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5-ht$_{5B}$ receptor mRNA in the raphe nuclei: Co-expression with serotonin transporter

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ABSTRACT

We used double-label *in situ* hybridization to examine the cellular localization of 5-ht_{5B} receptor mRNA in relation to serotonin transporter mRNA in the rat dorsal raphe (DR) and central superior nucleus (CS, median raphe nucleus). 5-ht_{5B} receptor mRNA hybridization signal was often found on serotonin transporter mRNA positive neuron profiles. The degree of cellular colocalization of these mRNAs notably varied among the different regions of the raphe nuclei. In the DR, cell bodies showing 5-ht_{5B} receptor mRNA expression were abundant in the medial portions of the nucleus, all of them being also labeled for serotonin transporter mRNA. In contrast, in the ventrolateral regions (lateral wings) of the DR, we observed serotonin transporter mRNA-positive cells, but they were devoid of 5-ht_{5B} receptor mRNA signal. In the CS, the level of coexpression of 5-ht_{5B} receptor mRNA with serotonin transporter mRNA was high in the intermediate portions of the nucleus, however we were unable to detect specific 5-ht_{5B} receptor mRNA hybridization signal in its caudal extent. Our results support the presence of 5-ht_{5B} receptor in serotonergic neurons in the DR and CS, suggesting an autoreceptor role for this receptor subtype.

KEY WORDS:
dorsal raphe; median raphe nucleus; autoreceptor; *in situ* hybridization

RUNNING TITLE: 5-ht_{5B} receptor mRNA in the raphe nuclei

Abbreviations:

5-HT: 5-hydroxytryptamine, serotonin
5-HTT: serotonin transporter
5-CT: 5-carboxamidotryptamine
CS: centralis superior nucleus
Dig: digoxigenin
DR: dorsal raphe nucleus
SSRIs: selective 5-HT reuptake inhibitors

INTRODUCTION

5-Hydroxytryptamine (serotonin; 5-HT) has a variety of functions in the brain (Leibowitz and Alexander, 1998; Barnes and Sharp, 1999) that are mediated by multiple receptor subtypes. These are currently classified into 7 families comprising 14 different receptors (Hoyer et al., 1994). Some of these subtypes have been cloned by nucleotide sequence homology with other previously identified 5-HT receptors and their function is not well known yet. Such is the case of the two members of the 5-ht_5 receptor family, namely the 5-ht_{5A} and 5-ht_{5B} subtypes (Plassat et al., 1992; Wisden et al., 1993; Matthes et al., 1993; Erlander et al., 1993; Rees et al., 1994; Grailhe et al., 2001). The genes coding for these receptors have been found in rat, mouse and human brain, however the human 5-ht_{5B} receptor gene does not encode a functional protein (Grailhe et al., 2001). The members of the 5-ht_5 family are G-protein-coupled receptors, but their transduction mechanisms have not yet been identified. Thus, most authors found that recombinant 5-ht_{5A} and 5-ht_{5B} receptors stably expressed in different cell lines do not alter the levels of cAMP nor inositol phosphates in response to 5-HT (Plassat et al., 1992; Wisden et al., 1993; Matthes et al., 1993; Erlander et al., 1993; Francken et al., 1998), however, Francken et al. (1998) reported that human 5-ht_{5A} receptors stably transfected in human embryonic kidney (HEK) 293 cells functionally couple to pertussis toxin-sensitive G (G_i/G_o) proteins and inhibit adenylate cyclase activity, whereas Grailhe et al. (2001) found that the human 5-ht_{5A} receptor expressed in *Xenopus* oocytes is able to couple to the inwardly rectifying K^+ channel GIRK1. The pharmacological profile of recombinant 5-ht_5 receptors is different from other 5-HT receptor families, although it shares some similarities with 5-HT_1 receptors, such as high affinity for 5-carboxamidotryptamine (5-CT) and several ergot alkaloid derivatives including LSD (Plassat et al., 1992; Wisden et al., 1993; Matthes et al., 1993; Erlander et al., 1993; Weiss et al., 1995; Bach et al., 1996; Grailhe et al., 2001). The lack of selective ligands for 5-ht_5 receptors has hindered a thorough pharmacological characterization, and the analysis of their localization and of their functional implications. Attempts to explore the autoradiographic distribution of 5-ht_5 receptors in the mouse brain have shown putative 5-ht_5 binding sites in the habenula, hippocampus, septum, olfactory bulb and raphe (Waeber et al., 1998; Grailhe et al., 1999). mRNA localization studies in the rat brain have demonstrated the presence of 5-ht_{5A} and 5-ht_{5B} receptor transcripts in

different areas, including piriform cortex, habenula and some hippocampal structures (Matthes et al., 1993; Erlander et al., 1993; Kinsey et al., 2001). Very high densities of 5-ht_{5B} receptor mRNA have been described in the inferior olivary nucleus and raphe nuclei. The occurrence of 5-ht_{5B} receptor mRNA in the dorsal raphe nucleus (DR) might be indicative of its presence in 5-HT neurons, since the DR is the brain area where most 5-HT-containing cell bodies of neurons that project to the forebrain are located (Dahlström and Fuxe, 1964; Steinbusch, 1981), implicating therefore an autoreceptor role of the 5-ht_{5B} receptor.

