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GABAergic lineage differentiation of AF5 neural progenitor cells *in vitro*

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Abstract

We have previously described an immortal rat CNS progenitor cell line, AF5, which is able to exit the cell cycle and assume a differentiated state with neuronal properties. The phenotypic specification of differentiated AF5 cells, however, is not known. When induced to differentiate by serum starvation in Neurobasal media, AF5 cells down-regulated glial fibrillary acidic protein (GFAP) and up-regulated expression of β-III-tubulin (TUJ-1), medium molecular weight neurofilament protein (NF-M) and neuronal growth-associated protein 43 (GAP43). Expression of the GABA lineage marker, GAD67, increased during differentiation, suggesting that AF5 cells adopt a GABAergic lineage. RT-PCR time-course analysis of the GABAergic neuron specification transcription factor, Pitx2, showed an increase in the Pitx2 transcript 48 hr after initiation of differentiation. In differentiated AF5 cells expression of the Pitx2 target gene products GAD 65 and GABA transporter-1 (GAT-1) was increased. Cellular GABA levels in differentiated AF5 cells were increased by about 26-fold, and GABA release into the medium was 150-fold higher as compared to undifferentiated cells. Therefore, AF5 cells can be induced to differentiate to a neuronal phenotype with a GABAergic lineage.

Keywords

Neuronal development; Glutamic acid decarboxylase; γ-aminobutyric acid; Differentiation; Immortal

Introduction

Multipotent central nervous system (CNS) progenitor cells are potentially useful for *in vitro* developmental studies and for *in vivo* cell-based therapies such as neural transplantation. AF5 cells are a CNS-derived cell line immortalized from primary fetal rat mesencephalic cells with a N-terminal fragment of the SV40 large T antigen (Truckenmiller et al. 1998). AF5 cells retain plasticity similar to primary neural stem cells, i.e. they proliferate as adherent cultures in an undifferentiated state when passaged at regular intervals, yet retain the ability to exhibit neuronal properties when allowed to become confluent and remain undisturbed for several weeks (Truckenmiller et al. 2002). This passive differentiation methodology does not, however, lend itself to experimental paradigms where the developmental properties of AF5 cells can be exploited. In addition, the ability to exhibit neuronal properties is not unique, as

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many other immortal cell lines also retain such potential, especially after transplantation into the brain (Renfranz et al. 1991; Snyder et al. 1992; Shihabuddin et al. 1995; Fricker-Gates et al. 2004). There are, however, very few neural cell lines that have the capacity to manifest lineage-specific functionality during *in vitro* culture. Cell lines which have the ability to differentiate and develop into neuronal cells with a consistent lineage in culture would provide a useful *in vitro* model for the study of neuronal development and phenotype-specific differentiation.

γ-aminobutyric acid (GABA) has multiple functions in the nervous system including roles as a neurotransmitter, neurotrophin and metabolite (Waagepetersen et al. 1999). In the CNS, GABA is the primary inhibitory neurotransmitter. As a neurotrophic agent, GABA plays a regulatory role during cell differentiation and early development, and as a metabolite it is a component of the GABA shunt of the tricarboxylic acid cycle. GABA is synthesized from glutamic acid by glutamic acid decarboxylase (GAD) that exists in two isoforms, GAD65 and GAD67. GAD65 is specific to GABAergic function; it is believed to maintain the pool of GABA in nerve terminals and is activated in response to increasing demand for the neurotransmitter. In contrast, GAD67 is constitutively expressed and synthesizes cytosolic GABA for non-vesicular release or metabolic purposes (Pinal and Tobin 1998). Expression of both isoforms of GAD and the GABA vesicular transporter, GAT1, are coordinated by the homeodomain transcription factor, Pitx2 (Eastman et al. 1999). Recent studies have demonstrated the role of Pitx2 in midbrain neuron development and GABAergic neuron specification (Westmoreland et al. 2001a, 2001b; Martin et al. 2002, 2004).

There are several cell lines that either inherently, or through genetic manipulation, express GAD67 and produce GABA *in vitro* (Giordano et al. 1993, 1996; Eaton et al. 1999; Conejero-Goldberg et al. 2000). While cell lines can also be manipulated to over-express GAD65 (Behrstock et al. 2000; Thompson et al. 2000), very little has been reported on inherent GAD65 expression or putative GABAergic function for immortalized cell lines during neuronal differentiation. Promoter studies have been done in the P19 embryonic carcinoma line (Pinal et al. 1997) and GAD65 expression has been reported in human fibroblasts, as well as neuroectodermal and insulinoma cell lines (Kono et al. 2001; Salazar et al. 2001; Varju et al. 2002). The neuronal cell line NTera-2N is negative for GAD65 mRNA (Yoshioka et al. 1997), and although PC12 cells have been found to modulate GAD65 and GAD67 expression in response to hypoxia, they are not otherwise GABAergic (Kobayashi and Millhorn 2001). In the present study we describe the propensity of an immortal, non-transformed rat mesencephalic cell line, AF5, to differentiate in response to high-confluence culture conditions and serum-starvation, to express GABA specific Pitx2 target genes and to exhibit neuronal and GABAergic lineage-specific properties.

