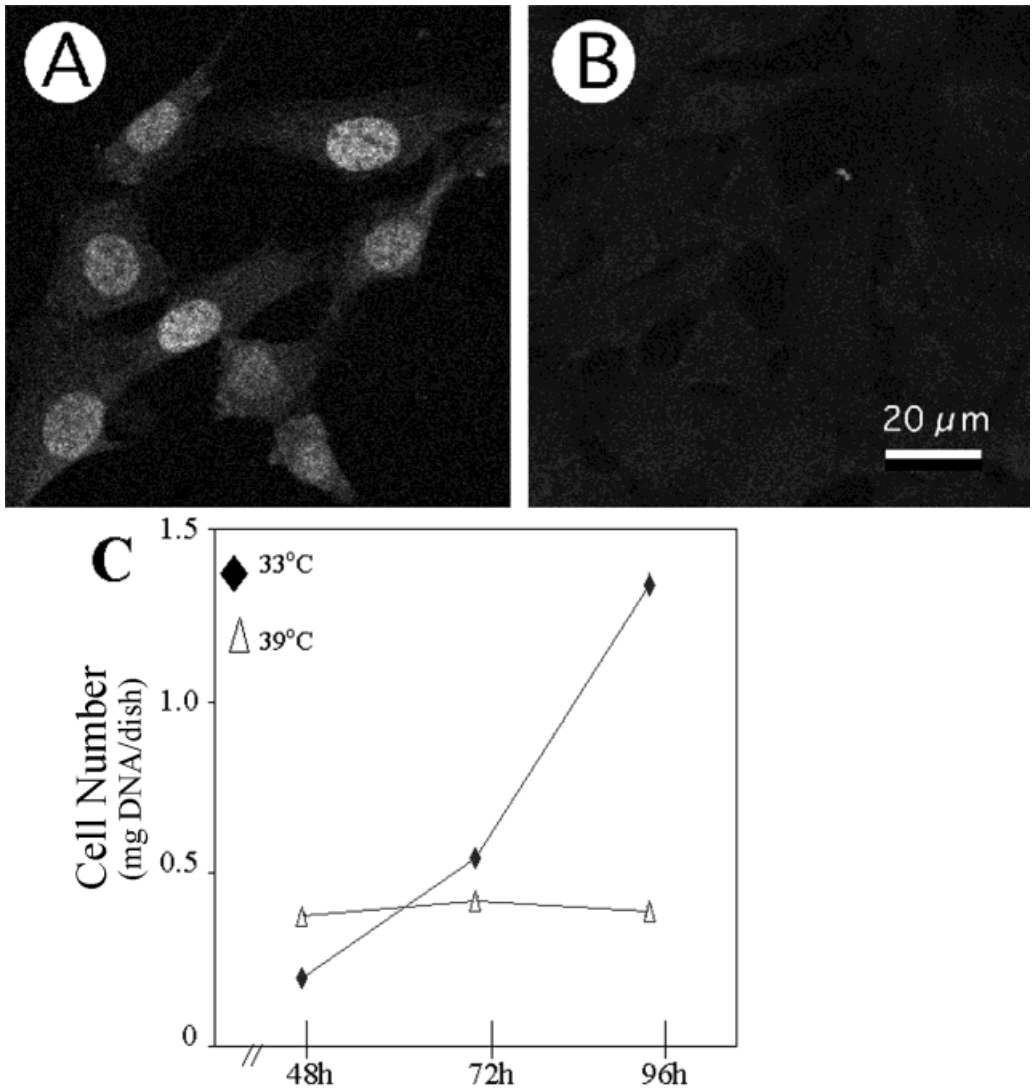

Fig. 2. T antigen distribution and temperature sensitive growth at 33°C and 39°C. Immunofluorescence microscopy demonstrated differences in large T antigen distribution between cells grown at 33°C and 39°C. At the permissive temperature, large T antigen is detectable in the nucleus (**A**). At the restrictive temperature, no detectable nuclear accumulation is apparent (**B**). Growth rates studied over 96 hr showed that cells continued to divide at 33°C (diamond), whereas at 39°C (triangle) growth was arrested (**C**). Scale bar = 20μm.

RESULTS

Mouse cortical astrocytes were conditionally immortalized using the temperature-sensitive mutant form of the SV40 large T antigen to produce the BAS8.1 cell line. At the proliferation-permissive temperature (33°C), cells can be transfected, cloned, and grown in large numbers for biochemical characterization. When cells are shifted to the non-permissive temperature (39°C, equivalent to the temperature of the rodent CNS), proliferation should stop, enabling the cells to be transplanted in a safe, non-tumorigenic state.

