British Journal of Pharmacology (1996) 119, 1393-1400

The effect of GABA stimulation on $GABA_A$ receptor subunit protein and mRNA expression in rat cultured cerebellar granule cells

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1 After 8 days *in vitro*, rat cerebellar granule cells were exposed to 1 mM γ-aminobutyric acid (GABA) for periods of 1, 2, 4, 6, 8 and 10 days. The effect of the GABA exposure on $GABA_A$ receptor α1, α6 and β2,3 subunit protein expression and α1 and α6 subunit steady-state mRNA levels, was examined using Western blotting and reverse transcriptase-polymerase chain reaction (RT-PCR), respectively.

2 GABA exposure for 2 days decreased α1 (35±10%, mean±s.e.mean), β2,3 (21±9%) and α6 (28±10%) subunit protein expression compared to control levels. The GABA-mediated reduction in α1 subunit expression after 2 days treatment was abolished in the presence of the $GABA_A$ receptor antagonist, Ru 5135 (10 μM).

3 GABA exposure for 8 days increased α1 (26±10%, mean±s.e.mean) and β2,3 (56±23%) subunit protein expression over control levels, whereas α6 subunit protein expression remained below control levels (by 38±10%). However, after 10 days GABA exposure, α6 subunit protein expression was also increased over control levels by 65±29% (mean±s.e.mean).

4 GABA exposure did not change the α1 or α6 subunit steady-state mRNA levels over an 8 day period, nor did it alter the expression of cyclophilin mRNA over 1-8 days.

5 These results suggest that chronic GABA exposure of rat cerebellar granule cells has a bi-phasic effect on $GABA_A$ receptor subunit expression that is independent of changes to mRNA levels. Therefore, the regulation of the $GABA_A$ receptor expression by chronic agonist treatment appears to involve post-transcriptional and/or post-translational processes.

Keywords: GABA; $GABA_A$ receptor regulation; cerebellar granule cells; down-regulation; up-regulation; receptor subunit protein; receptor subunit mRNA

Introduction

The γ-aminobutyric acid $(GABA)_A$ receptor is a ligand-gated ion channel receptor that mediates fast inhibitory transmission in the vertebrate central nervous system (Olsen & Toben, 1990). Structurally, the receptor is a heteroligomeric protein complex, probably composed of five subunits (Nayeem *et al.*, 1994). To date, 15 distinct subunits have been identified within the mammalian CNS; six α-subunits (α1-α6), three β-subunits (β1-β3), three γ-subunits (γ1-γ3), one δ subunit, and two ρ subunits (Sieghart, 1995). The number of different native $GABA_A$ receptor subtypes formed from these subunits is currently thought to be around ten, which vary in their relative abundance and location in the brain (Quirk *et al.*, 1994; McKernan & Whiting, 1996). The subunit composition markedly affects the pharmacology, function and regulation of the $GABA_A$ receptors, and probably underlies the functional diversity of $GABA_A$ receptors in mammalian brain (Sieghart, 1995).

Agonist-mediated receptor desensitization is a common feature of many biological systems, and appears to be an important mechanism for cells to control their responses to external stimuli (Lohse, 1993). $GABA_A$ receptors desensitize after a short period (s to min) of agonist-stimulation (Cash & Subbarao, 1987; Celentano & Wong, 1994) and are neurochemically characterized by an increase in affinity for GABA and the uncoupling of GABA/benzodiazepine interactions (Bristow & Martin, 1989). This rapid desensitization of the $GABA_A$ receptor is thought to result from phosphorylation of the receptor proteins (Sigel & Baur, 1988; Moss *et al.*, 1992; Krishek *et al.*, 1994). In addition, the chronic (hours-days) stimulation of the $GABA_A$ receptor with agonists leads to changes in $GABA_A$ receptor expression, as well as the desensitization of the receptor-mediated response (Hablitz *et al.*, 1989). Radioligand binding studies have shown that prolonged (chronic) exposure to GABA results in internalisation and down-regulation of $GABA_A$ receptors (Tehrani & Barnes, 1988; Hablitz *et al.*, 1989; Roca *et al*, 1990; Mehta & Ticku, 1992; Calkin & Barnes, 1994), as well as uncoupling between the GABA and benzodiazepine recognition sites (Roca *et al.*, 1990; Mehta & Ticku, 1992). In contrast, the chronic treatment of immature cerebellar granule cells with GABA causes an increase in $GABA_A$ agonist binding (Kim *et al.*, 1993).

Recently, it was shown that agonist-mediated $GABA_A$ receptor down-regulation of radioligand binding is accompanied by a removal of subunit proteins from the cell surface (Calkin & Barnes, 1994), which may involve receptor degradation by a non-lysosomal pathway (Borden & Farb, 1988). Furthermore, studies to understand the mechanisms underlying $GABA_A$ receptor down-regulation have observed a reduction in α1 (Montpied *et al.*, 1991), and α2 and α3 (Montpied *et al.*, 1991; Mhatre & Ticku, 1994) mRNA expression following agonist treatment. The interpretation of these findings with respect to the mechanisms of agonist-mediated receptor down-regulation is complicated by the different species (chick, mouse and rat) used in the previous investigations, and the ability of GABA to have different regulatory effects on $GABA_A$ receptor expression depending on the developmental stage (Schousboe & Redburn, 1995). Thus, the investigation of the mechanisms underlying agonist-mediated regulation of $GABA_A$ receptor expression and its relevance to, for example, tolerance or neuronal adaptation in the adult nervous system, must take into account the likely neurotransmitter or neurotrophic actions of GABA when interpreting findings from embryonic CNS tissues.

