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*Journal of Neurochemistry*, 2003, **84**, 743–752 doi:10.1046/j.0022-3042.2003.01557.x

# GABA$_B$ receptor mRNA in the raphe nuclei: co-expression with serotonin transporter and glutamic acid decarboxylase

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**Abstract**

We have used double-label *in situ* hybridization techniques to examine the cellular localization of GABA$_B$ receptor mRNA in relation to serotonin transporter mRNA and glutamic acid decarboxylase mRNA in the rat dorsal raphe, median raphe and raphe magnus nuclei. The degree of cellular co-localization of these markers notably varied among the different nuclei. In the dorsal raphe, cell bodies showing GABA$_B$ receptor mRNA were very abundant, the 85% being also labelled for serotonin transporter mRNA, and a low proportion (5%) showing glutamic acid decarboxylase mRNA. In the median raphe, the level of co-expression of GABA$_B$ receptor mRNA with serotonin transporter mRNA was significantly lower. Some cells were also identified that contained GABA$_B$ receptor mRNA in the absence of either one of the other mRNA species studied. Our results support the presence of GABA$_B$ receptors in serotonergic as well as GABAergic neurones in the dorsal and median raphe, providing the anatomical basis for the reported dual inhibitory/disinhibitory effect of the GABA$_B$ agonist baclofen on serotonergic function.

**Keywords:** dorsal raphe, double-labeling, GABA, GAD, *in situ* hybridization, median raphe.

*J. Neurochem.* (2003) **84,** 743–752.

5-Hydroxytryptamine (serotonin; 5-HT)-containing neurones are mainly localized in the midbrain and brainstem raphe nuclei (Dahlström and Fuxe 1964; Steinbusch 1981) and project to virtually all regions of the brain, being involved in a large number of physiological functions (Jacobs and Azmitia 1992; Leibowitz and Alexander 1998). The electrical activity of 5-HT neurones is under a fine control exerted by different neurotransmitter systems including, among others, serotonin itself, noradrenaline, dopamine, histamine, glutamate, γ-aminobutyric acid (GABA), glycine and several neuropeptides (for review see Jacobs and Azmitia 1992; Adell *et al*. 2002). The inhibitory neurotransmitter GABA plays an essential role in the regulation of 5-HT neurones, operating through local GABAergic interneurones as well as distal GABAergic afferents that project to the raphe nuclei (Gervasoni *et al*. 2000). Both GABA$_A$ and GABA$_B$ receptors are present in the raphe (Bowery *et al*. 1987; Price *et al*. 1987; Chu *et al*. 1990; Bischoff *et al*. 1999) and their stimulation appears to inhibit firing activity of 5-HT neurones (Gallager and Aghajanian 1976; Innis and Aghajanian 1987). Extra- and intracellular recordings have provided evidence that stimulation of GABA$_B$ receptors hyperpolarizes 5-HT neurones through the opening of a voltage-dependent, pertussis toxin-sensitive, inwardly rectifying potassium channel coupled to a G protein, which is also operated by 5-HT$_{1A}$ receptors (Innis and Aghajanian 1987; Colmers and Williams 1988; Innis *et al*. 1988; Williams *et al*. 1988). This would presuppose the localization of GABA$_B$ receptors in 5-HT neurones bearing 5-HT$_{1A}$ autoreceptors. Indeed, recent immunohistochemical studies have demonstrated the co-existence of GABA$_B$ receptor-like immunoreactivity (LI) with serotonergic cell markers in single raphe neurones (Wirtshafter and Sheppard 2001; Varga *et al*. 2002). In accordance with that, microdialysis studies have shown that under lights-off conditions the infusion of the GABA$_B$ agonist baclofen in the DR diminishes the release of 5-HT (Tao *et al*. 1996). However, during the

Received August 2, 2002; revised manuscript received October 28, 2002; accepted October 31, 2002.

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*Abbreviations used*: Dig, digoxigenin; DLL, dorsal nucleus of the lateral lemniscus; dMnR, median raphe nucleus, dorsal part; DR, dorsal raphe nucleus; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine, serotonin; IC, inferior colliculus; LI, like immunoreactivity; mlf, medial longitudinal fasciculus; MnR, median raphe nucleus; PBS, phosphate-buffered saline; RMg, nucleus raphe magnus; vMnR, median raphe nucleus, ventral part; VTg, ventral tegmental nucleus.

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lights-on period baclofen infusion in the DR and MnR increases 5-HT release, indicating a disinhibitory action (Abellán *et al.* 2000b). This view is also supported by intracellular recordings of dorsal raphe nucleus (DR) 5-HT neurones in midbrain slices, showing that baclofen at low concentrations ($IC_{50} = 72$ nM) counteracts $GABA_A$-mediated inhibitory post-synaptic currents in 5-HT neurones, whereas higher concentrations ($IC_{50} = 1.4$ μM) are required to elicit a direct inhibitory effect (Abellán *et al.* 2000b). These data have been interpreted as resulting from a dual action of baclofen, including an indirect (disinhibitory) effect mediated through $GABA_B$ pre-synaptic autoreceptors located on GABAergic terminals projecting to serotonergic neurones, and a direct (inhibitory) effect mediated through post-synaptic $GABA_B$ receptors located on 5-HT neurones. This hypothesis presumes the presence of $GABA_B$ receptors in serotonergic as well as in GABAergic cells.