To characterize our ability to control proliferation and transgene expression, we used the BAS8.1 cell line to genetically engineer a β-galactosidase-producing cell line (BASlinβgal) and a GABA-producing cell line (BASlin65), as described below. Immunofluorescence microscopy demonstrated differences in large T antigen distribution between cells grown at 33°C and 39°C. At the lower temperature, BASlin65 showed strong localization of large T antigen to the nucleus (Fig. 2A). In contrast, at the higher temperature, BASlin65 had no detectable nuclear accumulation of large T antigen, presumably due to large T antigen protein denaturation or increased degradation (Fig. 2B). When these cell lines were shifted from 33°C to 39°C, the cells stopped proliferating and took on a flatter, more extended shape. This cessation of growth is documented in Figure 2C, that shows 96 hr growth curves at both 33° and 39°.

To test for regulated expression of a foreign transgene, we transfected BAS8.1 cells with the reporter construct LIN(βgal) (Fig. 1B). Cytochemical analysis of BASlinβgal cells confirmed that β-galactosidase activity was expressed, and that this expression was tightly regulated by tetracycline (Fig. 3A and 3B). Quantitative analysis of enzymatic activity demonstrated that cells grown at 33°C in the absence of tetracycline had a β-galactosidase activity of 34 nmol ONPG cleaved/mg protein/hour. After 72 hr in the presence of tetracycline, β-galactosidase