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Cerebellar granule cells have been prominent in studies characterizing the trophic effects of GABA (Belhage *et al.*, 1988; 1990; Hansen *et al.*, 1991; Kim *et al.*, 1993). They provide a good model system to study the effect of GABA on the $GABA_A$ receptor expression in both immature and mature mammalian neurones, since there appears to be a developmental switch in the response to GABA at around 7 days *in vitro*, after which the trophic effects of GABA are lost (Belhage *et al.*, 1988; 1990). The loss of neurotrophic actions of GABA coincides with the appearance of other indicators of mature cerebellar granule cells (Balazs *et al.*, 1980; Gallo *et al.*, 1982; Gao & Fritschy, 1995). Thus, cerebellar granule cells at 8 DIV seem an appropriate neuronal cell to examine the agonist regulation of $GABA_A$ receptor expression (Beattie & Siegel, 1993), avoiding the complication of trophic and neurodifferentiative actions of GABA (Schousboe & Redburn, 1995), and therefore should provide information on how receptors are regulated in mature CNS neurones.

In this study, the effects of chronic GABA treatment on the $GABA_A$ receptor $\alpha 1$, $\beta 2,3$ and $\alpha 6$ subunit protein expression, and in parallel, the $\alpha 1$ and $\alpha 6$ mRNA levels, was investigated in 8 day *in vitro* cultured rat cerebellar granule cells, to assess the regulation of $GABA_A$ receptor expression in mammalian neurones at an advanced developmental stage. An abstract of the protein expression results of this study has been presented previously (Platt & Bristow, 1994).

Methods

Cell culture

Primary cerebellar granule cell cultures were prepared from 8 day postnatal rats by a procedure described previously (Brown & Bristow, 1996). Cells were seeded at a density of 8×10^6 ($\pm 2 \times 10^6$) cells per 25 cm^2 flask for Western blotting and RNA experiments. At 8 days *in vitro* (DIV), morphological examination of the cultures after staining for neurone-specific enolase and glial fibrilliary acidic protein (GFAP, not shown) revealed that more than 98% of the cells exhibited characteristic cerebellar granule cell morphology (GFAP positive cells were $1.6 \pm 0.3\%$, mean $\pm$ s.e.mean, $n = 3000$ cells).

Experimental treatment of cerebellar granule cells

At 8 DIV, cerebellar granule cells were treated by adding GABA (1 mM) to the growth medium and maintained for periods of 1, 2, 4, 6, 8 and 10 days. GABA was replaced every 2 to 3 days during media changes. Control cell cultures were maintained in growth medium for equivalent time periods. In some cultures the $GABA_A$ antagonist, Ru 5135 (10 μM), was used either alone or in combination with GABA.

Western blotting analysis

Extraction of protein samples from cerebellar granule cells for the detection of the $GABA_A$ receptor $\alpha 1$, $\alpha 6$ and $\beta 2/3$ subunits by immunoblotting was carried out as described previously (Brown & Bristow, 1996). Proteins were electrophoretically fractionated by a modification of the Laemmli (1970) technique using a 4% (w/v) stacking gel and 10% (w/v) resolving gel. Protein extracts from 1×10^5 cells were loaded per lane. Fractionated proteins were electroblotted onto nitrocellulose filters (Amersham) by use of a Mini Trans-Blot system (Bio-Rad) for 2 h at 100 V/250 mA by a modification of the procedure described by Towbin *et al.* (1979). Equal loading was confirmed by Coomassie Blue staining of the gels after the transfer step. The nitrocellulose filters were blocked overnight at 4°C in 0.2% (v/v) TWEEN-20, 5% (w/v) low-fat dried milk, in Tris-buffered saline (TBS) (20 mM Trizma base, 150 mM NaCl, pH 7.6) before incubating the membranes in primary antibody. Membranes were incubated for 2 h at 22 ± 2°C with either the $\alpha 1$ subunit antibody (3 μg protein ml^{-1}, Zimprich *et al.*, 1991), purified $\alpha 6$ subunit antibody (1:500 dilution Dr R.M. McKernan, personal communication) or the $\beta 2,3$ subunit monoclonal antibody bd 3 (1:5 dilution, Richards *et al.*, 1988) in blocking buffer. $\alpha 1$ and $\alpha 6$ subunit proteins were detected with donkey anti-rabbit horseradish peroxidase-conjugated antibody (1:5000 dilution in blocking buffer) and $\beta 2,3$ subunit protein was revealed with sheep anti-mouse horseradish peroxidase-conjugated antibody (1:5000 dilution in blocking buffer) for 1 h at 22 ± 2°C, followed by enhanced chemiluminescence (ECL) with Hyperfilm ECL (Amersham). Optical density readings were obtained with a Shimadzu CS-9000 scanning densitometer and were used to determine % differences in $\alpha 1$, $\alpha 6$, and $\beta 2,3$ subunit protein expression in treated as compared to control cerebellar granule cell cultures. The ECL signal was calibrated by exposing ECL-treated nitrocellulose blots (from gels loaded with protein extracts from 1×10^5 cells) to Hyperfilm for increasing time periods. This produced a linear response to ECL for up to 60 min exposure time. All experimental data that were used in this study fell within this linear range. Statistical analyses were performed by use of the Mann-Whitney U Test.