In order to investigate the presence of 5-ht_{5B} receptor mRNA in serotonergic cells, we used double-label *in situ* hybridization histochemistry to simultaneously visualize 5-ht_{5B} mRNA and 5-HT transporter (5-HTT) mRNA as a marker for 5-HT neurons. We examined the expression of these mRNAs throughout the entire rostrocaudal extent of the anterior raphe nuclei projecting to the forebrain, that is, the DR and central superior nucleus (CS; also named median raphe nucleus). Together, these data might provide some clues on the functionality of 5-ht_{5B} receptors in the rat brain.

MATERIAL AND METHODS

Specimens

Adult male Wistar rats (n=18) (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). Experimental procedures were approved by the required ethical committees and local authorities. The animals were kept in a controlled environment (12-hours light-dark circle and 22 ± 2ºC), with free access to food and water. The animals were sacrificed by decapitation, the brains rapidly removed, frozen on dry ice and stored at -20ºC. Tissue sections, 14 µm thick, were cut using a microtome-cryostat (HM500 M, Microm, Walldorf, Germany), thaw-mounted onto slides coated with APTS (3-aminopropyltriethoxysilane, Sigma, USA) and kept at –20ºC 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 of the 5-HTT mRNA

sequence (Blakely et al., 1991) and were synthesized on a 380 Applied Biosystem DNA synthesizer (Foster City Biosystem, Foster City, USA) and purified on a 20% polyacrylamide / 8 M urea preparative sequencing gel. For 5-ht_{5B} receptor mRNA one oligodeoxynucleotide complementary to bases 1065-1113, corresponding to the carboxy terminus of the 5-ht_{5B} receptor sequence (Matthes et al., 1993) was custom synthesized and purified by HPLC by Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). An additional oligonucleotide probe against the 5-ht_{5B} receptor mRNA (nucleotides 315-362) was used in preliminary experiments. Evaluation of the oligonucleotide sequences with basic local alignment search tool (BLAST) of EMBL and GenBank databases indicated that the probes do not show any significant similarity with mRNAs other than their corresponding targets in the rat.

Oligonucleotides were labeled either with ^{33}P, ^{32}P or with digoxigenin (Dig). For the radioactive method, oligonucleotides (2 pmol) were 3'-end-labeled with [^{33}P] or [^{32}P]α-dATP (>3000 Ci/mmol; Amersham) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany), purified by centrifugation using QIAquick Nucleotide Removal Kit (QIAGEN GmbH, Hilden, Germany). Alternatively, for the nonradioactive method, 100 pmol of oligonucleotide were labeled by 3'-end tailing with the same enzyme and Dig-11-dUTP (Roche Diagnostics GmbH) according to a previously described procedure (Schmitz et al., 1991). Dig-labeled oligonucleotides were purified by ethanol precipitation.

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) and have been published elsewhere (Serrats et al., 2003). Frozen tissue sections were first brought to room temperature, fixed for 20 min at 4ºC in 4% paraformaldehyde in phosphate-buffered saline (1x PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3x PBS at room temperature, twice for 5 min each in 1x PBS, and incubated for 2 min at 21ºC in a fresh solution of predigested pronase (Calbiochem, San Diego, 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 sec in 2 mg/ml glycine in 1x PBS. Tissues were finally rinsed in 1x PBS and dehydrated through a graded series of ethanol.

For hybridization, the radioactively labeled and/or the non-radioactively labeled probes were dissolved in a solution containing 50% formamide, 4x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate), 1x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 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-labeled probes in the hybridization buffer were in the same range (~ 1.5 nM). Tissue sections were covered with hybridization buffer containing the labeled probe/s, overlaid with Nescofilm coverslips (Bando Chemical, Kobe, Japan) and incubated overnight at 42ºC in humid boxes. The sections were then washed four times (45 min each) in a buffer containing 0.6M NaCl and 10 mM Tris-HCl (pH 7.5) at 60ºC.

Development of radioactive and non-radioactive hybridization signal

Slides that had been hybridized only with ^{33}P-labeled probes were directly dehydrated through graded series of ethanols and allowed to air-dry. In parallel, sections hybridized with Dig-labeled probe(s) were treated as described by Landry et al. (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 4ºC in the same solution with alkaline-phosphate-conjugated anti-digoxigenin-F(ab) fragments (1:5000; Roche Diagnostics GmbH). 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, USA) dissolved in 10 ml of alkaline buffer. The enzymatic reaction was stopped by extensive rinsing in alkaline buffer with the addition of 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-labeled oligonucleotides) were either exposed to Hyperfilm β-max (Amersham) or dipped into Ilford K5 nuclear track emulsion (Ilford, Mobberly, UK) diluted 1:1 with distilled water. The sections were exposed in the dark at 4°C for 6 weeks, and finally developed in Kodak D19 (Kodak, Rochester, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford).