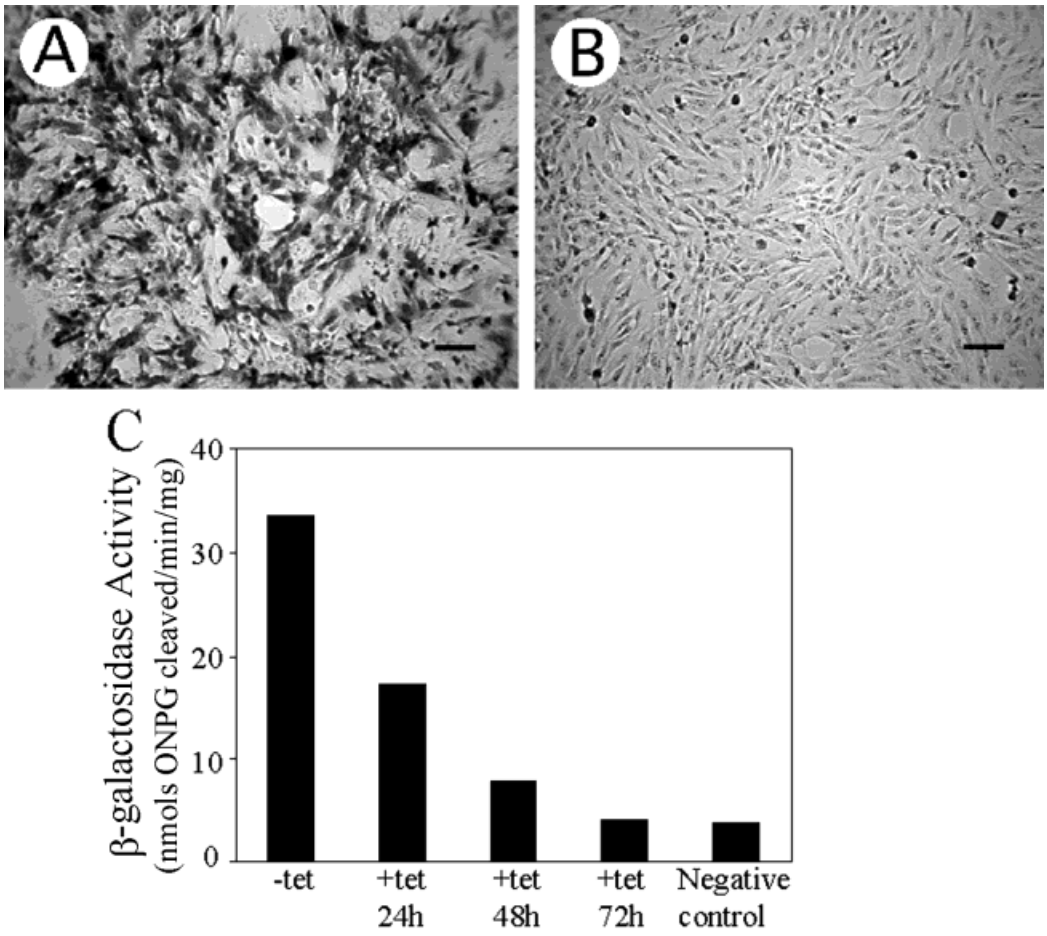

Fig. 3. Tetracycline regulates β-galactosidase expression. β-galactosidase activity in BASlin-βgal cells grown in the absence (**A**) and presence (**B**) of tetracycline. Cytochemical analysis of BASlinβgal cells showed that β-galactosidase activity was present and tightly regulated by tetracycline, confirming that our construct was effective in regulating expression of an associated insert. Cells grown at 33°C had a β-galactosidase activity of 34 nmol ONPG cleaved/mg protein/hour. Cells grown with tetracycline (1 μg/ml) had dramatically suppressed activity over 72 hr (**C**). Scale bar = 100 μM.

activity dropped dramatically (Fig. 3C). Similar β-galactosidase activity and tetracycline control were observed in cells grown at 39°C (data not shown).

We then determined whether a similar construct could drive the expression of the GABA-synthesizing enzyme, GAD. Because GAD_{65} has a reported role in synaptic release of GABA, whereas GAD_{67} likely plays a role in cellular metabolism, we chose this isoform because we are ultimately interested in transplanting our engineered cells into specific brain regions to provide a localized source of released GABA (Erlander and Tobin, 1991; Erlander et al., 1991; Kaufman et al., 1991). The parent astrocytic BAS8.1 cell line did not express significant amounts of endogenous GAD_{65}, as tested by RT-PCR (data not shown). We transfected these cells with the construct LIN(2.3RG65), in which rat GAD_{65} expression is driven by the same tetracycline-responsive element that we used to regulate β-gal (Fig. 1A). After transfection, we tested several clonal populations for GAD activity, that varied from 33 to 112 nmol CO_2/mg protein/hr. We chose one stable cell line, BASlin65, with intermediate GAD activity, to further characterize GAD_{65} and GABA production. Most of our characterization was done on proliferating cells grown at 33°C. We also performed additional studies on non-proliferating cells maintained at 39°C, at which temperature cells survived at least three weeks in culture.

Consistent with the results obtained for β-galactosidase expression in BASlinβgal cells, we demonstrated that the expression of GAD_{65} in BASlin65 cells was highly regulated by tetracycline. GAD_{65} polypeptide was clearly evident in rat brain extracts (Fig. 4, lane 1) as well as in BASlin65 cells grown in the absence of tetracycline (Fig. 4, lane 3). When grown in the presence of 1ug/ml tetracycline, however, the level of GAD_{65} expression decreased dramatically to undetectable levels (Fig. 4, lane 3), indistinguishable from BASlinβgal cells (Fig. 4, lane 2).

We also assayed GAD enzymatic activity in BASlin65 cells in the presence and absence of tetracycline, at both 33°C and 39°C (Fig. 5). GAD activity at 33°C was 40 nmol CO_2/mg protein/hr without PLP and 67 nmol CO_2/mg protein/hr with PLP, a 59% increase in GAD activity. This stimulation of GAD activity is consistent the known PLP-dependence of GAD_{65} (Erlander et al., 1991; Kaufman et al., 1991). Paralleling our results on GAD protein levels, GAD activity was tightly controlled by tetracycline, as shown by the dramatic decrease (>90%) in GAD activity in BASlin65 cells grown in the presence of

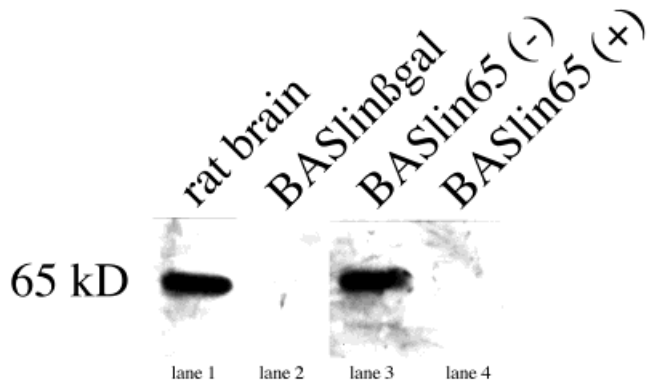

Fig. 4. Tetracycline regulates GAD_{65} production. In BASlin65 cells GAD_{65} polypeptide was detectable in rat brain extracts and in BASlin65 cells grown in the absence of tetracycline (**lanes 1** and **3**). In the presence of tetracycline (1 μg/ml), however, the level of GAD_{65} expression was undetectable and indistinguishable from the BASlinβgal cells (**lane 2** and **4**).