Analysis of cyclophilin mRNA

Total RNA was extracted from cerebellar granule cells after the appropriate treatment time with Trizol reagent (Gibco) according to Chomczynski & Sacchi (1987). The quantity of RNA was determined by absorbance at 260 nm. RNA (2 μg) in 10 mM NaOH, 1 mM EDTA was blotted onto Zeta-probe membranes (BioRad) which was then probed with a ^{32}P-labelled cyclophilin polymerase chain reaction (PCR) product, described below. The probe was purified with Wizard PCR Prep columns (Promega). Hybridisation was done at 65°C in 7% (w/v) SDS, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, for 4–6 h. For washing, the SDS was reduced to 5 (w/v), then 1% (w/v). The membrane was exposed to X-ograph Blue X-ray film (X-ograph Ltd.) and the optical densities (O.D.) of the autoradiograms were analysed with a 600 dpi scanner utilising Image 1.51 software, calibrated with O.D. standards (Kodak).

RT-PCR of $GABA_A$ receptor $\alpha 1$ subunit, $\alpha 6$ subunit and cyclophilin mRNA

cDNA samples were prepared from cerebellar granule cell RNA (2 μg, extracted as above) by a Pharmacia First Strand Synthesis Kit. An aliquot of this reaction was then used for PCR. Primer sequences for the $\alpha 1$ and $\alpha 6$ subunits were the same as those used by Bovolin *et al.*, 1992a and Zheng *et al.* (1993), respectively. Cyclophilin primers (upstream: TCTGAGCACTGGGGAGAAAGGATT; downstream: TCGGAGATGGTGATCTTCTTGCTG) were chosen to be the same length as the $GABA_A$ receptor subunit primers and with similar G/C content. The reaction mixture contained: $\alpha 1$ or $\alpha 6$ primers, 0.8 μM of each; cyclophilin primers, 0.1 μM of each; Tris-HCl 21 mM, pH 8.4; KCl 51 mM MgCl 4 mM, DTT 0.3 mM, bovine serum albumin 1.5 $\mu g\ ml^{-1}$, deoxynucleotide triphosphates 1.2 mM each (Pharmacia), [α-^{32}P]-dCTP 2 μCi (3000 Ci $mmol^{-1}$, Amersham) and *Taq* DNA polymerase 2.5 iu (Gibco). PCR products were separated by agarose gel electrophoresis (MetaPhor agarose, FMC Bioproducts). Bands were cut out and the incorporation of ^{32}P determined by Cherenkov counting. The amount of $\alpha 1$ and $\alpha 6$ product was expressed as a % of the cyclophilin product. The difference between the treated sample and its matched control was determined and expressed as a % of the control value. Statistical significance was determined by the Wilcoxon Matched-Pairs Signed-Rank Test.

Results

GABA treatment has a biphasic effect on $GABA_A$ receptor α1 subunit protein expression

Figure 1a shows a representative Western blot of rat cerebellar granule cell extracts probed with α1 subunit-specific antibody which detected a major protein band with an apparent molecular weight of 54±3 kDa (mean±s.d., $n=29$), in agreement with that previously obtained (Zezula *et al.*, 1991). In some experiments a lower molecular weight protein band (molecular weight approx. 45 kDa) was also seen, which paralleled the GABA-induced changes in the 54 kDa band. This probably represented a proteolytic fragment of the α1 subunit according to previous findings (Stephenson *et al.*, 1990; McKernan *et al.*, 1991). No immunoreactivity was observed when the α1 antibody was preincubated with its peptide. The α1 subunit protein expression appeared to increase in control cells cultured over the culture period of 10–20 days, in agreement with previous work (Thompson & Stephenson, 1994).

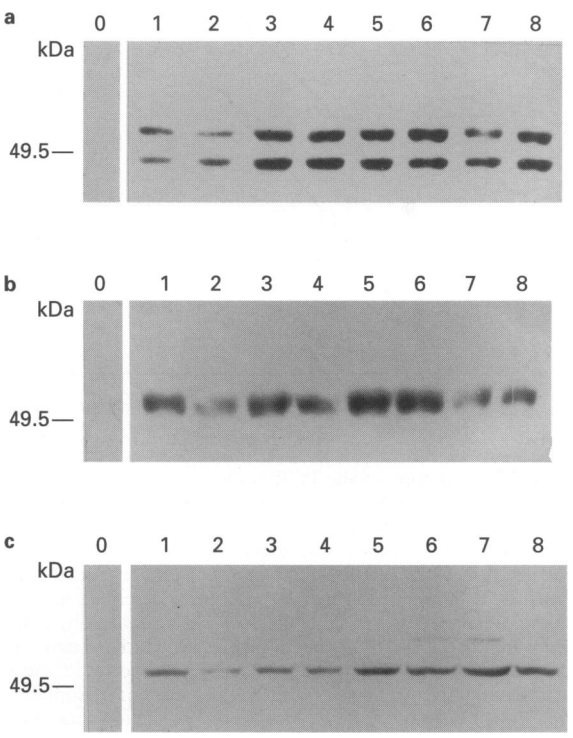

Figure 1 Representative Western blots of rat cerebellar granule cell protein extracts probed with $GABA_A$ receptor subunit-specific antibodies. After 8 days in culture, the cells were exposed to 1 mM GABA for 2–8 day periods. Protein extracts from 1×10^5 cells were loaded per lane and subjected to 10% SDS-polyacrylamide gel electrophoresis and Western blotting. Blots were probed with the appropriate antibody and visualised with Enhanced Chemiluminescence. The apparent molecular masses of the proteins were determined by calibrating the blots with prestained molecular weight markers as shown on the left hand side. (a) Rat cerebellar granule cell protein extracts probed with α1 subunit-specific antibody ($3\,\mu g\,ml^{-1}$). Lane 0: shows the immunoreactivity of α1 antibody preabsorbed with α1 peptide ($16\,\mu g\,ml^{-1}$); lane 1: 2 day control; lane 2: 2 day GABA treated; lane 3: 4 day control; lane 4: 4 day GABA treated; lane 5: 6 day control; lane 6: 6 day GABA treated; lane 7: 8 day control; lane 8: 8 day GABA treated. (b) Rat cerebellar granule cell blots probed with mAb bd 3 (1: 5 dilution). Lane 0: negative control; mAb bd 3 was replaced by hybridoma culture media. Lanes 1–8; as described in (a). (c) Rat cerebellar granule cell protein extracts probed with α6 antibody (1: 500 dilution). lane 0: negative control, α6 antibody was omitted from the primary antibody incubation step. Lanes 1–8; as described in (a).