Materials and methods

Cell culture

The AF5 cell line has been previously described (Truckenmiller et al. 1998, 2002). The cells were maintained in a 5% CO_2 incubator in Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12, 1:1, Gibco Life Technologies, Gaithersburg, MD) medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Differentiation medium consisted of Neurobasal media supplemented with serum-free human B27 (Neurobasal/B27 medium) (Gibco Life Technologies). To induce differentiation, AF5 cells were grown to 100% confluence, and maintained undisturbed in complete media for one to three weeks, or changed to Neurobasal/B27 media and further incubated for two to four days as indicated. AF5 cells were also incubated for two days in Neurobasal/B27 media supplemented with 50 μM dibutyryl cyclic AMP (dbcAMP) (Sigma-Aldrich, St. Louis, MO). Additional conditions tested included growth on neuritogenic BioCoat substrates including

poly-D/L-lysine, ornithine/laminin, collagen and fibronectin (Becton Dickinson, Franklin Lakes, NJ) in combination with either serum-containing or serum-free DMEM/F12 medium, anti-proliferative agents (50 μM dbcAMP, 10nM retinoic acid, Sigma), or various combinations of cell-cycle blockers (100 μM L-mimosine, 1.5μM aphidicolin, 500μM deferoxamine, Sigma). In addition, AF5 cells were co-cultured with primary neural cultures including E15-derived rat astrocytes or cortical neurons. In some experiments, morphological observations were assessed by using AF5 cells following transient transfection of the pTG plasmid, encoding an EGFP-Tau fusion protein (kind gift of Drs. B. Hoffer and A. Tomac, NIDA) using FuGene6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's suggested protocol.

Quantitative real time RT-PCR

Total RNA was extracted from AF5 cultures using RNA STAT-60 (TEL-TEST, Friendswood, TX). Two micrograms in 2 μl of total RNA was reverse transcribed to cDNA by a reaction containing 5 μl of first strand buffer, 5 μl of 10-mM dNTP mix, 1 μl of 25 μM oligo (dT) 15, 0.5 μl of RNase inhibitor (20 U/25 μl), and 1.0 μl of Moloney murine leukemia virus reverse transcriptase (MMLV, 100 U/25 μl). The reaction was performed at 42 °C for 1 hr, followed by heating at 75 °C for 10 min, and then cooling to 4 °C. To quantify expression of Pitx2, we applied quantitative real-time RT-PCR using a DNA Engine Opticon Fluorescence Detection System (MJ Research, Waltham, MA) in a total reaction volume of 20 μl containing SYBR Green PCR master mix essentially according to the manufacturer's protocol. The primer sequences and size of the PCR product for Pitx2 was sense CAAATGGAGAAAGCGGGAGC, antisense ATGGATGAGATGGAGTTGGGCG, 246 bp. PCR was performed as follows: 95 °C for 10 min for initial denaturation, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec. The results were analyzed using Opticon software. Relative expression was calibrated by normalizing to 18S ribosomal RNA (Ambion, Austin, TX) using 1.0 for the control.

Immunocytochemistry

Cells were immunostained as previously described (Truckenmiller et al. 1998). Briefly, adherent cells were fixed with ice cold 4% paraformaldehyde or 100% ethanol at room temperature for 10 min. Following three washes with phosphate buffered saline (PBS), non-specific binding was blocked for one hour in blocking buffer (10% normal goat serum and 0.1% bovine serum albumin). The cells were incubated overnight at 4 °C with primary antibody (anti-β-III-tubulin, 1:1000, Promega, Madison, WI; anti-GAD65, 1:100 or anti-GAD67, 1:1000, Chemicon, Temecula, CA; anti-nestin, 1:1000, Pharmingen, San Diego, CA) diluted in blocking buffer. After 3 washes with PBS, the cells were incubated with secondary antibodies (anti-rabbit or anti-mouse Alexa Fluor 594, 1:500, Molecular Probes, OR) diluted in blocking buffer, and washed. Some wells were counter-stained with DAPI (Sigma-Aldrich) to stain nuclei. The cells were covered with Slow Fade Light mounting solution (Molecular Probes, OR) and viewed with a Zeiss Axiovert inverted microscope with appropriate filters. Images were captured using a Photometrics Cool Snap digital camera and IPLab software.