Specificity of the probes

The specificity of the oligonucleotide probe used to detect 5-ht_{5B} receptor mRNA was verified by Northern blot and RT-PCR analyses. Total RNA was extracted from different regions of the rat brain using Rneasy Maxi kit (QIAGEN). RNA samples (30 µg) were electrophoresed on a 1% agarose gel, blotted onto a nylon membrane and UV cross-linked. The blot was stained with 0.04% (w/v) methylene blue in 0.5 M sodium acetate pH 5.2 for 10 min, and later bleached in water for 5 min. Pre-hybridization and hybridization were performed in a solution containing 5x SSPE (0.75 M NaCl, 0.05 M NaH_2PO_4, 0.004 M EDTA), 50% formamide, 5x Denhardt's, 0.2% SDS, 500 µg/ml ssDNA at 42ºC. The ^{32}P-labeled 5-ht_{5B} receptor probe was added at a concentration of 5 x 10^6 dpm/ml, hybridized overnight and washed in a buffer containing 2x SSC, 0.1% SDS at 42ºC. The blots were exposed to X-ray film (Biomax MR, Kodak) for 4 weeks.

The RT-PCR procedure was performed as described before (Vilaró et al., 2002). The sequences of the PCR primers used were: sense primer 5' GGCTGCAGCGTTGCCAGGTGAGC, bases 901-923 from XM_213869, and antisense primer 5' CCACCATCATGGCGGCTCGCTTC, bases 1178-1200 from XM_213869. PCR reaction products (10 µl each) were run on a 3.5% MS-12 (Pronadisa Hispanlab, Madrid, Spain) agarose gel and transferred to Hybond-N nylon membranes (Amersham). Membranes were incubated with the ^{33}P-labeled 5-ht_{5B} oligonucleotide probe, which was supposed to hybridize within the region amplified by the PCR primers. Several control experiments were carried out to determine the specificity of the signal obtained by *in situ* hybridization. 1) The thermostability of hybrids obtained was checked for every probe. 2) For a given oligonucleotide probe, the hybridization signal was completely blocked by competition of the labeled probe in the presence of 50-fold excess of the same unlabeled oligonucleotide. 3) For each mRNA species, at least two different oligonucleotide probes were assayed separately. Hybridization signal was considered specific only when the regional and cellular labeling obtained with both probes was independently identical. 4) To assure the specificity of the nonradioactive hybridization signal, we compared the results obtained with the same probe radioactively labeled.

Data analysis

Hybridized tissue sections were examined with a Wild 420 macroscope (Leica, Heerbrugg, Germany), with a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) and with an Axioplan Zeiss microscope (Carl Zeiss, Oberkochen, Germany), equipped with bright- and dark-field condensers for transmitted light. A Darklite illuminator (Micro Video Instruments Inc, Avon, USA) was used to improve the visualization of autoradiographic silver grains and capture of dark-field images. Micrography was performed using a digital camera (DXM1200 3.0, Nikon) and analySIS Software (Soft Imaging System GmbH, Münster, Germany). Alternatively, microphotographs were taken with Kodak Ektachrome 64T (Kodak). The figures were prepared for publication using Adobe PhotoShop software (Adobe Software, san Jose, CA).

5-HTT mRNA positive neurons were identified as cell profiles showing dark staining (alkaline phosphatase reaction product) surrounding or covering the nucleus. 5-ht_{5B} receptor mRNA hybridization signal was considered positive when accumulations of silver grains over cell profiles were at least 3-fold higher than average background. Cell counting was performed manually at the microscope with the help of analySIS Software.

The average densities of 5-ht_{5B} receptor mRNA in different brain regions were evaluated semiquantitatively on film autoradiograms with the aid of an image analysis system (MCID M4, Imaging Research, St. Catherines, Ontario, Canada).

RESULTS

Controls of specificity

The specificity of the probe used to detect 5-ht_{5B} receptor mRNA in *in situ* hybridization studies was assessed by Northern blotting and RT-PCR. In the Northern blot (Fig. 1A), our oligonucleotide probe labeled two bands which would correspond to 5-ht_{5B} mRNA species of 3000 and 1900 nucleotides reported by Erlander et al. (1993), however, we did not observe the third band of 1500 nucleotides found by these authors. As expected, 5-ht_{5B} mRNA was detected in samples from hippocampus and brainstem, but not in cerebellum. In the RT-PCR assay, our 5-ht_{5B} receptor probe recognized a single band of the expected size (300 bp) (Fig. 1B,C).

The probes directed against the mRNAs encoding the 5-ht_{5B} receptor mRNA and 5-HT transporter fulfilled the criteria of specificity previously described. Figure 2 illustrates the hybridization signal obtained for 5-ht_{5B} receptor mRNA at two different brain levels and the effect of incubation with an excess of the same, unlabeled probe, which completely blocks labeling.