Treatment of the cerebellar granule cell cultures with 1 mM GABA had significant effects on α1 subunit protein expression as shown in Figure 2. Exposure of the cells to GABA for 2 days resulted in a significant decrease by 35±10% (mean±s.e.mean, $n=15$, $P<0.01$) in α1 subunit protein expression below control cell levels. This GABA-induced down-regulation of α1 subunit protein was completely inhibited by the addition of 10 μM Ru 5135 (3α-hydroxy-16-imino-5 β-17-aza-androstan-11-one) to the cell culture media during the 2 day GABA treatment period (16±16% over control; mean±s.e.mean, $n=3$, $P<0.05$).

Prolonging the GABA treatment period to 4 days led to a significant increase in α1 subunit protein expression over control cell levels (15±8%; mean±s.e.mean, $n=8$, $P<0.05$). This trend continued, with 20–40% increases in α1 subunit protein expression observed after 6 and 8 day treatment with 1 mM GABA. In general, a decreased subunit expression, though of smaller magnitude, was observed with 100 μM GABA (K, Platt, unpublished) and infers that the GABA-induced reduction in $GABA_A$ receptor subunit protein expression is a concentration-dependent effect (Brown & Bristow, 1996).

GABA treatment has a biphasic effect on $GABA_A$ receptor β2,3 subunit protein expression

Probing rat cerebellar granule cell extracts with mAb bd 3, which recognises an epitope on both the β2 and β3 subunits (Haring *et al.*, 1985), revealed a protein band of 55±4 kDa (mean±s.d., $n=22$, Figure 1b), which corresponds to that previously obtained (Haring *et al.*, 1985; Richards *et al.*, 1988). Replacing mAb bd 3 with hybridoma culture media resulted in no immunoreactivity, confirming method specificity.

Significant reductions (20–25%) below control cell levels in β2,3 subunit protein expression were observed when the cells were treated with 1 mM GABA for 2 and 4 days (Figure 2). In contrast, exposure of the cells to 1 mM GABA for longer periods (6–10 days) resulted in a 60–70% increase over control levels ($P<0.01$) in β2,3 subunit protein expression.

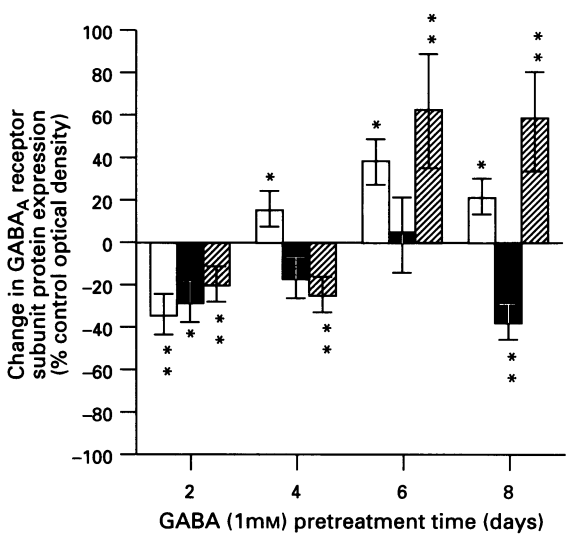

Figure 2 Time course of changes in the level of $GABA_A$ receptor subunit protein expression determined by Western blotting analysis in rat cultured cerebellar granule cells following treatment with 1 mM GABA. Optical density readings of the luminescence detection films were taken by a scanning densitometer and were used to calculate percentage differences in subunit protein expression in GABA treated as compared to control cell cultures. Open columns, α1 subunit; solid columns; α6 subunit; hatched columns, β2,3 subunit. Values are mean±s.e.mean obtained from 3 to 15 separate cultures. *$P<0.05$, **$P<0.01$ (Mann-Whitney U Test).