In order to validate this model we have now examined the localization of $GABA_B$ receptors in relation to 5-HT and GABAergic cells of the raphe nuclei at different midbrain and upper pons levels. We have used double *in situ* hybridization histochemical techniques to identify the phenotype of cells expressing $GABA_B$ receptor mRNA using serotonin transporter (5-HTT) mRNA and glutamic acid decarboxylase (GAD; EC 4.1.1.15) mRNA as markers of 5-HT and GABAergic cells, respectively.

Preliminary reports of the data have been presented in abstract form (Serrats *et al.* 2001, 2002).

## Materials and methods

### Specimens

Adult male Wistar rats ($n = 15$, 200–250 g b.w.) were purchased from Iffa Credo (Lyon, France). Animal care followed the Spanish legislation on 'Protection of animals used in experimental and other scientific purposes' in agreement with European regulations (O.J. of EC L358/1 18/12/1986). The animals were kept in a controlled environment (12-h light–dark cycle and 22 ± 2°C), with free access to food and water. The animals were killed by decapitation, the brains rapidly removed, frozen on dry ice and stored at − 20°C. Tissue sections, 14 μm thick, were cut using a microtome-cryostat (Microm HM500 OM, Walldorf, Germany), thaw-mounted onto slides coated with APTS (3-aminopropyltriethoxysilane, Sigma, St Louis, MO, USA) and kept at −20°C until use.

### Hybridization probes

The oligodeoxyribonucleotide probes used were as follows: for the 5-HTT mRNA, two probes were always used together, 5-HTT-probe 1, complementary to bases 51–98, and 5-HTT-probe 2, complementary to bases 612–659 (Blakely *et al.* 1991); the probe for GAD mRNA was complementary to bases 1537–1590 (Kobayashi *et al.* 1987); the probe used for $GABA_B$-R1 mRNA was a pan probe which should recognize both $GABA_B$-R1a and $GABA_B$-R1b subtypes and was complementary to bases 2814–2858 (Kaupmann *et al.* 1997). The probes for 5-HTT and GAD mRNA were synthesized on a 380 Applied Biosystem DNA synthesizer (Foster City Biosystems, Foster City, CA, USA) and purified on a 20% polyacrylamide/8 M urea preparative sequencing gel. The $GABA_B$ receptor mRNA probe was custom synthesized and high-performance liquid chromatography (HPLC) purified by Amersham Pharmacia Biotech (Little Chalfont, UK).

Oligonucleotides were labelled either with $^{33}P$ or with digoxigenin (Dig). For the radioactive method, oligonucleotides (2 pmol) were 3′-end-labelled with [$^{33}P$]α-dATP (> 2500 Ci/mmol; DuPont-NEN, Boston, MA, USA) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany), purified by centrifugation using QIAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany) and eluted in 200 μL of elution buffer. Alternatively, for the non-radioactive method, 100 pmol of oligonucleotide were labelled by 3′-end tailing with the same enzyme and Dig-11-dUTP (Boehringer Mannheim, Mannheim, Germany) according to a previously described procedure (Schmitz *et al.* 1991). Dig-labelled oligonucleotides were purified by ethanol precipitation and resuspended in 200 μL of 1 × TE (10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0).

### *In situ* hybridization histochemistry procedure

The protocols for single- and double-label *in situ* hybridization were based on previously described procedures (Tomiyama *et al.* 1997; Landry *et al.* 2000). Frozen tissue sections were first brought to room temperature, fixed for 20 min at 4°C in 4% paraformaldehyde in phosphate-buffered saline (1 × PBS: 8 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3 × PBS at room temperature, twice for 5 min each in 1 × PBS, and incubated for 2 min at 21°C in a fresh solution of pre-digested pronase (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/mL in 50 mM Tris–HCl pH 7.5, 5 mM EDTA. The enzymatic activity was stopped by immersion for 30 s in 2 mg/mL glycine in 1 × PBS. Tissues were finally rinsed in 1 × PBS and dehydrated through a graded series of ethanol.

For hybridization, the radioactively labelled and/or the non-radioactively labelled probes were diluted in a solution containing 50% formamide, 4 × SSC (1 × SSC: 150 mM NaCl, 15 mM sodium citrate), 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulphate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 μg/mL yeast tRNA and 500 μg/mL salmon sperm DNA. The final concentrations of radioactive and Dig-labelled probes in the hybridization buffer were in the same range (approximately 1.5 nM). Tissue sections were covered with hybridization buffer containing the labelled probe(s), overlaid with Nescofilm coverslips (Bando Chemical Ind, Kobe, Japan) and incubated overnight at 42°C in humid boxes. The sections were then washed four times (15 min each) in 1 × SSC at 55°C, and once in 1 × SSC at room temperature for 30 min.

### Development of radioactive and non-radioactive hybridization signal

Slides that had been hybridized only with [$^{33}P$]-labelled probes were directly dehydrated through ethanols and allowed to air dry. In parallel, sections hybridized with Dig-labelled probe(s) were treated as described by Landry *et al.* (2000). Thus, after washing, the slides were immersed for 30 min in a buffer containing 0.1 M Tris–HCl pH 7.5, 1 M NaCl, 2 mM $MgCl_2$ and 0.5% bovine serum albumin

(Sigma) and incubated overnight at 4°C in the same solution with alkaline-phosphate-conjugated antidigoxigenin-F(ab) fragments (1 : 5000; Boehringer Mannheim). Afterwards, they were washed three times (10 min each) in the same buffer (without antibody), and twice in an alkaline buffer containing 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl and 5 mM $MgCl_2$. Alkaline phosphatase activity was developed by incubating the sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Gibco–BRL, Gaithersburg, MD, USA) diluted in 10 mL of alkaline buffer. The enzymatic reaction was stopped by extensive rinsing in the alkaline buffer supplemented with 1 mM EDTA. The sections were then briefly dipped in 70% and 100% ethanol and air-dried.