Immunoblot analysis

Lysates of cells cultured under undifferentiated or differentiated conditions were processed to isolate either cytosolic or membrane fractions. Lysate protein concentrations were estimated using a modified Bradford assay (BioRad) and equal amounts of each sample were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes containing cytosolic proteins were probed for GAD67 (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:1000), β-III-tubulin (Promega, Madison, WI; 1:1000), GFAP (DAKO, Carpinteria, CA; 1:1000), neuronal GAP43 (Santa Cruz, 1:2000) medium molecular weight Neurofilament

(Chemicon, 1:1000) or α-tubulin (Sigma-Aldrich) overnight at 4°C. Membranes containing cellular membrane fractions were similarly probed for GAD65 (Chemicon, 1:1000) or GAT-1 (Chemicon, 1:1000). Appropriate HRP-conjugated secondary antibodies (Biorad) were used at a dilution of 1:3000 and membranes were developed using chemiluminescent detection methods (Santa Cruz Biotechnologies).

GABA assays

AF5 cells were grown to sub-confluence in DMEM/F12 media (undifferentiated) or 24 or 48 hr days post-confluence in Neurobasal/B27 medium with 10% FCS. Lysates were harvested by scraping following the addition of a solution consisting of 0.1% Triton X-100 and 30 mM HEPES, pH 7.2. GABA levels in the lysates and culture medium were measured by HPLC using o-phthalaldehyde-sulfite derivatization (Jacobs 1987). GABA levels were normalized to total cellular protein measured using the BCA assay.

Statistical analysis

All values are expressed as means ± standard error of the mean (SEM). Groups were compared using analysis of variance (ANOVA) followed by *post-hoc* tests for individual comparisons. The criterion for statistical significance was $p < 0.05$.

Results

Differentiating conditions

A number of culture conditions, including treatment with mitotic inhibitors or neurotrophins, in combination with several neuritogenic substrates, induced partial differentiation with incomplete commitment toward neuronal phenotypes, as measured by changes in neuron-specific protein expression. Poly-D/L-lysine, ornithine/laminin, collagen and fibronectin in combination with serum-free medium, anti-proliferative agents (50 μM dbcAMP, 10 nM retinoic acid), or various combinations of cell-cycle blockers (L-mimosine, aphidicolin, deferoxamine) did not produce consistent differentiation (data not shown). In the presence of the anti-proliferative agent, dbcAMP, AF5 cells ceased dividing and extended short processes; however, no neuronal marker expression was detectable (data not shown). Figure 1 a-c shows a morphological change when AF5 cells were co-cultured with astrocytes isolated from E15 embryonic rat. This morphological change was not accompanied by neuron-associated protein expression and was not consistently reproducible. Co-culture of AF5 cells with rat cortical neurons rarely resulted in up-regulation of the neuron-specific marker β-III-tubulin; however, this was not associated with any morphological change, including neurite extension or a decrease in the ratio of cytosolic to nuclear volume (see Fig 1e, f).

The two treatment regimens which produced the most consistent differentiation were maintenance for several weeks under confluent conditions, as previously described (Truckenmiller et al. 2002), or incubation in serum-free Neurobasal/B27 medium, with or without the addition of the anti-proliferative agent, dbcAMP (Fig. 2). Figure 2a and 2b show that growth in Neurobasal/B27 media for four days stimulated a morphological change including decreased cytosolic volume and neuritogenesis. Addition of dbcAMP resulted in decreased nestin expression, increased GAD67 expression, some neurite extension, and cytosolic compaction (Fig. 2c-f). Long-term maintenance at high confluence decreased nestin and increased β-III-tubulin expression, as previously described (Truckenmiller et al. 2002), in addition to stimulating the expression of GAD65 (Fig. 2g, h). Thus, the phenotypic analysis of differentiating AF5 cells was carried out using growth to confluence followed by culture in Neurobasal/B27 medium alone.

Neuronal and GABAergic marker expression

Immunoblot analysis was performed for cultures either passaged every three days (proliferating) or allowed to become confluent and switched to Neurobasal/B27 media for four days (differentiated). In differentiated cultures, glial fibrillary acidic protein (GFAP) protein was decreased, and increases in β-III-tubulin, neuronal GAP43 and medium molecular weight neurofilament protein (NF-M) were seen (Fig. 3a).

Proliferating AF5 cells expressed basal levels of GAD67. When differentiated, however, GAD67 was markedly up-regulated (Fig. 3b). GAD67 expression, however, is also found in other neural subtypes, including glia, and is not necessarily indicative of a GABAergic lineage commitment.

GABA-specific transcription factor Pitx2 and target gene expression

There was a significant increase in Pitx2 transcription as AF5 cells differentiated in Neurobasal/B27 media (Fig. 4A). The increase in the Pitx2 transcript was transient, peaking at 48 hr, and attenuating by 4 days, consistent with its role as a developmentally-regulated transcription factor.