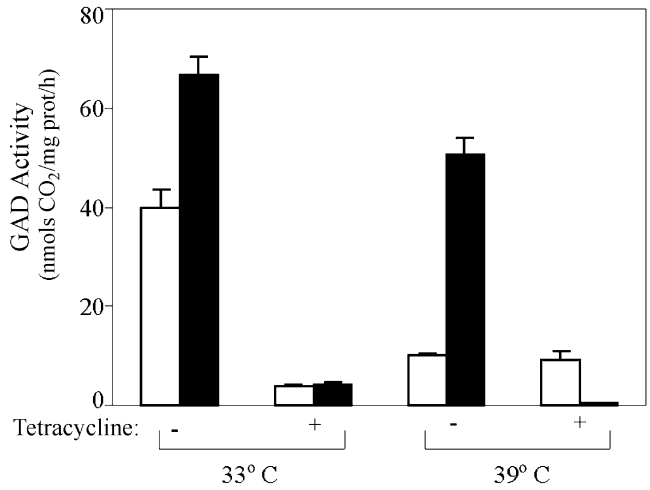

Fig. 5. Tetracycline regulates GAD enzymatic activity in BASlin65 at both 33°C and 39°C. Cell extracts were incubated with ^{14}C-glutamate in the presence (solid bar) and absence (open bar) of the cofactor pyridoxal phosphate (PLP). GAD activity at 33°C was 40 nmol CO_2/mg protein/hour without PLP, and 67 nmol CO_2/mg protein/hour with PLP. GAD activity was tightly controlled by tetracycline, as shown by the dramatic decrease (>90%) in GAD activity in BASlin65 cells grown in the presence of 1 μg/ml tetracycline. GAD activity and regulation were similar at 33°C and at 39°C, as BASlin65 grown at 39°C also demonstrated tetracycline-regulatable, PLP-responsive GAD activity. Error bars = SEM.

1 μg/ml tetracycline. GAD activity and regulation were similar at 33°C and at 39°C, as BASlin65 grown at 39°C also demonstrated tetracycline-regulatable, PLP-responsive GAD activity (Fig. 5). GAD activity remained high to at least 30 passages of BASlin65 cells.

Immunofluorescence localization studies of GAD_{65} protein in the transfected astrocytes indicated that GAD_{65} is distributed in a distinct perinuclear localization and punctate staining pattern (Fig. 6B). This appearance suggests that the GAD_{65} is associated with membrane vesicles in the cytoplasm of the astrocytic cells, consistent with the known ability of GAD_{65} to interact with membranes (Solimena et al., 1994). Moreover, the perinuclear localization of the GAD_{65} corresponded to that described for synaptic vesicle proteins expressed in non-neuronal cells (Feaney et al., 1993). In contrast, immunofluorescence analysis with an anti-GABA antibody showed that the distribution of GABA was diffuse throughout the cytoplasm (Fig. 6C), as would be expected for cells that lack the vesicular GABA transporter. Cells grown at 39°C showed similar distributions of GAD_{65} and GABA immunoreactivity (data not shown). Again, both GAD_{65} and GABA immunoreactivity showed tight regulation by tetracycline (Fig. 6E and 6F).

Because our goal was to generate a cell line that could serve as a vehicle for GABA delivery to distinct regions of the brain, we examined the ability of BASlin65 cells to synthesize and release GABA. The total GABA content of BASlin65 cells, as measured by HPLC, was 26 nmol GABA/10^6 cells (Fig. 7). Under resting conditions, about 7 nmol GABA/10^6 cells/hr was released from these cells into the extracellular environment (i.e., about 30% of the total cellular content/hour). As expected, GABA production, like GAD activity and GAD immunoreactivity, was tightly regulated by tetracycline (Fig. 7). We have thus demonstrated that BASlin65 cells can release significant amounts of GABA into the extracellular medium, and that this release can be regulated by tetracycline.