Sections incubated with radioactive probes (alone or in combination with Dig-labelled oligonucleotides) were dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Chesire, UK) diluted 1 : 1 with distilled water. The sections were exposed in the dark at 4°C for 1 week, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford).

### Specificity of the probes

Several control experiments were carried out to determine the specificity of the hybridization signal: (i) the thermo-stability of hybrids obtained was checked for every probe; (ii) for a given oligonucleotide probe, the hybridization signal was completely blocked by competition of the labelled probe in the presence of 50-fold excess of the same unlabelled oligonucleotide; (iii) to assure the specificity of the non-radioactive hybridization signal, we compared the results obtained with the same probe radioactively labelled.

### Analysis of the results

Tissue sections were examined in bright- and dark-field in a Wild 420 macroscope (Leica, Heerbrugg, Germany) and in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with bright- and dark-field condensers for transmitted light and with epi-illumination. Micrography was performed using a digital camera (DXM1200 3.0, Nikon) and analySIS Software (Soft Imaging System GmbH, Münster, Germany). Bright- and dark-field images were captured with transmitted light. Alternatively, silver grains in the emulsion layer were visualized with epi-illumination passing through a filter (pol-cube) that selects for polarized light reflected off the silver grains and captured as a separated image. The pol image was inverted with Adobe Photoshop software (Adobe Software, Mountain View, CA, USA). The figures were prepared for publication using Adobe Photoshop software.

Cell counting was performed manually at the microscope with the help of analySIS Software. Analysis of variance and post-hoc Tukey's test were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A $p$-value < 0.05 was considered statistically significant.

## Results

The presence of 5-HTT, GAD and GABA$_B$ mRNA in cells of the DR, median raphe nucleus (MnR) and raphe magnus (RMg) of the rat was examined at 11 different antero-posterior coronal levels comprised between planes − 7.0 and − 9.3 mm from bregma (Paxinos and Watson 1998). A macroscopic view of the localization of these three mRNA species in the DR and MnR at level 4 (approx. − 8.3 mm from bregma) is illustrated in Fig. 1. Within the MnR, two separate populations of cells were recognized, according to their anatomical location, morphological characteristics and mRNA content (see below). We will designate these two subnuclei as dorsal (dMnR) and ventral (vMnR) portions of the MnR (see Fig. 1).

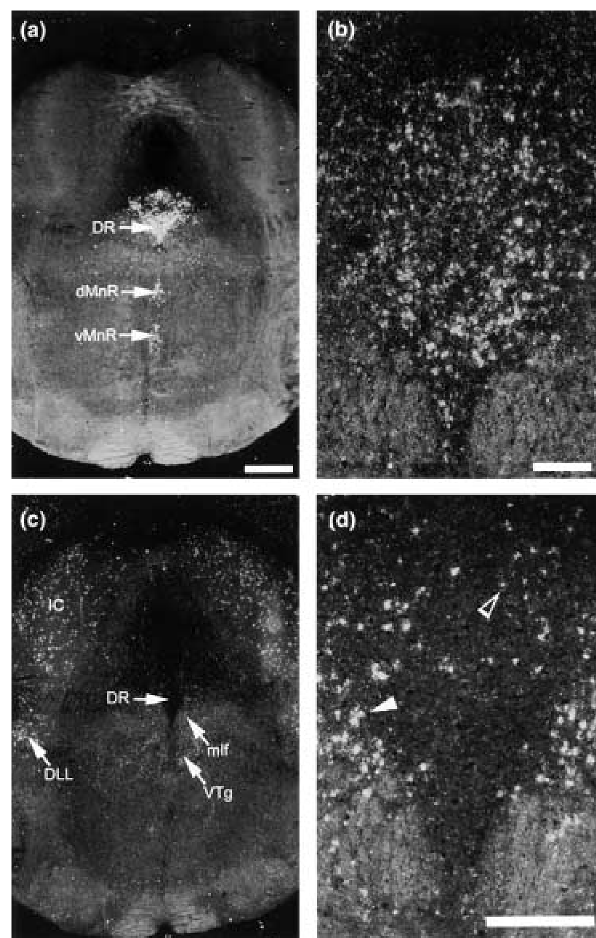


**Fig. 1** Macroscopic dark-field images from serial coronal sections of the rat midbrain showing the radioactive labeling of mRNAs encoding 5-HTT (a), GABA$_B$ receptor (b) and GAD (c and d). (a) Abundant 5-HTT mRNA labeling is observed in the dorsal raphe (DR) and dorsal (dMnR) and ventral (vMnR) parts of the median raphe. (b) GABA$_B$ receptor mRNA is also detected in the DR. (c) Very strong GAD mRNA labeling can be seen in the inferior colliculus (IC), dorsal nucleus of the lateral lemniscus (DLL) and areas surrounding the raphe nuclei such as the ventral tegmental nucleus (VTg) and cells in the medial longitudinal fasciculus (mlf). Positive cells in the raphe nuclei are not clearly discernible at this magnification. (d) Detail from section in (c), showing numerous cells containing GAD mRNA, in the ventromedial portion of the DR. Some heavily labelled cells (arrowheads) are abundant in the ventrolateral edges of the nucleus, while in cells located more dorsally in the DR, the hybridization signal is weaker (open arrowheads). Bar = 1 mm (a and c), 100 μm (b and d).