AF5 cells were also tested for expression of two Pitx2 target genes, GAD65 and GAT-1. Figure 4b indicates that proliferating, undifferentiated AF5 cells are negative for GAD65 and contain low levels of GAT-1 protein, but became GAD65 positive and markedly up-regulated GAT-1 after differentiation for 4 days.

GABA production

Undifferentiated AF5 cells under log-phase growth conditions contained trace levels of intracellular GABA (~0.5 nmol/mg protein). GABA levels increased ~26-fold in differentiated cells treated with fresh Neurobasal/B27 media for 24 hr (Fig. 5a). Intracellular GABA was also elevated at 48 hr post-differentiation, but diminished as compared to 24 hr, perhaps due to increased GABA efflux. The increase in intracellular GABA was not due to the media change, as no GABA increase was observed in undifferentiated cells exposed to fresh DMEM/F12, even after 48 hr. Figure 5b shows GABA efflux levels in the media of differentiated AF5 cells after 24 and 48 hr in fresh Neurobasal/B27 media, attaining levels >150-fold that of undifferentiated AF5 cells. Again, this effect is not a result of the media change, as undifferentiated cells treated with fresh DMEM/F12 media for 48 hr did not efflux appreciable amounts of GABA into the medium (< 2nmol/mg protein).

Discussion

AF5 cells expressed a GABAergic phenotype when confluent and incubated in a neuronal supportive media, Neurobasal/B27. Under these conditions, AF5 cells down-regulated neuronal precursor and glial-specific markers and up-regulated neuron-specific protein expression. They also expressed proteins specific for GABAergic neuronal function and produced GABA.

There have been reports that neural precursor cells can be differentiated *in vitro* to GABAergic phenotypes (Carpenter et al. 1999; Reynolds et al. 1992). In fact, it has been suggested that a GABAergic phenotype is a “default” differentiation pathway for neuronal precursor cells (Sah et al. 1997; Jain et al. 2003;). In nearly all of these cases, however, GABAergic lineage has been defined either by the expression of GAD67 or the presence of GABA immunoreactivity, which is not thought to indicate synaptic GABAergic function. In contrast, the AF5 cell line can be readily induced to differentiate in a manner that results in the expression of a relatively

complete GABAergic phenotype, including expression of Pitx2, GAD65, and GAD67, GAT-1, and the production of GABA.

Currently, there is a paucity of relevant cell lines suitable for *in vitro* diagnostic and/or *in vivo* therapeutic use, as the majority of studies are performed in either tumor cell lines requiring supra-physiological treatments to differentiate or primary cells requiring extensive manipulation and sub-culturing techniques to yield consistent results (Trojanowski et al. 1997; Vaudry et al. 2002). Since AF5 progenitor cells are stable *in vitro* (Truckenmiller et al. 2002) and can be readily induced to undergo GABAergic differentiation *in vitro*, these cells may provide a useful *in vitro* model for studies of GABAergic neuronal differentiation and neurodegenerative/neurocognitive disease models related to GABAergic function.