DISCUSSION

In this study, we used a cell line conditionally immortalized with a temperature-sensitive mutant of SV40 large T-antigen. These cells are advantageous for transplantation studies because cell growth occurs only at permissive temperatures, whereas at non-permissive temperatures cells cease mitosis but retain metabolic activity (Jat and Sharp, 1989; Whittemore and White, 1993). We have demonstrated that this astrocytic cell line is likely nontumorigenic, because at 39°C division ceases, whereas production of GAD_{65} and GABA is unaffected. Because the cells were healthy in culture at 39°C, they are likely to remain viable after transplantation, thus providing some of the many astrocyte-derived factors described previously (Kesslak et al., 1986; Smith et al., 1986; Smith and Silver, 1988; Cunningham et al., 1991; Franklin et al., 1991; La Gamma et al., 1993; Mucke et al., 1993; Takshima et al., 1994; Bradbury et al., 1995).

We engineered this conditionally immortalized astrocytic cell line to produce β-galactosidase (BASlinβgal) or GAD_{65} (BASlin65) in a tetracycline-regulatable manner. In view of previous reports that some cell lines with tetracycline-sensitive constructs have a low responsiveness to tetracycline (Ackland-Berglund and Leib, 1995; Howe et al., 1995), it was important to demonstrate that gene expression in the BASlinβgal and BASlin65 cell lines could be tightly suppressed. Both enzymatic assays and histochemical analysis showed that the BASlinβgal cell

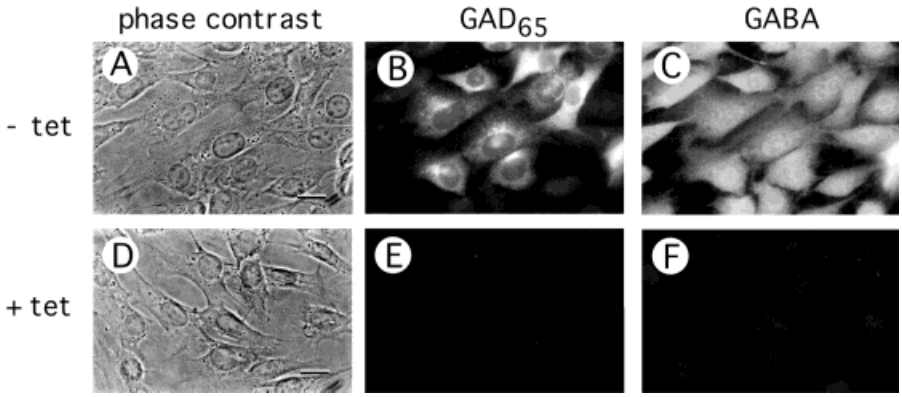

Fig. 6. Perinuclear and punctate distribution of GAD_{65} and diffuse distribution of GABA. Panels **A** and **D** are phase contrast images of the BASlin65 cells shown in panels **B**, **C** and panels **E**, **F**, respectively. GAD_{65} is present in the cytoplasm, with a distinct perinuclear localization and punctate staining pattern (B). In contrast, immunofluorescence analysis with an anti-GABA antibody showed that the distribution of GABA was diffuse throughout the cytoplasm (C). Both GAD_{65} and GABA immunoreactivity showed tight regulation by tetracycline (E, F). Scale bars = 20 μM.

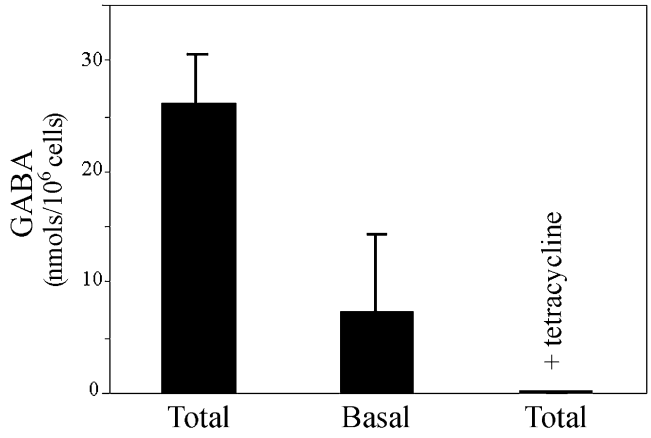

Fig. 7. BASlin65 cells synthesize and release GABA. The total GABA content of BASlin65 cells, measured by HPLC, was 26 nmol GABA/10^6 cells. GABA release after a 60 min exposure to a balanced salt solution was also measured by HPLC. Under resting conditions, about 7 nmol GABA/10^6 cells/hr was released from these cells into the extracellular environment. GABA production was tightly regulated, as BASlin65 cells grown in tetracycline produced an undetectable level of GABA. Error bars = SEM.

line is highly responsive to tetracycline. A high level of β-galactosidase expression and tight controllability in the engineered BASlinβgal reporter cell line verify that our constructs and methods were successful. Western blot analysis, GAD assays, immunofluorescence experiments, and HPLC studies showed that GAD_{65} is expressed and GABA is produced in BASlin65 grown in the absence of tetracycline, but that this expression dramatically decreases in the presence of tetracycline. The tight regulation of gene expression permits the evaluation of the effects of GABA delivery independent of cell transplantation effects.