As an additional control of specificity of the hybridization probe, the presence of 5-ht_{5B} receptor mRNA was examined in brain areas other than the raphe nuclei. Labeled cells were found in few brain structures, including the pyramidal cell layer of the CA1 field of the hippocampus, medial habenula, intermediate layers of the entorhinal cortex, parasubiculum, and inferior olivary nucleus (not shown). The granule cells of the dentate gyrus and the lateral habenula presented a weak hybridization signal. A semiquantitative evaluation of the content of 5-ht_{5B} receptor mRNA in several brain regions is reported in Table 1.

Dorsal Raphe

5-HTT mRNA hybridization signal was strong and allowed a clear visualization of individual cell profiles in the DR and its surroundings (Fig. 3). Between 70 and 300 labeled cell bodies per section were counted in the DR, depending on the anteroposterior level. Different subpopulations of 5-HTT mRNA-positive cells could be identified within the DR, which exhibited differences in cell shape and density. Thus, most 5-HTT mRNA positive cells were located in the ventromedial and dorsomedial parts of the DR, just dorsal to the fasciculus longitudinalis medialis. In these two subnuclei, 5-HTT mRNA expressing cells were predominantly ovoid and densely packed, whereas in the lateral wings labeled cell profiles were either ovoid or spindle-shaped and showed a more scattered distribution (Figs.3,4).

Throughout the entire anteroposterior extent of the rat DR, 5-ht_{5B} receptor mRNA signal was also strong, and it was found exclusively in the medial portion of the nucleus, corresponding to its ventromedial, dorsomedial, interfascicular and caudal parts (Figs. 3C, 4B). In contrast, no specific 5-ht_{5B} receptor mRNA hybridization signal could be detected in the lateral wings of the nucleus (Figs. 3C, 4A).

Dual labeling was performed to examine concomitant expression of 5-ht_{5B} receptor and 5-HTT mRNAs on single tissue sections. Coexpressing cells were counted at a single-cell resolution at different anteroposterior levels of the DR and the results are depicted in schematic representations in Fig. 5. No double-labeled cells were found in

the lateral wings of the DR (Fig. 4A, 5). In contrast, a high degree of coexpression of both mRNAs was observed in the medial portion of the nucleus (Fig. 4B, 5). The level of colocalization in the medial part of the DR varied significantly ($p<0.0001$) throughout the rostrocaudal axis, ranging from 62%-85% of the population of 5-HTT mRNA positive cells of this subregion (see Fig. 6A). Within the boundaries of the DR, no 5-ht_{5B} receptor mRNA hybridization signal could be observed in the absence of 5-HTT mRNA labeling in our experimental conditions.

Central Superior Nucleus

5-HTT mRNA-positive cells in the CS displayed less dense packing than in DR, and were scattered around the midline, ventrally to the medial longitudinal fasciculus. 5-ht_{5B} receptor mRNA hybridization signal showed a pattern of expression similar to the 5-HTT mRNA in the CS.

The same dual labeling approach mentioned above was used to localize cells which coexpressed 5-ht_{5B} receptor mRNA and 5-HTT mRNA in the CS (Fig. 4C, D, 5). The proportions of 5-HTT mRNA-positive cells expressing 5-ht_{5B} receptor mRNA varied depending on the level studied: at anterior portions of the CS, ~20% of serotonergic cells coexpressed 5-ht_{5B} receptor mRNA, whereas at intermediate regions the degree of coexpression reached 75%. In contrast, no colocalization was observed in serotonergic cells of the posterior parts of the CS (see Figs. 4C,D, 5 and 6B). Similar to the DR, 5-ht_{5B} receptor mRNA labeling was always associated with 5-HTT mRNA in the CS.

DISCUSSION

The main finding of this study is the detection of 5-ht_{5B} receptor mRNA in some populations of 5-HTT mRNA-positive cells in the DR and CS. As 5-HTT mRNA is specifically expressed in serotonergic cells of the raphe nuclei, it can be considered a marker of 5-HT cell phenotype (Rattray et al., 1999). Therefore, our data indicate that 5-ht_{5B} receptor mRNA is present in some 5-HT neurons, suggesting an autoreceptor role for this receptor subtype.

Although the presence of 5-ht_{5B} receptor mRNA in the rat DR has been previously described (Matthes et al., 1993; Erlander et al., 1993), it remained to be established whether or not this receptor is expressed by the proper serotonergic neurons. Using a double *in situ* hybridization procedure we have now identified two types of serotonergic cells in the anterior raphe nuclei: double-labeled cells coexpressing

5-ht$_{5B}$ receptor and 5-HTT mRNAs, and 5-HTT mRNA-positive cells lacking 5-ht$_{5B}$ receptor mRNA. It is noteworthy that the localization of double-labeled cells was restricted to the medial portions of the DR (including the dorsomedial, ventromedial, interfascicular and caudal cell groups) and the intermediate CS. Double-labeled cells represented the large majority of 5-HT cells in these subregions, although the degree of coexpression varied depending on the rostrocaudal level of the nucleus. In contrast, 5-HTT mRNA-positive cells in the lateral wings of the DR were apparently devoid of 5-ht$_{5B}$ mRNA. These observations denote an intranuclear heterogeneity in the expression of 5-ht$_{5B}$ receptor mRNA in the anterior raphe nuclei. Such intranuclear differences in the expression of a putative autoreceptor raise the possibility for a differential functionality of serotonergic cells in these subregions of the anterior raphe nuclei.