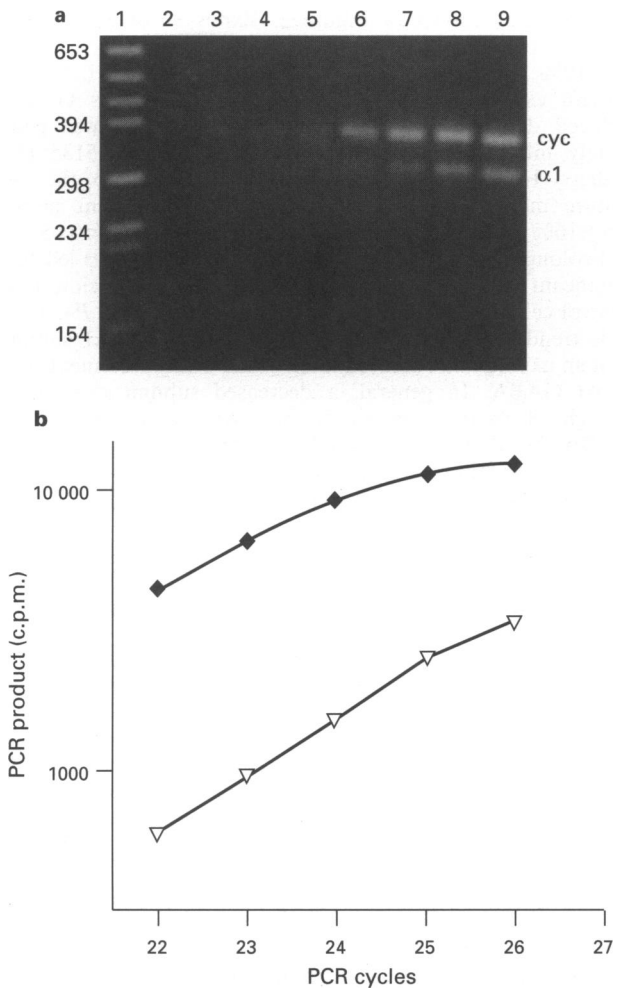

Figure 3 Optimization of the PCR protocol to measure α1 subunit and cyclophilin mRNA. (a) Representative reverse transcriptase polymerase chain reaction (RT-PCR) amplification profiles for cyclophilin (cyc) and α1 subunit (α1) transcripts. Total RNA was extracted from cerebellar granule cells as detailed in the methods and 2 μg RNA were used for cDNA synthesis. PCR amplification was carried out as described in methods for 19–26 cycles (lanes 2–9). The PCR products were visualized by ethidium bromide staining. Lane 1 shows the DNA size markers (numbers refer to base pairs). The size of the α1 and cyclophilin products were 304 and 366 base pairs, respectively. (b) Cumulative graph of the cDNA products of cyclophilin (◆) and α1 subunits (▽) in the same incubations at the indicated number of PCR cycles. The amount of PCR product was measured by radioactive counting of the bands as described under methods. The results are expressed as means ± s.e.mean of counts per minute from >3 separate experiments; c.p.m. = counts per minute (Cherenkov counting).

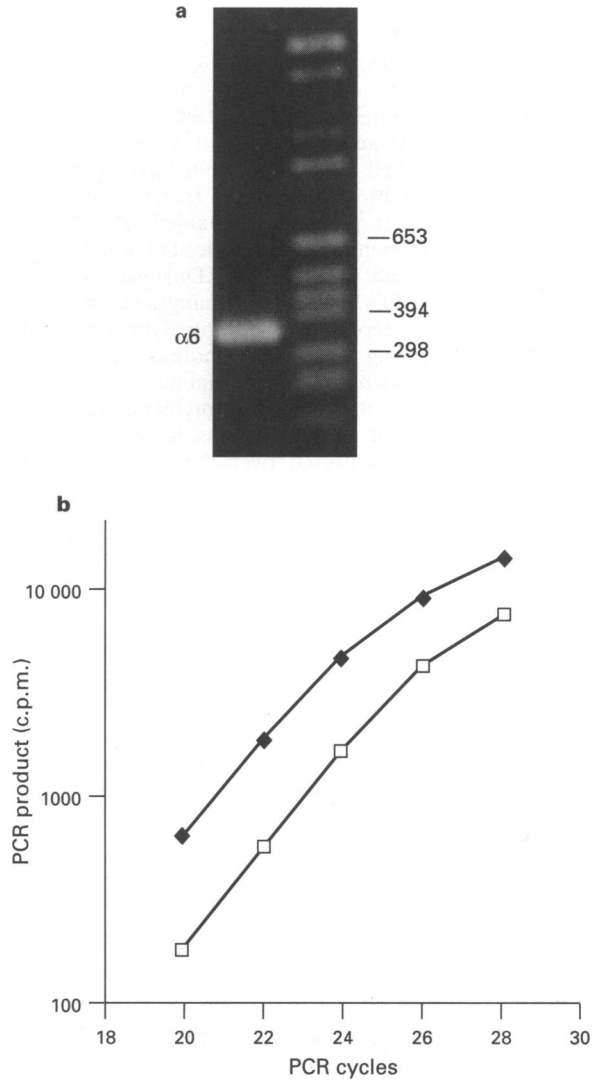

Figure 4 Optimization of the PCR protocol to measure α6 subunit and cyclophilin mRNA. (a) Representative reverse transcriptase polymerase chain reaction (RT-PCR) amplification for the α6 subunit (α6) transcript. Total RNA was extracted from cerebellar granule cells as detailed in the methods and 2 μg RNA were used for cDNA synthesis. PCR amplification was carried out as described in methods for 23 cycles (left lane). The PCR product was visualized by ethidium bromide staining. The size of the α6 product is 348 base pairs. DNA size markers (numbers refer to base pairs) are shown in the right lane. (b) Cumulative graph of the cDNA products of cyclophilin (◆) and α6 (□) subunits in the same incubations at the indicated number of PCR cycles. The amount of PCR product was measured by radioactive counting of the bands as described under methods. The results are expressed as mean ± s.e.mean of counts per minute from >3 separate experiments; c.p.m. = counts per minute (Cherenkov counting).

The effect of chronic GABA stimulation on $GABA_A$ receptor α6 subunit protein expression

Western blotting analysis of rat cerebellar granule cell extracts with α6 antibody revealed a protein of apparent molecular weight 56 ± 2 kDa (mean ± s.d., $n = 18$, Figure 1c), in agreement with a previously published value (Pollard *et al.*, 1993). Immunoreactivity was absent when control strips of nitrocellulose were incubated in blocking buffer not containing α6 antibody during the primary antibody incubation step.

As shown in Figure 2, chronic stimulation of $GABA_A$ receptors had significant effects on α6 subunit protein expression. Treatment of cells with 1 mM GABA for 2 and 8 days resulted in 30–40% reductions below control cells in α6 subunit protein levels, whereas exposure to 1 mM GABA for 4 and 6 day periods failed to have any significant effect. In contrast, a 65 ± 29% (mean ± s.e.mean, $n = 4$, $P < 0.01$) increase over control cells in α6 subunit protein expression was observed after 10 days treatment with 1 mM GABA.