The GAD_{65} protein is not only expressed, but it is also enzymatically normal because we have demonstrated its PLP-dependence resembles that of endogenous GAD_{65} (Erlander et al., 1991; Kaufman et al., 1991). The localization pattern of GAD_{65} in the engineered BASlin65 cell line also implies that this introduced enzyme retains properties characteristic of endogenous GAD_{65}. Immunofluorescence studies showed that GAD_{65} in BASlin65 cells can associate with membranes, a previously described characteristic of GAD_{65} rather than GAD_{67} (Kaufman et al., 1991). Moreover, the staining was not associated with all membranes, but instead was localized to particular regions of the BASlin65 cells, suggesting that the GAD_{65} associated selectively with certain intracellular membranes. In particular, in BASlin65 cells it seems that GAD_{65} behaves like a synaptic vesicle protein because the localization of GAD_{65} in these astrocytic cells is similar to the pattern reported for synaptic vesicle proteins expressed in other non-neuronal cells (Feaney et al., 1993).

As a consequence of the tetracycline-regulated expression of GAD_{65}, GABA production was also turned on and off in these cells. We have demonstrated tetracycline-regulated GABA production by quantitative HPLC analysis, that indicates that BASlin65 cells produce more GABA than other reported GABA-ergic cell lines, a finding that increases the utility of these cells as candidates for brain transplantation studies (Giordano et al., 1993; Ruppert et al., 1993; Giordano et al., 1996). Cells grown in the presence of tetracycline do not produce GABA at levels detectable by either immunofluorescence or HPLC analysis, suggesting that it should be possible to switch GABA production and release off or on in brain transplants by providing or withdrawing tetracycline.

In addition to synthesizing GABA, the BASlin65 cells also release GABA into the extracellular environment. The diffuse staining pattern of GABA suggests that BASlin65 cells do not package GABA into storage vesicles. This is consistent with the astrocytic phenotype of this cell line, because glial cells do not contain either the synaptic vesicles nor the vesicular GABA transporter that neurons use to store and release GABA (McIntire et al., 1997). Indeed, we have obtained preliminary immunofluorescence findings showing that BASlin65 cells do not express the vesicular GABA transporter protein.

The absence of the vesicular GABA transporter has important implications for GABA secretion, because release of GABA from the cytoplasm, rather than from vesicles, would not be expected to depend on increased intracellular Ca^{2+}. In fact, preliminary HPLC data suggests that GABA release from these cells is Ca^{2+}-

independent because release was unaffected by the addition of A23187, a Ca^{2+} ionophore, or by EGTA, a Ca^{2+} chelator. On the other hand, preliminary transport studies using ^{3}H-GABA showed that uptake by BASlin65 was blocked with β-alanine, a specific blocker of the glial GABA transporter GAT-3 (Liu et al., 1993). Therefore, although BASlin65 cells do not seem to have Ca^{2+}-stimulated vesicular release, it is possible that some GABA may be released from the cells through reversal of one of the plasma membrane GABA transporters (Gallo et al., 1991; Atwell et al., 1993).

In conclusion, we have generated an immortalized astrocytic cell line that expresses GAD_{65} and produces GABA in a tetracycline-regulatable fashion. These cells will help us understand the functions of GAD_{65} and GABA in a neural cell line, independent of other GABA-associated proteins such as the vesicular GABA transporter. The non-tumorigenic nature of this cell line, and the ability to control gene expression, make it an excellent choice for grafting experiments. BASlin65 cells allow significant basal release of GABA without the depolarization and Ca^{2+} influx typically required for neuronal release of neurotransmitters. Hence, these cells are attractive as a local source of GABA in transplantation studies, because they will not require specific connections or activation to trigger GABA release. We have recently obtained results that BASlin65 cells do not form tumors in vivo and survive for at least 4 weeks after transplantation into the rat brain. Furthermore, when grafted into the substantia nigra, these GABA-producing cells significantly affect the development of behavioral seizures in kindled rats (Thompson et al., 2000). Because of their safety, their tight controllability, their ability to produce and release GABA, and their potential to survive in long-term grafts, BASlin65 cells are an excellent tool for experimental studies of GABA in normal neuronal circuitry and in Parkinson's disease, Huntington's disease, and epilepsy.