Abundant evidence is available in the literature indicating the existence of different neuronal subgroups in the DR, with distinct morphological and neurochemical features and particular neuroanatomic organization of their afferent and efferent connections (Steinbusch, 1981; Imai et al., 1986). For instance, cells located in the lateral wings of the DR are larger and less densely packed than cells in the other subnuclei (Steinbusch, 1981; Törk, 1990). Differential subregional distributions of neuronal afferents to the DR and CS have been reported by several groups (Peyron et al., 1998; Fite et al., 1999; Kirby et al., 2000; Goto et al., 2001). Also, a topographic organization of efferent pathways arising from the mesencephalic raphe nuclei is supported by numerous studies (Villar et al., 1988; Vertes, 1991; Gonzalo-Ruiz et al., 1995; Simpson et al., 1998; Vertes et al., 1999; Janusonis et al., 1999; Kirifides et al., 2001). Several groups agree in ascribing the origin of cortical 5-HT inputs to neurons located in medial portions of the DR. Thus, 5-HT pathways innervating the medial prefrontal, the piriform, and primary somatosensory (barrel field) appear to arise from the midline portion of the DR (Van Bockstaele et al., 1993), ventromedial DR (Datiche et al., 1995), and midline regions of the rostral DR (Kirifides et al., 2001), respectively. These latter observations are of particular interest in the context of the present work, since we found 5-ht$_{5B}$ receptor mRNA signal in most 5-HT cells in the medial aspect of the DR. Therefore, it could be speculated that 5-ht$_{5B}$ autoreceptors would operate in serotonergic projections to the cortex but not in projections to some subcortical structures, including some visual centers and trigeminal somatosensory nuclei, which receive serotonergic inputs from the lateral wings of the DR.

In addition to the raphe nuclei, 5-ht$_{5B}$ receptor mRNA is found in few other brain structures. As shown by *in situ* hybridization histochemistry (Matthes et al., 1993; Erlander et al., 1993; Kinsey et al., 2001), very high concentrations of 5-ht$_{5B}$ receptor mRNA are found in the medial nucleus of the habenula, inferior olivary nucleus and pyramidal cell layer of the CA1 field (but not CA3) of the hippocampus. The densities of 5-ht$_{5B}$ receptor mRNA are high in the DR and intermediate in the CS, as well as in the parasubiculum and the entorhinal cortex. Only low densities are present in the dentate gyrus and lateral nucleus of the habenula. The physiological functions of the 5-ht$_{5B}$ receptor are still unknown, but some speculations can be put forward in basis to the neuroanatomical localization of its mRNA. For instance, the presence of 5-ht$_{5B}$ receptor mRNA in the inferior olive and hippocampus might suggest a role of this receptor in motor coordination and learning and memory (Scoville and Milner, 1957; Parent and Carpenter, 1996). Likewise, 5-ht$_{5B}$ receptors expressed in the habenula might be involved in the modulation of exploratory behavior (Lee and Huang, 1988). In fact, LSD, which has high affinity for recombinant 5-ht$_{5B}$ receptors (Wisden et al., 1993), has been reported to alter locomotor and exploratory behavior (Mittman and Geyer, 1991). Unfortunately, until specific antisera or selective ligands for the 5-ht$_{5B}$ receptor are available, it will be very difficult to properly study the physiological and functional role of this receptor in the mammalian brain.

The presence of 5-ht$_{5B}$ receptor mRNA in some populations of serotonergic neurons in the DR and CS described here suggests an autoreceptor role for this neuroreceptor. The physiological function of 5-ht$_{5B}$ autoreceptors would most likely depend on their cellular location on cell bodies, dendrites and/or terminals of 5-HT neurons. To our knowledge, one single report has addressed the issue of the anatomical distribution of 5-ht$_{5B}$ receptor binding sites (Waeber et al., 1998). According to these authors, putative 5-ht$_{5B}$ receptors (both auto- and heteroreceptors) would be localized predominantly at the somatodendritic level. So far, the existence of three 5-HT autoreceptors (5-HT$_{1A}$, 5-HT$_{1B}$ and 5-HT$_{1D}$ subtypes) is well established (Hoyer et al., 1994; Stamford et al., 2000). The 5-HT$_{1A}$ autoreceptor is expressed in the somatodendritic compartment of 5-HT neurons, where it controls cell firing as well as 5-HT synthesis and release, both locally and in projection areas, whereas 5-HT$_{1B}$ and 5-HT$_{1D}$ autoreceptors are located on 5-HT nerve terminals where they modulate 5-HT release. In the case of 5-ht$_{5B}$ autoreceptors, it seems unlikely that they would control electrical activity of serotonergic neurons, because the decrease in firing of