The three mRNA species were labelled in tissue sections using either radioactive or non-radioactive probes. Both methods yielded identical results concerning the average number of positive cells and their local distribution. When [$^{33}$P]- and Dig-labelled probes were used simultaneously in double *in situ* hybridization assays, the radioactive signal obtained (density of silver grains per cell) was clearly weaker than in single [$^{33}$P]-probe assays. In contrast, no clear-cut differences in signal intensity could be observed for Dig-labelled oligonucleotides when used alone or in combination with radioactive probes.

### Distribution of 5-HTT mRNA

A strong 5-HTT mRNA signal was observed in the DR, MnR and RMg (see Fig. 1a). Most 5-HTT mRNA-positive cells were located within the boundaries of the DR, which contained between 70 and 480 labelled cell bodies per section, depending on the level. 5-HTT mRNA expressing cells in the DR were densely packed in the dorsomedial and ventromedial parts of the nucleus, and showed a more scattered distribution in its lateral wings. The ventromedial part of the DR contained the largest number of positive cell bodies in the DR. 5-HTT mRNA-labeling (either radioactive or non-radioactive) per cell in the MnR and RMg was lighter than in the DR. In the MnR, positive perikarya were grouped in two clusters: the dorsal (dMnR) and the ventral (vMnR) groups (see Fig. 1a), whereas in the RMg, positive cells were dispersed throughout the nucleus. Besides the raphe nuclei, sparse cell profiles presenting also 5-HTT mRNA hybridization signals were seen in the reticular formation of the pons and lowest midbrain.

### Distribution of $GABA_B$ receptor mRNA

$GABA_B$ receptor transcript (Fig. 1b) was visualized in the DR, MnR and RMg using either [$^{33}$P]- or Dig-labelled probes. The strongest $GABA_B$ receptor mRNA signal corresponded to the DR, both in terms of number of positive cells and intensity of labeling. Besides the raphe nuclei, in the sections examined, very strong $GABA_B$ receptor mRNA signal was also seen in Purkinje cells of the cerebellum (not shown). Weaker labeling was observed in the periaqueductal gray, inferior colliculus, granule cell layer of the cerebellum and cerebral cortex (not shown).

### Distribution of GAD mRNA

In the brain sections analysed, a strong GAD mRNA hybridization signal was found in the superficial gray layer of the superior colliculus, inferior colliculus, dorsal nucleus of the lateral lemniscus and Purkinje cells (not shown). Lower levels of GAD mRNA were present in many other structures such as the neocortex, molecular layer of the cerebellum and areas surrounding the DR and MnR (including the ventral tegmental nucleus, medial longitudinal fasciculus and periaqueductal gray, among others). Within the raphe nuclei, GAD mRNA-containing cells could be identified in the DR (Figs 1c and d), MnR and RMg. Positive cells were most abundant in the ventrolateral portion of the DR and in its lateral parts. In the DR and MnR we could clearly distinguish two types of labelled cells, depending on the degree (intermediate or low) of GAD mRNA expression.

### Co-localization of 5-HTT mRNA and $GABA_B$ receptor mRNA (Figs 2a–c and 3)

The coexistence of $GABA_B$ receptor mRNA and 5-HTT mRNA in the DR, MnR and RMg was examined by double *in situ* hybridization using two [$^{33}$P]-labelled probes for 5-HTT and a Dig-labelled probe for $GABA_B$ receptor mRNA in the coronal levels selected. The number of cells labelled for $GABA_B$ receptor mRNA and double-labelled cells counted in these nuclei are summarized in Fig. 3.

In the DR (Figs 2a and 3a), 84 ± 6% (mean ± SD) of the cells presenting $GABA_B$ receptor mRNA signal were found to co-express 5-HTT mRNA. Thus, approximately 15% of $GABA_B$ receptor mRNA-positive cells in the DR were devoid of 5-HTT mRNA signal. The proportions of single- versus double-labelled cells did not display significant differences along the rostro-caudal axis.

In the MnR, the dMnR and vMnR parts were analysed independently. In the dMnR, 72 ± 14% of the $GABA_B$ receptor mRNA-positive cells also contained 5-HTT mRNA, whereas in the vMnR the degree of co-expression was lower (50 ± 10%). The differences among the DR, dMnR and vMnR concerning the ratios of double-labelled cells versus $GABA_B$ receptor mRNA-positive cells were highly significant (one-way ANOVA, $p < 0.0001$). Tukey's post-hoc test indicated statistically significant differences ($p < 0.001$) in all pair comparisons (DR vs. vMnR; DR vs. dMnR; vMnR vs. dMnR). In neither the DR nor the MnR could we detect cells that expressed 5-HTT mRNA in the absence of $GABA_B$ receptor mRNA.

Finally, we analysed the expression of 5-HTT and $GABA_B$ receptor mRNA in the RMg. In this nucleus we identified 5–10 cells positive for 5-HTT mRNA in each section, which did not express $GABA_B$ receptor mRNA in most cases. Only in a few sections could single cells be identified which co-expressed both mRNAs.