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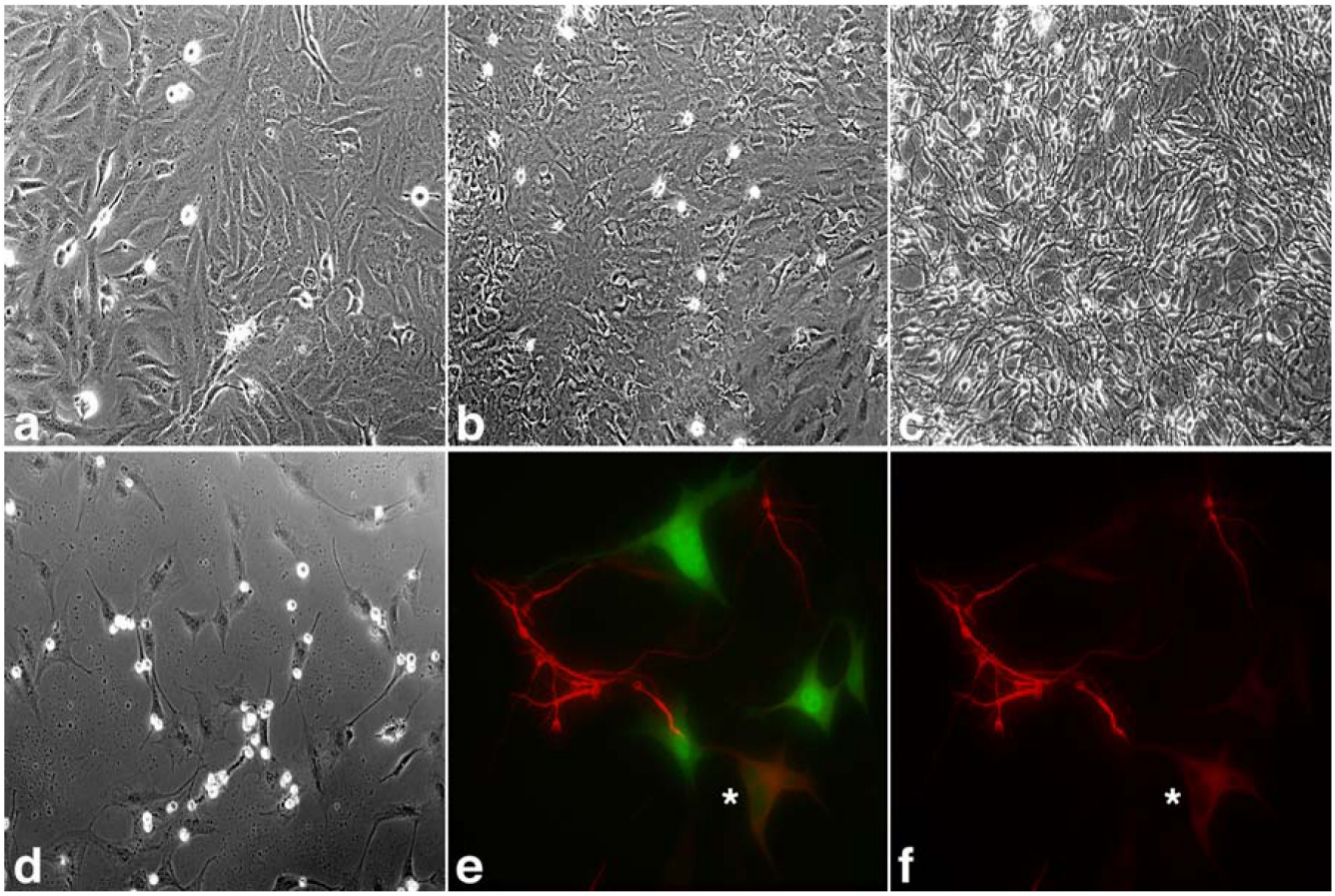

Fig. 1.

a) Confluent AF5 cells, b) Confluent E15 derived rat astrocytes, c) coculture of rat astrocytes and AF5 cells resulted in morphological changes. d) AF5 cells treated with 50μM dbcAMP for several days resulted in cell death and minor neurite extension. e) AF5 cells labeled with GFP (green) and cultured in the presence of rat cortical neurons did not undergo morphological changes. f) β-III-tubulin (red) expression increased in a minority of cells. *β-III-tubulin expression detected in a single AF5 cell.