these neurons elicited by treatment with selective 5-HT reuptake inhibitors (SSRIs) is fully reversed by selective 5-HT_{1A} receptor antagonists (Gartside et al., 1995; Arborelius et al., 1995). In contrast, a possible role of 5-ht_{5B} receptors on the regulation of 5-HT synthesis could be inferred from the observation that the reduction in 5-HT synthesis induced by SSRIs is not reversed neither by the 5-HT_{1A} antagonist WAY 100,635 nor by the 5-$HT_{1B/D}$ antagonist GR 127,935, whereas these compounds do prevent the inhibition of 5-HT synthesis caused by 5-HT_{1A} or 5-$HT_{1B/D}$ selective agonists, respectively (Moret and Briley, 1997; Barton and Hutson, 1999). In addition it is noteworthy that methiothepin, which shows a relatively high affinity for the recombinant 5-ht_{5B} receptor (Matthes et al., 1993), increases the synthesis of 5-HT (Moret and Briley, 1997). It cannot be excluded that the 5-ht_{5A} subtype might also participate in the mentioned effects on 5-HT synthesis, since 5-ht_{5A} receptor–like immunoreactivity has been found in several raphe nuclei (Oliver et al., 2000). Further work is necessary to ascertain whether 5-ht_{5B} receptors do act as autoreceptors and determine their functional relevance.

In summary, our study shows the presence of 5-ht_{5B} receptor mRNA in some 5-HTT mRNA-positive cells of the rat DR and CS. This localization suggests that 5-ht_{5B} receptors might act as autoreceptors in specific populations of serotonergic neurons. In addition, the present report provides further evidence for the existence of clearly differentiated cell populations in the DR in terms of neurochemical characteristics. However, the actions of this putative autoreceptor and the relevance of the subregional variability in the expression of 5-ht_{5B} receptor mRNA within the DR remain to be established.

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Table 1. Estimated densities of 5-ht$_{5B}$ receptor mRNA in different regions of the rat brain.

Brain region	5-ht$_{5B}$ receptor mRNA
Cortex	
Primary olfactory cortex	-
Frontal cortex	-
Parietal cortex	-
Temporal cortex	-
Retrosplenial cortex	-
Entorhinal cortex, intermediate layers	++
Entorhinal cortex, external layers	+
Basal ganglia and olfactory tubercle	-
Hippocampus	
CA1 (pyramidal cell layer)	++++
CA2 (pyramidal cell layer)	-
CA3 (pyramidal cell layer)	-
Dentate gyrus (granule cell layer)	+
Subiculum	-
Parasubiculum	++
Thalamus	
Medial habenula	++++
Lateral habenula	+
Other thalamic nuclei	-
Hypothalamus	-
Amygdala	-
Midbrain and brainstem	
Superior and inferior colliculi	-
Parabigeminal nucleus	-
Dorsal raphe nucleus	+++
Central superior nucleus	++
Inferior olivary nucleus	++++

Data were obtained by microdensitometric analysis of film autoradiograms. Density of 5-ht$_{5B}$ receptor mRNA hybridization signal: +: low; ++: intermediate; +++: high; ++++: very high; – not detected.