### Co-localization of GAD mRNA and $GABA_B$ receptor mRNA (Figs 2d–f and 4)

The co-existence of GAD mRNA and $GABA_B$ receptor mRNA was examined using a [$^{33}$P]-labelled probe for GAD mRNA and Dig-labelled probe for $GABA_B$ receptor mRNA. In the raphe nuclei, few double-labelled cells could be identified, which accounted for 5.82 ± 2.17% of the total population of $GABA_B$ receptor mRNA-containing cells in the DR (Fig. 4). A similar situation was observed in the MnR and RMg, although a quantitative analysis was not performed in these nuclei due to the difficulties in determining their

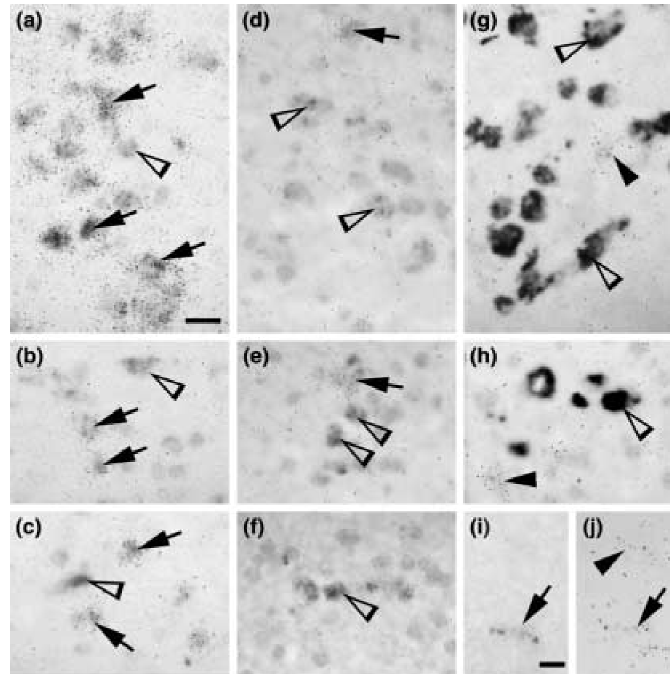


**Fig. 2** Photomicrographs illustrating the simultaneous detection of different mRNA species using [$^{33}$P]-labelled oligonucleotides (silver grains) and Dig-labelled oligonucleotides (dark precipitate). Open arrowheads point to Dig-labelled cell profiles, filled arrowheads point to radioactively labelled cell profiles, arrows point to double-labelled cells. (a–f) Bright field images show GABA$_B$ receptor mRNA (dark precipitate) in combination with radioactive labeling of 5-HTT mRNA (a–c) or GAD mRNA (d–f) in the DR (a and d), dMnR (b and e) and vMnR (c and f). (g–j) Images show 5-HTT mRNA (dark precipitate) in combination with radioactive labeling of GAD mRNA, in the DR (g), MnR (h) and RMg (i and j). Bright field (i) and inverted polarized-light image (see text for details, j) from the same field in RMg showing co-localization of 5-HTT mRNA and GAD mRNA. Bars: (a–f) bar in (a) = 20 μm; (i and j), bar in (i) = 20 μm.

precise limits in the absence of serotonergic markers. We could not unequivocally identify GAD mRNA-positive cells devoid of GABA$_B$ receptor mRNA signal within the raphe nuclei examined. This type of cell was present in the periaqueductal gray, where GAD mRNA could be found with or without GABA$_B$ receptor mRNA. In other midbrain regions, such as the dorsal nucleus of the lateral lemniscus and inferior colliculus, abundant GAD mRNA-containing cells were found, without detectable GABA$_B$ receptor mRNA signal.

### Co-localization of GAD mRNA and 5-HTT mRNA (Figs 2g–j)

Double detection of GAD mRNA and 5-HTT mRNA was carried out using a [$^{33}$P]-labelled probe for GAD mRNA and two Dig-labelled probes for 5-HTT mRNA. We were unable to clearly identify GAD/5-HTT mRNA double-labelled cells in the DR and MnR. In contrast, in the RMg, we could find Dig-labelled cell bodies that presented concomitant radioactive labeling. These double-labelled cells were scarce (only 1–2 per section), representing the 15–25% of 5-HTT mRNA-positive cells in the RMg, and were mainly located in the central portion of the nucleus, and occasionally in its lateral parts.

### Co-localization of GAD mRNA, GABA$_B$ receptor mRNA and 5-HTT mRNA (Fig. 5)

GAD mRNA, GABA$_B$ receptor mRNA and 5-HTT mRNA were simultaneously labelled using [$^{33}$P]-labelled probes for GAD and 5-HTT mRNA, and Dig-labelled probe for GABA$_B$ receptor mRNA. Using this approach, we intended to visualize cells expressing GABA$_B$ receptor mRNA that were neither serotonergic nor GABAergic. As shown in Fig. 5, we could detect some cells presenting GABA$_B$ receptor mRNA hybridization signal, which were devoid of noticeable radioactive labeling.

## Discussion

We have examined the distribution of cells containing GABA$_B$ receptor mRNA, GAD mRNA and 5-HTT mRNA in the rat DR, MnR and RMg. The use of double-label *in situ* hybridization has allowed us to analyse in detail the co-localization of these mRNAs in cells of the serotonergic nuclei of the midbrain and upper pons. The present study is a step further in the knowledge of the expression of GABA-ergic markers in the raphe nuclei and extends previous studies from this laboratory on the occurrence of those mRNA species in the DR and MnR (Abellán *et al*. 2000a).