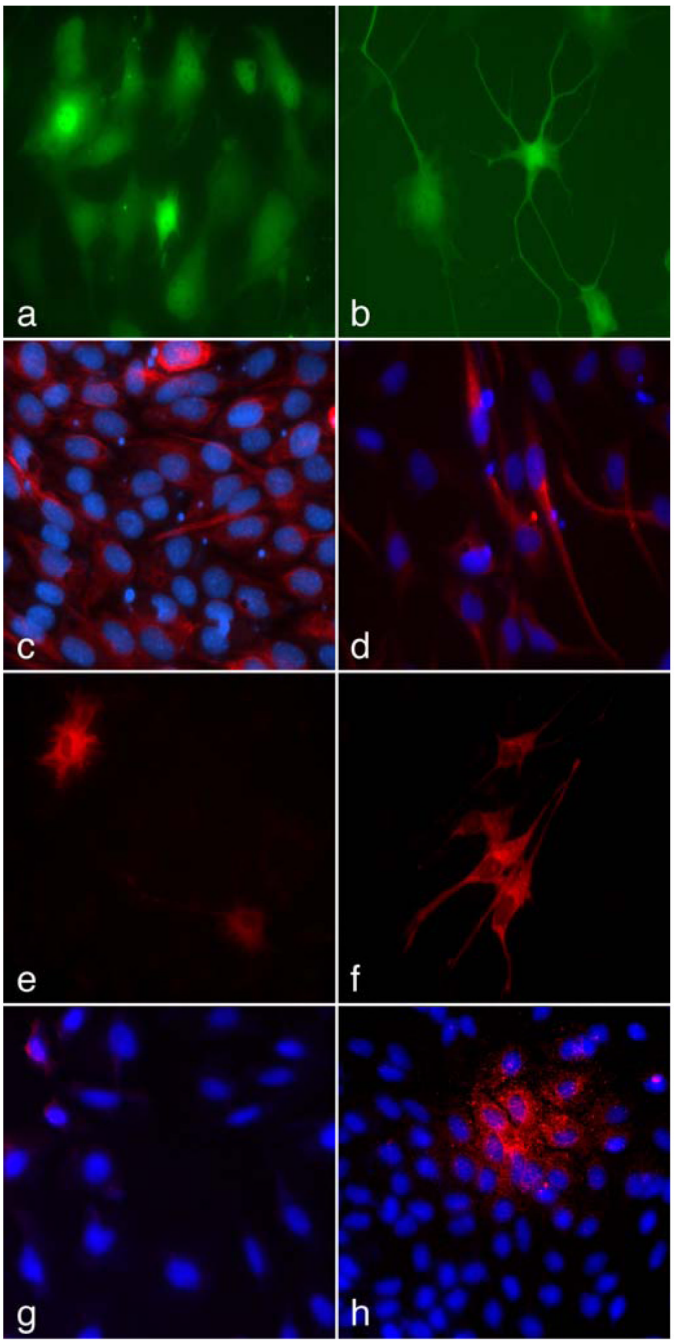

Fig. 2.

Phenotypic Changes in AF5 cells. (a, b) Cells transiently transfected with a plasmid vector encoding an enhanced green fluorescent protein/tau fusion protein (EGFP-Tau; pTG) and cultured under normal proliferative conditions or incubated in Neurobasal/B27 for 4 days. This resulted in morphological changes including neurite extension and cytosolic compaction. (c) Cells under proliferative conditions expressed high levels of nestin protein (red). (d) The number of cells that are nestin positive decreased with Neurobasal/B27 incubation supplemented with dbcAMP for 48 hr; nuclei were counterstained with DAPI (blue). (e, f) 48 hr in Neurobasal/B27/dbcAMP also modulated both cellular morphology and GAD67 expression. (g) Cells under logarithmic growth conditions were negative for GAD65, but (h)

when allowed to grow undisturbed in complete media for 1 week, began to express GAD65 (red); nuclei were counterstained with DAPI (blue).

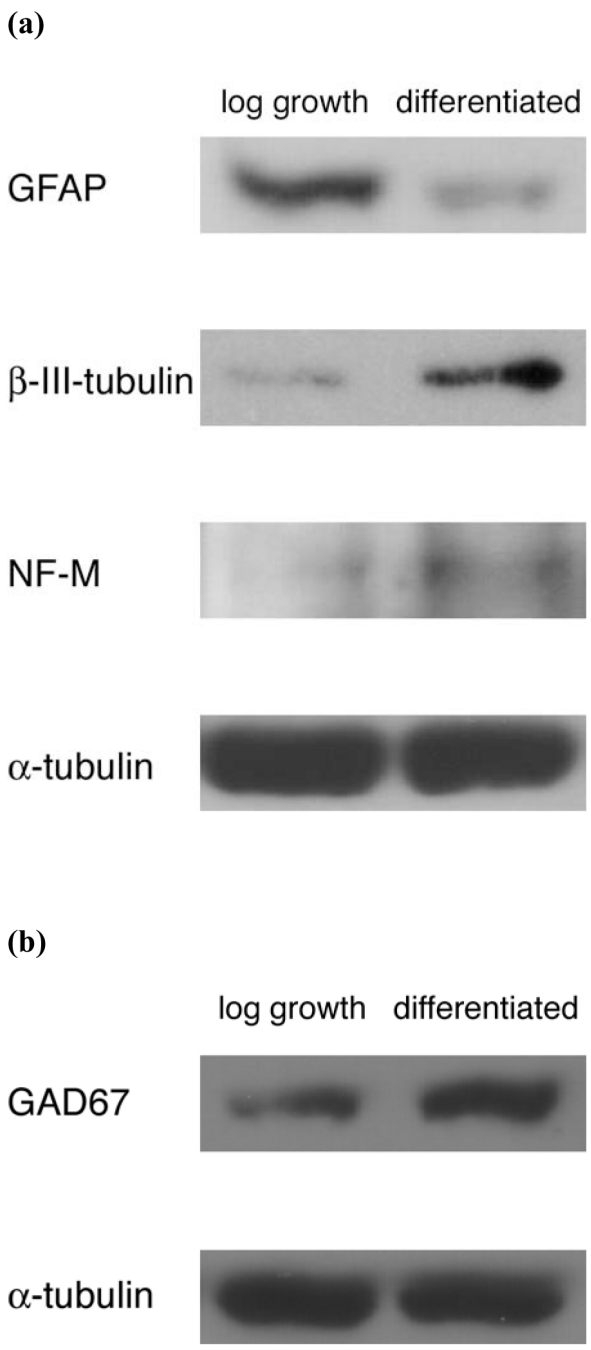

Fig. 3.