FIGURES

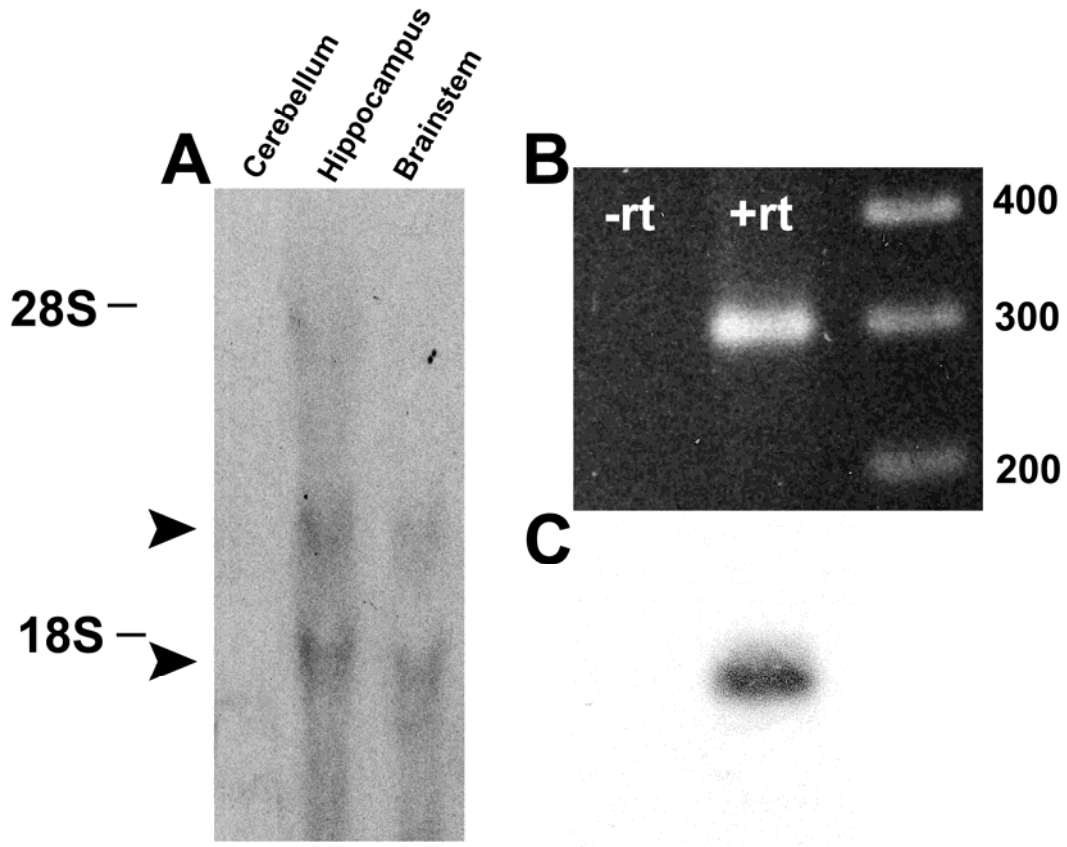

Fig. 1. A: Northern blot analysis of the expression of 5-ht$_{5B}$ receptor mRNA. Total RNA samples from cerebellum, hippocampus and brainstem (30 µg each) were used. Two bands (arrowheads) of ~3,000 and ~1,900 nucleotides are detected in lanes from hippocampus and brainstem, while no signal can be observed in cerebellum. **B,C:** Expression of mRNA coding for 5-ht$_{5B}$ receptor detected by RT-PCR. Presence or absence of reverse transcriptase is indicated, respectively, by +rt and -rt. Sizes in basepairs of the relevant molecular weight markers are indicated. Ethidium bromide staining (**B**) of the RT-PCR products and (**C**) the hybridization signal obtained with the ^{33}P-labeled 5-ht$_{5B}$ receptor oligonucleotide probe.