GABA treatment does not alter the cyclophilin mRNA levels

To obtain accurate mRNA measurements and to control for any possible non-specific effects of GABA treatment on the levels of granule cells RNA, an internal standard RNA was required. Cyclophilin is an abundant cytosolic protein in most eukaryotic cells and is calculated to represent 1% of the mRNA found in the cytosol of animal cells (Milner & Sutcliff, 1983). A single PCR product was obtained for

cyclophilin (366 base pairs (b.p.), Figure 3a), for the α1 subunit (304 b.p., Figure 3a), and for the α6 subunit (348 b.p., Figure 4a). Furthermore, upon incubation with Hinf I (α1 and cyclophilin) or Hpa II (α6) the digestion products for cyclophilin (303, 63 b.p.), α1 (174, 130 b.p.), and the α6 product (175, 165, 8 b.p.), were of the expected size, indicating that the products are derived from the intended targets. Figure 5 shows the unchanging expression of cyclophilin mRNA after GABA treatment for 1–8 days, confirming the validity of using cyclophilin as a standard mRNA marker. To establish the appropriate number of PCR cycles to amplify α1 and α6 when cyclophilin primers were present in the same reaction, the PCR product from α1 and cyclophilin (Figure 3b) and α6 and cyclophylin (Figure 4b) was measured at increasing number of PCR cycles. Over 22–25 PCR cycles for the α1 and cyclophylin (Figure 3b), and over 20–24 cycles for the α6 and cyclophilin (Figure 4b), the products were rising in the log-linear amplification range and showed a constant relationship to each other. Thus, 23 PCR cycles were used throughout the study to measure the mRNA levels of the individual subunits.

GABA treatment does not change α1 or α6 mRNA expression over 1–8 days of treatment

Figure 6 shows that GABA treatment does not alter the α1 steady-state mRNA levels in GABA-treated compared to control cultures at 1, 2 or 8 days. Similarly, the expression of the α6 subunit mRNA compared to control cultures is unaffected by GABA (Figure 6).

Discussion

The results of this investigation suggest that chronic GABA treatment of rat 8 DIV cultured cerebellar granule cells has a biphasic effect on $GABA_A$ receptor subunit protein expression. Initially, the response of the cells is to down-regulate the α1, β2,3 and α6 subunits, followed by an up-regulation after longer stimulation periods. The switch from GABA-induced down-regulation to increased $GABA_A$ receptor subunit protein expression occurred over a different time course for each of the subunits investigated, suggesting that their expression may be differentially regulated. The process, at least for the α1- and α6-subunits, appears to be independent of alterations in the steady-state mRNA levels, and thus is likely to involve post-transcriptional and/or post-translational mechanisms.

Agonist-mediated down-regulation of $GABA_A$ receptors has been inferred from the reduction in radioligand binding sites associated with the $GABA_A$/benzodiazepine receptor complex after chronic exposure to GABA (Maloteaux *et al.*, 1987; Hablitz *et al.*, 1989; Roca *et al.*, 1990; Mehta & Ticku, 1992). Under similar conditions, the function of the $GABA_A$ receptor is decreased (Hablitz *et al.*, 1989) and uncoupling between the GABA and benzodiazepine binding sites has been described (Roca *et al.*, 1990; Mehta & Ticku, 1992). More recently, in studies in embryonic chick neurones (Calkin & Barnes, 1994; Baumgartner *et al.*, 1994) and foetal mouse cerebral cortical neurones (Mhatre & Ticku, 1994) GABA-mediated down-regulation of certain $GABA_A$ receptor subunit proteins has been observed. However, GABA is known to have trophic and differentiative actions in immature neurones (Meier *et al.*, 1991) and may serve different functions during ontogeny (Schousboe & Redburn, 1995), making the interpretation of results from embryonic or foetal neurones difficult to relate to possible changes that occur in the adult CNS. To overcome this potential problem, this study has addressed the effect of GABA treatment on $GABA_A$ receptor subunit polypeptide and mRNA expression in rat cerebellar granule cells, at an advanced developmental age (8 DIV). At this stage in cerebellar granule cell development, GABA is presumably acting purely as a neurotransmitter, and is without the complication of trophic or neurodifferentiative actions (Schousboe & Redburn, 1995).

The results of this investigation showed that treatment of rat 8 DIV cerebellar granule cells with GABA results in a transient decrease in α1, β2,3 and α6 subunit protein expres-

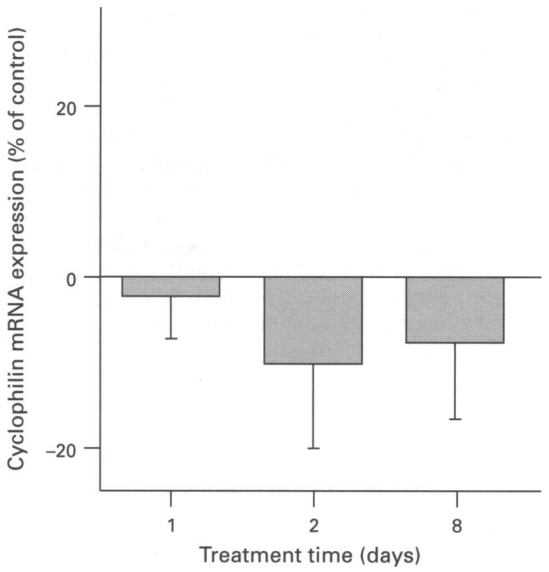

Figure 5 The effect of GABA treatment on the expression of cyclophilin mRNA in rat cerebellar granule cells. Total RNA was extracted from GABA (1 mM) treated and control cerebellar granule cells after the indicated treatment time as detailed in the methods. The quantity of RNA was determined by absorbance at 260 nm and equal amounts (2 μg) were blotted onto Zeta-probe membranes which was then hybridized with a ^{32}P-labelled cyclophilin probe. After hybridization and washing, as detailed in methods, the membrane was exposed to X-ray film and the optical density of the autoradiogram was analysed with an flatbed 600 dpi scanner and Image 1-51 software. The results are expressed as means ± s.e.mean of the % difference from the corresponding control cells, derived from 7–9 separate experiments.