(a) Neural marker expression during differentiation. AF5 cells were incubated to sub-confluence in DMEM/F12 media (log growth) or in Neurobasal/B27 media post-confluence for 4 days (differentiated). Immunoblot analysis indicates downregulation of glial fibrillary acidic protein (GFAP) and increased expression of neuron-specific β-III-tubulin, neuronal GAP43 and neurofilament-M proteins (NF-M). Immunoblot analysis of α-tubulin is shown to indicate protein loading. (b) AF5 cells upregulated the GABAergic marker, GAD67 after 4 days in Neurobasal/B27 media indicating a potential for expression of a GABAergic phenotype.

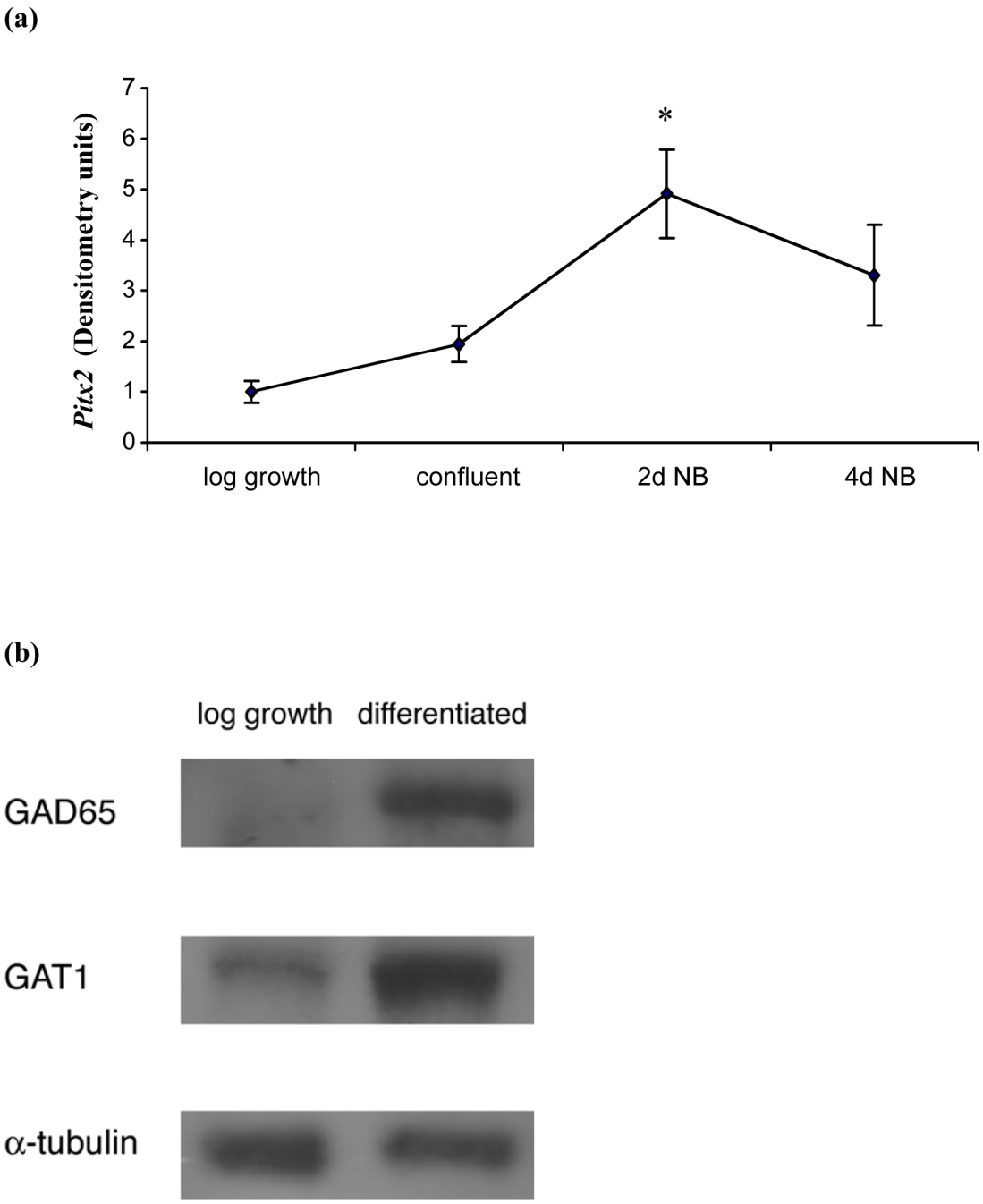

Fig. 4.