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REFERENCES

Ackland-Berglund CE, Leib DA. 1995. Efficacy of tetracycline-controlled gene expression is influenced by cell type. Biotechniques 18:197–199.

Atwell D, Barbour B, Szatkowski M. 1993. Nonvascular release of neurotransmitter. Neuron 11:401–407.

Bongarzone ER, Foster LM, Byravan S, Verity AN, Landry CF, Schonmann V, Amur-Umarjee S, Campagnoni AT. 1996. Conditionally immortalized neural cell lines: potential models for the study of neural cell function. Methods: a companion to methods in enzymology. Methods 10:489–500.

Bradbury EJ, Kershaw TR, Marchbankds RM, Sinden JD. 1995. Astrocyte transplants alleviate lesion induced memory deficits independently of cholinergic recovery. Neuroscience 65:955–972.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem 72:248–254.

Bredesen DE, Hisanaga MD, Sharp FA. 1990. Neural transplantation using temperature-sensitive immortalized neural cells: a preliminary report. Ann Neurol 27:205–207.

Cunningham LA, Hansen JT, Short MP, Bohn MC. 1991. The use of genetically altered astrocytes to provide nerve growth factor to adrenal chromaffin cells grafted into the striatum. Brain Res 561:192–202.

Erlander MG, Tillakaratne NJK, Feldblum S, Patel N, Tobin AJ. 1991. Two genes encode distinct glutamate decarboxylases. Neuron 7:91–100.

Erlander MG, Tobin AJ. 1991. The structural and functional heterogeneity of glutamic acid decarboxylase: a review. Neurochem Res 16:215–226.

Feaney MB, Yee AG, Delvy ML, Buckley KM. 1993. The synaptic vesicle proteins SV2, synaptotagmin and synaptophysin are sorted to separate cellular compartments in CHO fibroblasts. J Cell Bio 123:575–584.

Fisher LJ, Jinnah HA, Kale LC, Higgins GA, Gage FH. 1991. Survival and function of intrastriatally grafted primary fibroblasts genetically modified to produce L-DOPA. Neuron 6:371–380.

Franklin RJM, Crang AJ, Blakemore WF. 1991. Transplanted type-1 astrocytes facilitate repair of demyelinating lesions by host oligodendrocytes in adult rat spinal cord. J Neurocytol 20:420–430.

Frederiksen K, Jat PS, Valtz N, Devy D, McKay R. 1988. Immortalization of precursor cells from the mammalian CNS. Neuron 1:439–448.

Gallo V, Patrizio M, Levi G. 1991. GABA release triggered by the activation of neuron-like non-NMDA receptors in cultured type 2 astrocytes is carrier-mediated. Glia 4:245–255.

Giordano M, Takashima H, Herranz A, Poltorak M, Geller HM, Marone M, Freed WJ. 1993. Immortalized GABA-ergic cell lines derived from rat striatum using a temperature-sensitive allele of the SV40 large T antigen. Exp Neurol 124:395–400.

Giordano M, Takashima H, Poltorak M, Geller HM, Freed WJ. 1996. Constitutive expression of glutamic acid decarboxylase (GAD) by striatal cell lines immortalized using the tsA58 Allele of the SV40 large T antigen. Cell Transplant 5:563–575.

Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Gottlieb DI, Chang YC, Schwob JE. 1986. Monoclonal antibodies to glutamic acid decarboxylase. Proc Natl Acad Sci USA 83:8808–8812.

Hosford DA, Clark S, Cao Z, Wilson WA, Lin FH, Morrisett RA, Hion A. 1992. The role of $GABA_B$ receptor in absence seizures of lethargic (lh/lh) mice. Science 257:398–401.

Hosimaru M, Ray J, Sah DW, Gage FH. 1996. Differentiation of the immortalized adult neuronal progenitor cell line HC2S2 into neurons by regulatable suppression of the v-*myc* oncogene. Neurobiology 93:1518–1523.

Howe JR, Skryabin BV, Belcher SM, Zerillo CA, Schmauss C. 1995. The responsiveness of a tetracycline-sensitive expression system differs in different cell lines. J Biol Chem 270:14168–14174.