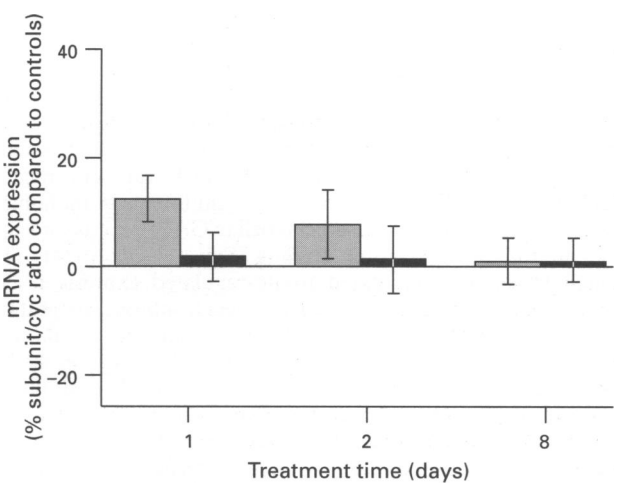

Figure 6 The effect of GABA treatment on the expression of $GABA_A$ receptor α1 (stippled columns) and α6 (solid columns) subunit mRNA in rat cerebellar granule cells. Total RNA was extracted from GABA (1 mM) treated and control cerebellar granule cells after the indicated treatment time as detailed in methods and 2 μg of RNA was used for cDNA synthesis. PCR amplification of the α subunits with cyclophilin was carried out as described in methods for 23 cycles. The amount of PCR product was measured by radioactive counting of the bands as described under methods. The α subunit PCR products were normalized by expressing them as a ratio of the cyclophilin product in the same reaction. The results are shown as means ± s.e.means of the % differences from the corresponding control cells and are derived from 12–18 (α1) and 8–17 (α6) separate experiments.

sion, that is consistent with the GABA-induced reduction in α2, and α3 subunit polypeptides in mouse cerebral cortical neurones (Mhatre & Ticku, 1994), and corresponds to the time course for loss of radioligand binding sites (Maloteaux *et al.*, 1987; Hablitz *et al.*, 1989; Roca *et al.*, 1990; Montpied *et al.*, 1991; Mehta & Ticku, 1992). The GABA-induced down-regulation of α1 subunit protein expression was inhibited by the $GABA_A$ receptor antagonist Ru 5135, supporting a $GABA_A$ receptor-mediated effect, as previously observed with bicuculline (Brown & Bristow, 1996).

The mechanisms that underlie the agonist-mediated down-regulation of $GABA_A$ receptors are poorly understood (Sieghart, 1995), and probably vary with the intensity of agonist stimulation and in the species or neuronal cell type. Our observations would suggest that the down-regulation of α1 and α6 subunit proteins in rat cerebellar granule cells is independent of changes in steady-state mRNA levels, contrasting with previous studies in foetal mouse (Hirouchi *et al.*, 1992; Mhatre & Ticku, 1994) and embryonic chick (Montpied *et al.*, 1991) cerebral cortex neurones. The current findings presented therefore support a translational or post-translational mechanism that underlies the reduction of receptor subunit protein expression. An increased degradation of the proteins is the most likely interpretation of these findings. The technique of Western blotting employed here will detect both cell-surface and internalised proteins, and thus a reduction in protein must result from degradation of the protein (or epitope) and/or reduced synthesis of the protein subunits, rather than a sequestration of the receptors in internal stores. Previous work has also observed a rapid GABA-induced degradation of receptor proteins (Calkin *et al.*, 1994) and a receptor turnover rate (18 h, Borden & Farb, 1988) appropriate to account for a decline in subunits detectable by 2 days of treatment. In addition, earlier studies have shown that GABA-induced down-regulation of $GABA_A$ receptor binding was not a result of a non-specific decrease in protein synthesis (Roca *et al.*, 1990). The process may be different from that in embryonic and foetal neurones due to the more advanced developmental stage of 8 DIV cerebellar granule cells, perhaps representing the process used when the cells are responding to GABA as a neurotransmitter, rather than as a neurotrophic agent (Schousboe & Redburn, 1995). However, even in chick embryo cerebellar neurones, acute GABA stimulation appears to induce a down-regulation of $GABA_A$ receptor binding without any detectable reduction in subunit mRNA levels (Calkin *et al.*, 1994).