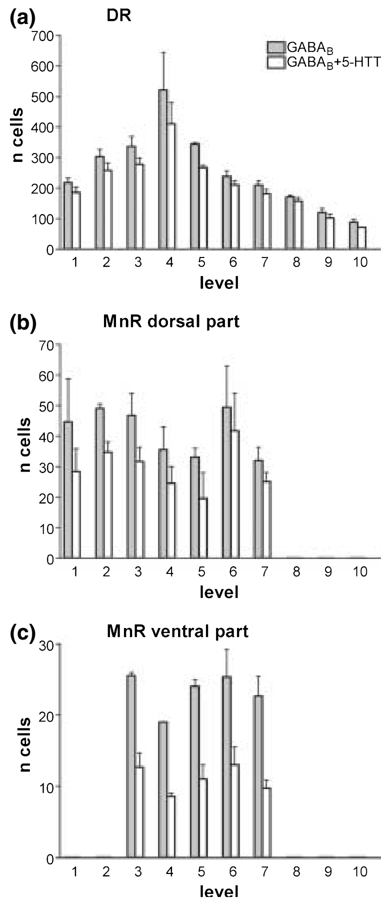


**Fig. 3** Degree of co-localization of $GABA_B$ receptor mRNA with 5-HTT mRNA in the DR (a), dorsal MnR (b) and ventral MnR (c). Bars indicate the total number of cell profiles expressing $GABA_B$ receptor mRNA (▨) or double-labelled cell profiles (□) per section. Cells were counted in 14 μm-thick coronal sections taken at 10 different antero-posterior levels (1–10). Data are mean ± SD and were obtained from three animals.

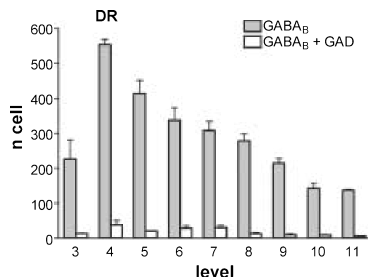


**Fig. 4** Degree of co-localization of $GABA_B$ receptor mRNA with GAD mRNA in the DR. Bars indicate the total number of cell profiles expressing $GABA_B$ receptor mRNA (▨) or double-labelled cell profiles (□) per section. Cells were counted in 14 μm-thick coronal sections taken at anatomical levels 3–11, as described. Data are mean ± SD and were obtained from three animals.

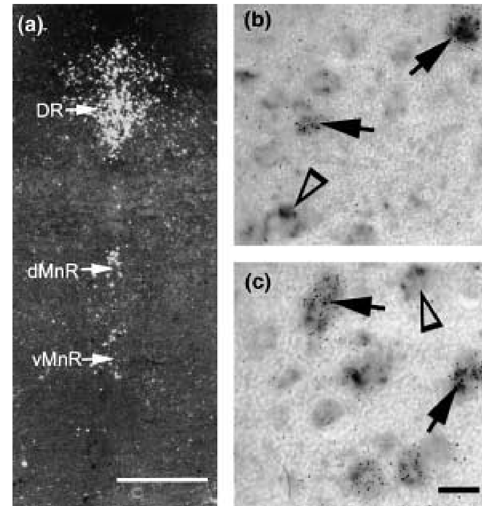


**Fig. 5** Triple-labeling *in situ* hybridization with [$^{33}$P]-labelled oligonucleotide probes complementary to 5-HTT mRNA and GAD mRNA (silver grains) and Dig-labelled oligonucleotide probe complementary to $GABA_B$ receptor mRNA (dark precipitate). (a) Dark-field image showing intense radioactive labeling in the DR and MnR (dMnR and vMnR portions) corresponding to 5-HTT plus GAD mRNA. (b and c) Bright-field micrographs displaying single-labelled versus double-labelled cells in the DR (b) and MnR (c). Arrows point to cells showing non-radioactive and radioactive signal. Open arrowheads point to Dig-labelled cell profiles ($GABA_B$ receptor mRNA), demonstrating the existence of $GABA_B$ receptor mRNA-positive cells which do not express neither 5-HTT mRNA nor GAD mRNA. Bars: 1 mm (a) and 20 μm (b and c).

Our reults concerning the cellular localization of 5-HTT, $GABA_B$ receptor and GAD mRNA in the rat raphe nuclei are in good agreement with published data. As expected, 5-HTT mRNA hybridization signal in the mesencephalon and upper pons was essentially restricted to the raphe nuclei. Thus, 5-HTT mRNA signal was strong in cell bodies in the DR, MnR and RMg, as it has been shown previously in rat, mouse, cat and humans (Fujita *et al*. 1993; Rattray *et al*. 1994, 1999; Charnay *et al*. 1996; McLaughlin *et al*. 1996; Bengel *et al*. 1997). In accordance with those studies, the highest densities of 5-HTT mRNA corresponded to the DR. We could also detect 5-HTT mRNA in cells scattered throughout the pontomesencephalic reticular formation, presumably 5-HT-containing neurones present in this area (Vertes and Crane 1997). The localization of 5-HTT mRNA-positive cell profiles observed here entirely correlates with the known location of serotonergic cell bodies (Dahlström and Fuxe 1964; Steinbusch 1981; Rattray *et al*. 1999). Despite the fact that 5-HTT mRNA has been detected in cultured astrocytes and in adult rat forebrain using RT–PCR (Bel *et al*. 1997), in our preparations we did not observe 5-HTT mRNA hybridization patterns that could correspond to glia. According to Rattray *et al*. (1999), 5-HTT mRNA is exclusively expressed in serotonergic neurones, and furthermore, virtually all

5-HT-containing neurones contain 5-HTT mRNA (but see Brown and Molliver 2000). Therefore, the presence of specific 5-HTT mRNA hybridization signal can be considered as a specific marker of serotonergic phenotype.