AF5 cells modulated the GABAergic-specific Pitx2 transcription factor and target protein expression. (a) Real-time PCR time-course analysis was carried out on AF5 cells under normal proliferating conditions, growth to confluence or differentiated for 2 and 4 days in Neurobasal/B27 medium. A significant increase in Pitx2 transcription factor mRNA was detected 2 days after induction of differentiation. (b) AF5 cells were incubated to sub-confluence in DMEM/F12 media (log growth) or in Neurobasal/B27 media post-confluence for 4 days (differentiated). Immunoblot analysis indicates that GAD65 protein was undetectable in AF5 cells during logarithmic growth in DMEM/F12 media, and accumulated during differentiation.

AF5 cells contain basal levels of GAT-1, which was up-regulated in response to differentiating conditions. Also shown is analysis of α-tubulin to indicate protein loading.

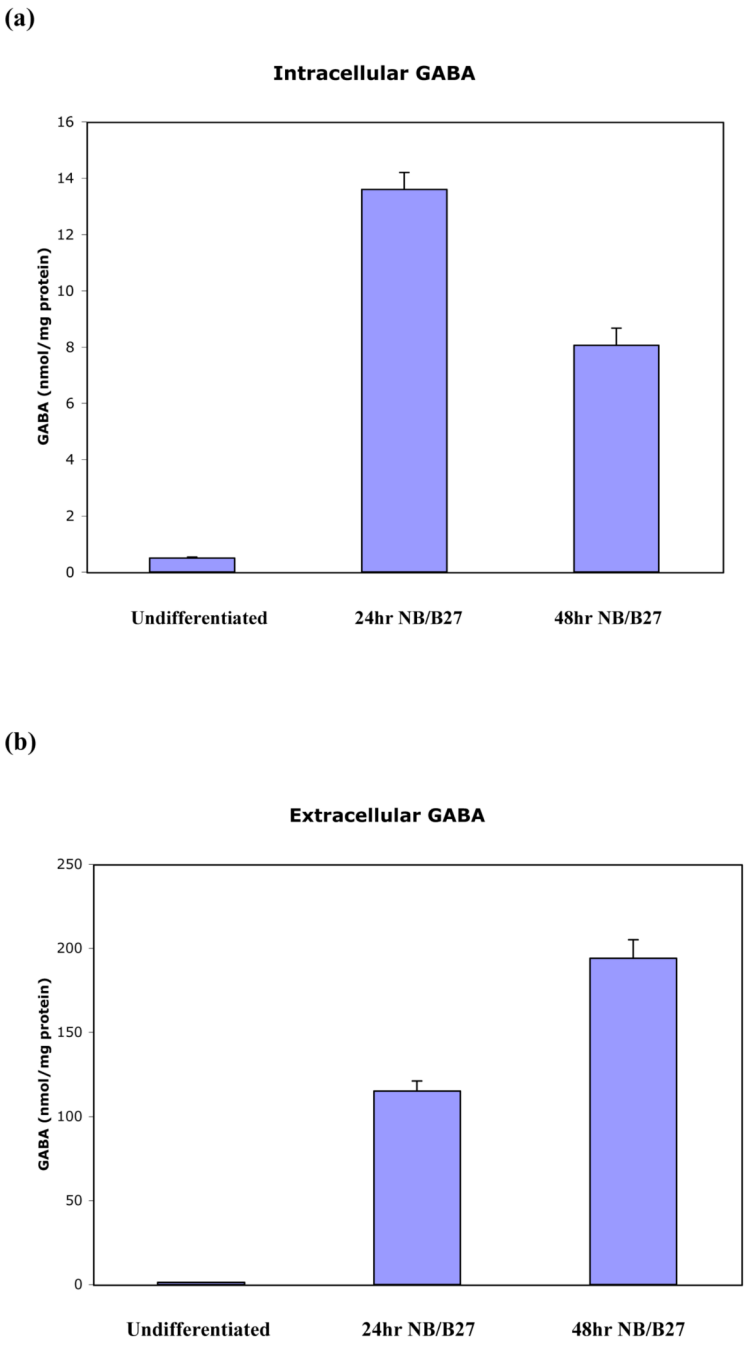

Fig. 5.

AF5 cells contain and release GABA. (a) AF5 cells cultured after confluence in Neurobasal media contained ~26-fold more GABA than undifferentiated cells, as measured by HPLC. Values of 13.6 +/- 0.6 and 8.07 +/- 0.06 nmol/mg protein were measured in differentiated cells following 24-hour and 48-hour exposure to fresh NB/B27 medium, respectively. (b) Differentiated AF5 cells also released >150-fold more GABA into the media than undifferentiated cells, generating media GABA levels of 115.0 +/- 6.0 and 194.0 +/- 11.0 nmol/mg protein after 24 and 48 hr in Neurobasal/B27 media, respectively.