Exposure of cerebellar granule cell cultures to GABA for extended periods (>2 days) showed a shift to up-regulation of α1, β2,3 and α6 subunit protein expression. These findings are consistent with the experiments in which rats chronically treated with the GABA transaminase inhibitor, ethanolamine *O*-sulphate, showed increased cerebellar GABA levels and up-regulated $GABA_A$ receptor binding (Sykes *et al.*, 1984). The switch from down-regulated to up-regulated expression occurred over a different time course for each subunit, suggesting that their expression is under differential temporal regulation, perhaps similar to that occurring in developing cerebellar granule cell neurones (Beattie & Siegel, 1993). This situation could result in a disparity in the composition of $GABA_A$ receptors on the neuronal cell surface after chronic treatment periods. For example, after 8 days of treatment, when the levels of α6 are reduced whilst the levels of α1 and β2,3 are elevated, there may be a switch in the expression of the α1-, α6-, α1α6- or β2,3-containing receptors, leading to an alteration in the affinity or efficacy of the $GABA_A$ receptor to agonists (Puia *et al.*, 1991; Kleingoor *et al.*, 1993; Ebert *et al.*, 1994; Ducic *et al.*, 1995). A change from receptors containing α6 subunits to more containing α1 would be expected to increase the EC_{50} for GABA (Kleingoor *et al.*, 1993; Zheng *et al.*, 1993), and result in an apparent desensitization of the GABA response. Likewise, an increase in the proportion of receptors containing β2,3 subunit can influence $GABA_A$ receptor function (Verdoorn *et al.*, 1990; Ebert *et al.*, 1994; Sieghart, 1995). The situation of a reduced $GABA_A$ receptor function, by whatever mechanism, would presumably be desirable to neurones in a high GABA environment. The prediction of a change in $GABA_A$ receptor affinity is also consistent with the observation that 8 day GABA-stimulation of cerebellar granule cells induces the formation of low affinity $GABA_A$ receptors (Meier *et al.*, 1984).

The receptor subunit proteins measured in this study are the predominant subunits expressed in cerebellar granule cells (Bovolin *et al.*, 1992b), but in the mature cerebellum the predicted receptor isoforms contain other subunits, for example, γ1, γ2, α2, α3 (Quirk *et al.*, 1994; Khan *et al.*, 1996). In addition, it is known from functional studies in transfected cells that different α, β and γ subunit combinations alter GABA potency (Sigel *et al.*, 1990; Luddens *et al.*, 1994) and efficacy (Ebert *et al.*, 1994). Therefore, the switching of the α and β subunits alone may be a too simplistic mechanism to account for the response of cerebellar granule cells to chronic GABA stimulation.

It is conceivable that the increased or decreased subunit protein expression is not reflected in a comparable alteration in the receptor isoforms expressed on the neuronal cell surface, but that it is the result of changes in intracellular subunit storage. This possibility, as far as we are aware, has yet to be addressed. However, preliminary immunocytochemical data from this laboratory with bd-17 antibody, suggest an increased cell-surface expression of the β2,3 subunit after 8 days of GABA treatment (Platt & Bristow, unpublished), lending support to increased cell-surface $GABA_A$ receptor expression containing the β2,3 subunit.

In immature cerebellar granule cell neurones, GABA induces an up-regulation of $GABA_A$ receptor subunit mRNA (Kim *et al.*, 1993), but this is due to the trophic effects of GABA (Meier *et al.*, 1991; Schousboe & Redburn, 1995), and is only evident up until 7 DIV (Belhage *et al.*, 1988). It seems unlikely that the increased $GABA_A$ receptor subunit protein expression observed here is due to the trophic actions of GABA, as the age of the granule cells correspond to post-natal day 16 (P16) at the start of the treatment, with the increase being seen at the equivalent of >P20. At P20 the cells should have developed to a mature cerebellar granule cell phenotype (Gao & Fritschy, 1995). Furthermore, the morphology of the cells at 8 DIV (P16) to 16 DIV (P24) correlate with that of post-migratory cerebellar granule cells (Palay & Chan-Palay, 1974; Hatten *et al.*, 1984). Therefore, it seems more probable that the increase in expression of $GABA_A$ receptor subunit protein expression represents a novel mechanism of $GABA_A$ receptor regulation. This finding, however, is not the only example of agonist-induced up-regulation of ligand-gated ion channel receptors, since an increase in L-[^{3}H]-nicotine binding is also observed in the brain after chronic nicotine treatment (Schwartz & Kellar, 1983).

The mechanisms underlying this GABA-stimulated increase in $GABA_A$ receptor subunit protein expression are unknown, but the results presented here do not support a role for mRNA levels in regulating the protein expression. Similarly, other workers have not observed increased steady-state mRNA levels after long-term agonist treatment (Hirouchi *et al.*, 1992). Two plausible explanations for the up-regulated protein expression are increased translation of the $GABA_A$ receptor subunit mRNA and/or a decreased receptor turnover, but further studies are required to clarify which (if any) of these mechanisms are employed.

In conclusion, the response of rat cerebellar granule cells to chronic GABA stimulation, at an age equivalent to post-migratory granule cells *in vivo*, ranges from an initial transient down-regulation of subunit proteins to an increased expression, both of which appear independent of changes in steady state mRNA levels. Although the agonist-mediated down-regulation is consistent with previous studies, the up-regulation and the RNA-independence of the pathways contrast with previous observations in embryonic chick and mouse cerebral cortical neurones. This suggests possible species, brain region and developmental age differences in the mechanisms underlying the responses of cultured neurones to chronic GABA treatment.

The authors wish to express their thanks to Dr R.M. McKernan, Dr J.G. Richards and Professor W. Sieghart for their kind gifts of $GABA_A$ receptor subunit-specific antibodies. Many thanks also go to Dr P. Sharpe and Dr Tidd for the use of their scanning densitometers. The expert technical support of Mr A. Oneguebu and Mrs D. Price is gratefully acknowledged. This work was funded by The Medical Research Council (G9213132N), The Nuffield Foundation, and the University of Manchester Research Support Fund.

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(Received August 5, 1996
Revised September 5, 1996
Accepted September 6, 1996)