In addition to 5-HTT mRNA, the raphe nuclei are well known to contain high concentrations of 5-HT transporter protein, as evidenced by ligand binding autoradiography (Rainbow *et al*. 1982; Dawson and Wamsley 1983; D'Amato *et al*. 1987), and more recently using immunohistochemical methods (Sur *et al*. 1996).

The DR showed also a prominent signal for $GABA_B$ receptor mRNA. This is in agreement with published studies reporting high levels of expression of $GABA_B$ receptor mRNA in monoaminergic regions of the brainstem, including the DR (Bischoff *et al*. 1999; Lu *et al*. 1999; Abellán *et al*. 2000a). In fact, the DR has been reported to be one of the regions most enriched in $GABA_B$ receptor mRNA in the rat brain (Bischoff *et al*. 1999; Lu *et al*. 1999). According to these authors the signal we observed in the DR would primarily correspond to the $GABA_B$-R1a mRNA subtype, which appears to be more abundant in this region, although it also contains the $GABA_B$-R1b subtype. The presence of $GABA_B$ receptor binding sites in the raphe nuclei is well established, as shown by receptor autoradiography with [$^3$H]GABA or [$^3$H]baclofen as ligands (Bowery *et al*. 1987; Price *et al*. 1987; Chu *et al*. 1990; Bischoff *et al*. 1999). Immunohistochemical studies have also shown very intense GABAB-R1-LI in cell bodies in the DR and MnR (Margeta-Mitrovic *et al*. 1999; Wirtshafter and Sheppard 2001; Varga *et al*. 2002). Thus, there is a good correlation between $GABA_B$ binding sites, $GABA_B$-LI and $GABA_B$ mRNA in the raphe nuclei of the midbrain and upper pons, indicating that the $GABA_B$ receptor can be, at least in part, of local origin in these nuclei.

Two isoforms of GAD, namely GAD65 and GAD67, have been identified, which are encoded by different genes (Kobayashi *et al*. 1987; Wyborski *et al*. 1990; Erlander *et al*. 1991). The mRNAs coding for the two GADs present a very close regional distribution in the brain and, in the rat, almost all GABA neurones contain mRNA for both GAD65 and GAD67 (Feldblum *et al*. 1993; Esclapez *et al*. 1994). In the present work we have visualized GABAergic neurones using a probe against GAD67 mRNA because in general it appears to be more abundant than GAD65 mRNA (Esclapez *et al*. 1994). The distribution observed in the sections examined is in good agreement with previous studies reporting GAD67 mRNA in the cerebellum, particularly in Purkinje cells, and structures such as the inferior colliculus and nucleus raphe magnus (Wuenschell *et al*. 1986; Julien *et al*. 1987; Esclapez *et al*. 1993, 1994; Feldblum *et al*. 1993; Stornetta and Guyenet 1999). Our data are also in accordance with immunohistochemical studies which have revealed the presence of GAD-LI and GABA-LI in the brainstem raphe nuclei of the rat, mainly in cells clustered in the lateral portions of the DR (Nanopoulos *et al*. 1982; Belin *et al*. 1983; Mugnaini and Oertel 1985; Millhorn *et al*. 1987, 1988; Kachidian *et al*. 1991; Wang and Nakai 1993; Esclapez *et al*. 1994; Ford *et al*. 1995; Stamp and Semba 1995; Gervasoni *et al*. 2000).

When comparing the expression of 5-HTT, $GABA_B$ receptor and GAD mRNA in the raphe nuclei, the most abundant mRNA species in terms of number of labelled cells was the $GABA_B$ receptor mRNA. Conversely, GAD mRNA was present only in a reduced number of raphe cells and it was mainly found beyond the boundaries of the raphe nuclei.

By using double *in situ* hybridization we have visualized the presence of $GABA_B$ receptor mRNA in different populations of cells within the raphe nuclei, including serotonergic (5-HTT mRNA-positive), GABAergic (GAD mRNA-positive) and also a minority of non-serotonergic non-GABAergic cells. One of our goals has been to evaluate the proportions of single- versus double-labelled cells within the raphe nuclei.

The degree of coexistence of $GABA_B$ receptor and 5-HTT mRNAs in the raphe significantly varied from one nucleus to the other. Thus, whereas in the DR and MnR all serotonergic neurones exhibited $GABA_B$ receptor mRNA signal, in the RMg these mRNA species were rarely found together. Double-labelled cells represented a large proportion (84%) of $GABA_B$ receptor mRNA-positive cells of the DR. This ratio remained constant among the different cell groups (ventral and dorsal portions and lateral wings) and along the rostrocaudal extent of the nucleus. In contrast, in the MnR, we could discriminate between two groups of $GABA_B$ receptor mRNA-positive cells: a ventral cluster (vMnR) where only the 50% of cells co-expressed 5-HTT mRNA, and a dorsal group (dMnR) with characteristics closer to the DR. Cytoarchitectural differences between cells located in the dorsal and ventral portions of the MnR have been described before (Vertes and Crane 1997).

In addition to serotonergic cells, $GABA_B$ receptor mRNA was also detected in all GAD mRNA-positive cell profiles visualized within the boundaries of the raphe nuclei studied here. This finding suggests that all GABAergic neurones in the raphe express $GABA_B$ autoreceptors. This is in agreement with data from Liang *et al*. (2000) who reported that all GABA neurones in the rat brain contain $GABA_B$ receptor mRNA, with the unique exception of the dorsal lateral geniculate nucleus. However, it should be mentioned that in the tissue sections examined here we could observe GAD mRNA in cells apparently devoid of $GABA_B$ receptor mRNA in structures such as the periaqueductal gray, dorsal nucleus of the lateral lemniscus or the inferior colliculus. These midbrain nuclei were not the focus of the present study and were not exhaustively examined. It should be noted also that limitations in the sensitivity of our *in situ* hybridization method might hinder the visualization of mRNAs present in low densities.