Jat PS, Sharp PA. 1989. Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the non-permissive temperature. Mol Cell Biol 9:1672–1681.

Jiao S, Gurevich V, Wolff JA. 1993. Long-term correction of rat model of Parkinson's disease by gene therapy. Nature 362:450–453.

Kaufman DL, Houser CR, Tobin AJ. 1991. Two forms of the GABA synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. J Neurochem 56:720–723.

Kesslak JP, Nieto Sampedro M, Globus J, Cotman CW. 1986. Transplants of purified astrocytes promote behavioral recovery after frontal cortex ablation. Exp Neurol 92:377–390.

Labarca C, Paigen K. 1980. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem 102:344–352.

La Gamma EF, Wiesinger G, Lenn NJ, Strecker RE. 1993. Genetically modified primary astrocytes as cellular vehicles for gene therapy in the brain. Cell Transplant 2:207–214.

Lindroth P, Mopper K. 1979. High performance liquid chromatography determination of subpicomole amounts of amino acid by precolumn fluorescence derivatization with *o*-phthalaldehyde**.** Anal Chem 51:1667–1674.

Liu Q, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson N. 1993. Molecular characterization of four pharmacologically distinct γ-aminobutyric acid transporters in mouse brain. J Biol Chem 268:2106–2112.

McIntire SL, Reimer RJ, Schuske KK, Edwards RH, Jorgensen EM. 1997. Identification and characterization of the vesicular GABA transporter. Nature 389:870–876.

Mucke L, Rockenstein EM. 1993. Prolonged delivery of transgene products to specific brain regions by migratory astrocyte grafts. Transgenics 1:3–9.

Ruppert C, Sangdrasagra A, Anton B, Evans C, Schweitzer ES, Tobin AJ. 1993. Rat-1 fibroblasts engineered with GAD_{65} and GAD_{67} cDNAs in retroviral vectors produce and release GABA. J Neurochem 61:768–771.

Salle GL, Robert JJ, Berrard S, Rodoux V, Stratfold-Perricaudet LD, Perricaudet M, Mallet J. 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. Science 256:988–990.

Schubert D, Heinemann S, Carlisle W, Rarikas H, Kimes B, Patrick J, Steinbach HH, Culp W, Brandt BL. 1974. Clonal cell lines from the rat central nervous system. Nature 249:224–227.

Segovia J, Fisher LJ, Sandrasagra A, Ruppert C, Anton B, Evan CJ, Gage FH, Tobin AJ. 1992. A possible therapeutic application of cell lines engineered to produce GABA. Soc Neurosci (Abstracts) 18:781.

Smith GM, Silver J. 1988. Transplantation of immature and mature astrocytes and their effect on scar formation in the lesioned central nervous system. Prog Brain Res 78:353–360.

Smith GM, Miller RH, Silver J. 1986. Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. J Comp Neurol 251:23–43.

Solimena M, Dirkx R, Radzynski M, Mundigl O, DeCamilli P. 1994. A signal located within amino acids 1–27 of GAD65 is required for its targeting to the Golgi complex region. J Cell Biol 126:331–341.

Takeshima T, Johnston JM, Commissiong JW. 1994. Mesencephalic type 1 astrocytes rescue dopaminergic neurons from death induced by serum deprivation. J Neurosci 14:4769–4779.

Thompson K, Anantharam V, Behrstock S, Bongarzone E, Campagnoni AT, Tobin AT. 2000. Conditionally immortalized cell lines, engineered to produce and release GABA, modulate the development of behavioral seizures. Exp Neurol 161:481–489.

Tumilowicz JJ, Nichols WW, Cholon JJ, Greene AE. 1970. Definition of a continuous human cell line derived from neuroblastoma. Cancer Res 30:2110–2118.

Tuszynski MH, Um HS, Amarl KG. 1990. Nerve growth factor infusion in the primate brain reduces lesion-induced cholinergic neuronal degeneration. J Neurosci 10:3604–3614.

Whittemore SR, Holets VR, Keane RW, Levy DJ, McKay RDG. 1991. Transplantation of a temperature-sensitive, nerve growth factor secreting, neuroblastoma cell line into adult rats with fimbria-fornix lesions rescues cholinergic septal neurons. J Neurosci Res 28:156–170.

Whittemore SR, White LA. 1993. Target regulation of neuronal differentiation in a temperature-sensitive cell line derived from medullary raphe. Brain Res 615:27–40.