It has been shown that GABA-immunoreactive terminals contact 5-HT neurones in the DR (Wang *et al*. 1992). This observation together with the present data showing the occurrence of $GABA_B$ receptor mRNA in both or 5-HTT mRNA-positive and GAD-containing cells in the raphe nuclei provides anatomical support to the hypothesis expressed by Abellán *et al*. (2000b) to explain the dual effect of the $GABA_B$ agonist baclofen on 5-HT cells. Thus, post-synaptic $GABA_B$ receptors located on serotonergic neurones could mediate the inhibition of 5-HT release caused by baclofen (Tao *et al*. 1996), whereas the activation of pre-synaptic $GABA_B$ receptors located in GABAergic cells would result in a final disinhibitory action on serotonergic neurones (Abellán *et al*. 2000a, 2000b). It should be pointed out that, according to our observations, GAD/$GABA_B$ receptor mRNA-containing cells in the raphe nuclei are not abundant. Therefore the reported pre-synaptic $GABA_B$ effects are likely to be mediated mainly by receptors located in nerve terminals from afferent GABAergic projection neurones. It has been demonstrated that the DR receives GABAergic inputs both from local interneurones located in the DR itself and periaqueductal gray and from neurones located in distant regions from the forebrain to the medulla (Gervasoni *et al*. 2000).

In this study we show the existence of $GABA_B$ receptor mRNA-positive cells in the DR that do not display 5-HTT nor GAD mRNA hybridization signal, and therefore appear to be neither GABAergic, nor serotonergic. Such cells can be part of other neurochemical identities which have been described within the boundaries of the raphe nuclei, such as dopaminergic neurones (Björklund and Hökfelt 1984), nitric-oxide containing neurones (Xu and Hökfelt 1997; Léger *et al*. 1998), or neuropeptide-containing neurones (Kirifides *et al*. 2001). For instance, $GABA_B$ receptor-LI has been shown in tyrosine hydroxylase immunoreactive cell bodies of the DR (Wirtshafter and Sheppard 2001).

Our double-label experiments showed also the co-existence of GAD mRNA and 5-HTT mRNA in single raphe cells. The co-localization of these two mRNAs was limited to the RMg, and we have not been able to unequivocally detect such double-labelled cells in the DR and MnR. The presence of GABAergic markers in DR and MnR 5-HT neurones is controversial as different authors have reported discrepant results from immunohistochemical studies (Nanopoulos *et al*. 1982; Belin *et al*. 1983; Kachidian *et al*. 1991; Wang *et al*. 1992). In an attempt to clarify this issue, Stamp and Semba (1995) have quantified cells immunoreactive for both 5-HT and GAD in the rat brainstem and found very few 5-HT/GAD double-labelled cells in the DR and MnR, accounting for less than 1% of all 5-HT-stained neurones in these nuclei. The present data essentially agree with the view that a very small proportion of 5-HT cells – if any – also express GAD. Experimental factors (e.g. different technical approaches, additional presence of GAD65, use of colchicine; Cortés *et al*. 1990) may account for this small difference.

In contrast, a substantial percentage of cells in the RMg (15–25%) were 5-HTT/GAD mRNA double-labelled, consistently with previous findings. Thus, the co-existence of 5-HT with GABA or GAD is well documented from immunohistochemical studies (Belin *et al*. 1983; Millhorn *et al*. 1987, 1988; Kachidian *et al*. 1991; Stamp and Semba 1995). This percentage is considerably higher than that reported by Stamp and Semba (1995) who found GAD-LI in less than 4% of 5-HT neurones, but is closer to data from Millhorn *et al*. (1988). The RMg is an important component of the antinociceptive system (Basbaum and Fields 1978), and in particular, on the basis of retrograde transport studies showing projections of 5-HT/GABA neurones to the spinal cord, it has been speculated that these neurones might have a role in the modulation of nociception (Millhorn *et al*. 1987).

In conclusion, we have detected the presence of $GABA_B$ receptor mRNA in serotonergic and also in GABAergic cells in the raphe nuclei, a finding that provides the anatomical basis for the dual effect of baclofen on the serotonergic neurones observed in electrophysiological and microdialysis studies. We have also visualized DR neurones expressing $GABA_B$ receptor mRNA which are neither serotonergic nor GABAergic. Our findings emphasize also the neurochemical diversity of the different raphe nuclei, as we demonstrate distinct patterns of co-expression of 5-HTT mRNA, $GABA_B$ receptor mRNA and GAD mRNA in the DR, MnR and RMg.

## Acknowledgements

This work was supported by grant SAF200-0212 from 'Ministerio de Ciencia y Tecnología' (MCYT). Support from DURSI – 'Generalitat de Catalunya' to the Department of Neurochemistry of IIBB-CSIC (IDIBAPS) as a 'Grup de Recerca Consolidat' (5092-CSIC-01) is also acknowledged (grant 2001-SGR-00355). Jordi Serrats is a recipient of a fellowship 'Beca de Formació de Personal Investigador' from 'Institut d'Investigacions Biomèdiques August Pi i Sunyer' (IDIBAPS).

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