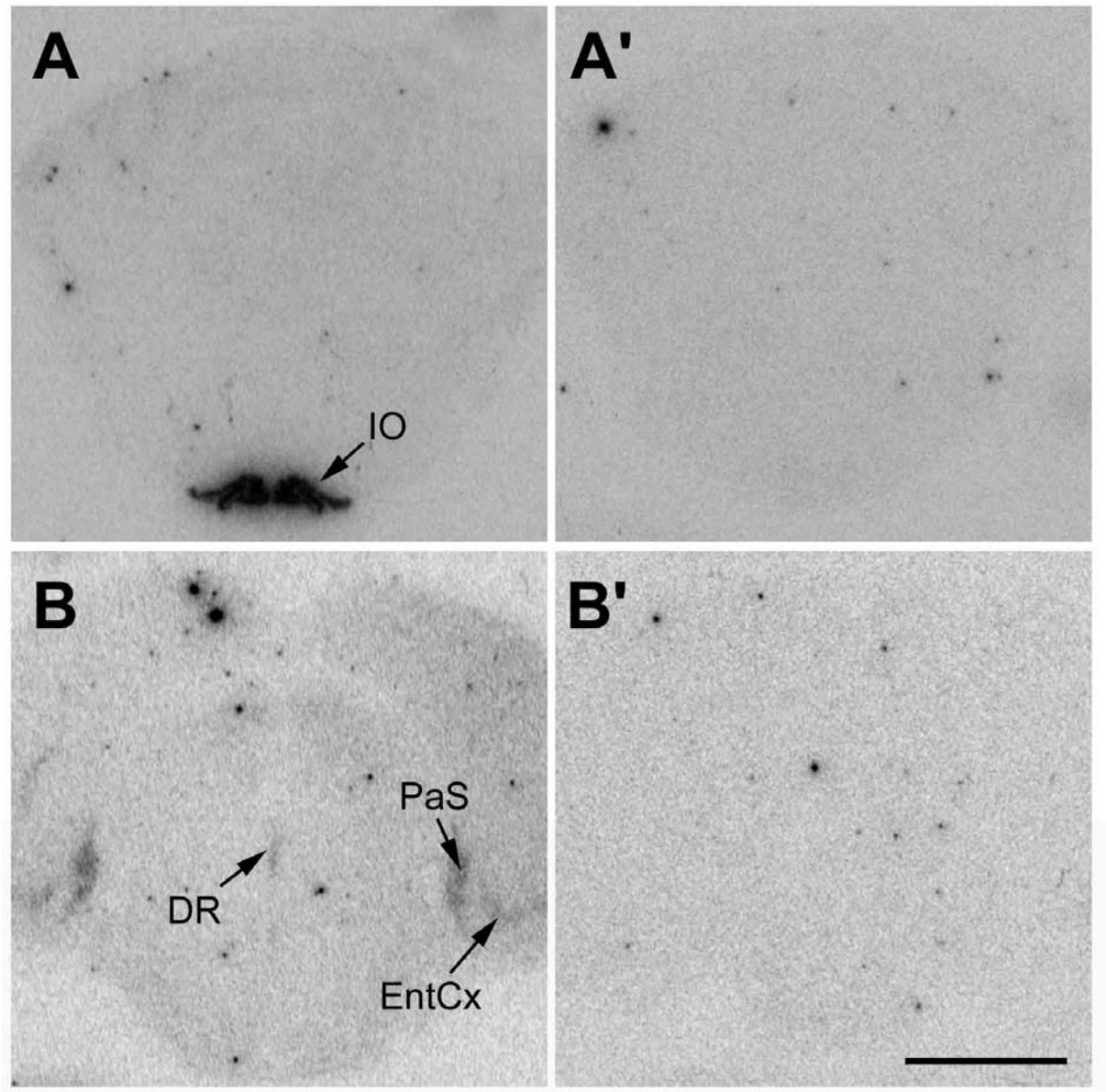

Fig. 2. Film autoradiograms showing the hybridization pattern obtained from brain sections taken at two different levels using the ^{32}P-labeled 5-ht_{5B} receptor probe alone (**A, B**) or in the presence of an excess of the unlabeled oligonucleotide (**A', B'**). Note that the labeling in inferior olive (IO), dorsal raphe (DR), entorhinal cortex (EntCx) and parasubiculum (PaS) is strong in standard incubation conditions (**A, B**) and it is blocked in the corresponding control sections (**A', B'**). Scale bar = 3 mm.

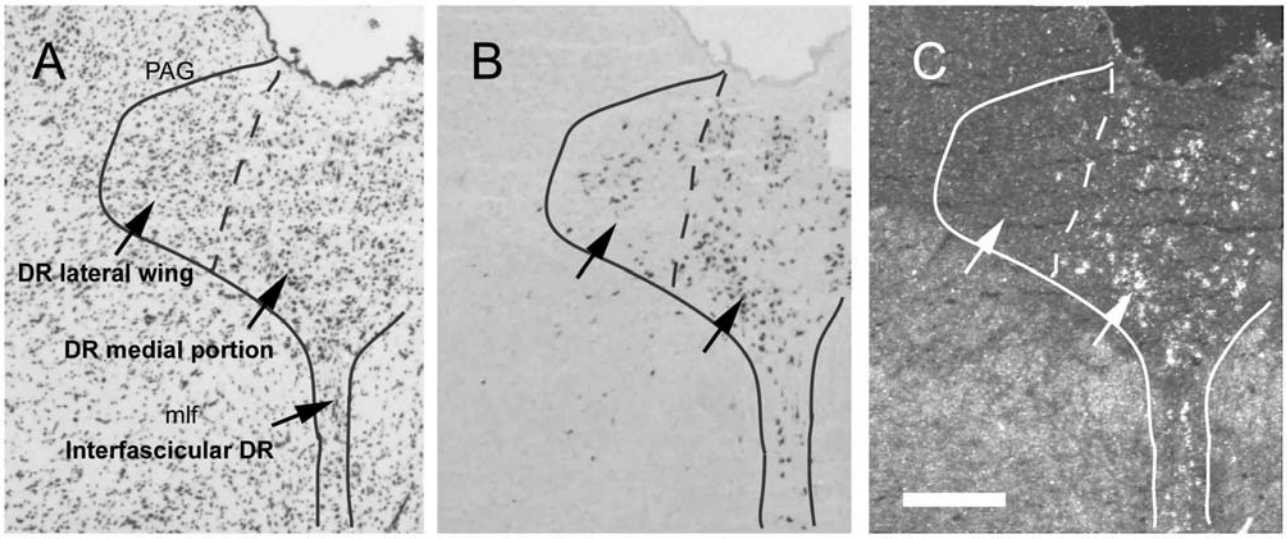

Fig. 3. Visualization of 5-HTT and 5-ht_{5B} receptor mRNAs in the dorsal raphe. **A:** A consecutive section to **B** and **C** stained with cresyl violet for anatomical reference. The approximate limits of the DR and its subdivisions –lateral wings and medial portion- are depicted. **B:** A bright-field photomicrograph where numerous cell profiles expressing 5-HTT mRNA are visualized using digoxigenin-labeled oligonucleotide probes. **C:** A dark-field photomicrograph from an emulsion-dipped tissue section displaying 5-ht_{5B} receptor mRNA signal in the DR. Note that 5-ht_{5B} receptor mRNA signal is restricted to the medial portion of the DR (including dorsomedial, ventromedial and interfascicular parts), while the lateral wings show no specific hybridization signal. DR, dorsal raphe; mlf, medial longitudinal fasciculus; PAG, periaqueductal gray. Scale bar = 0.5 mm.

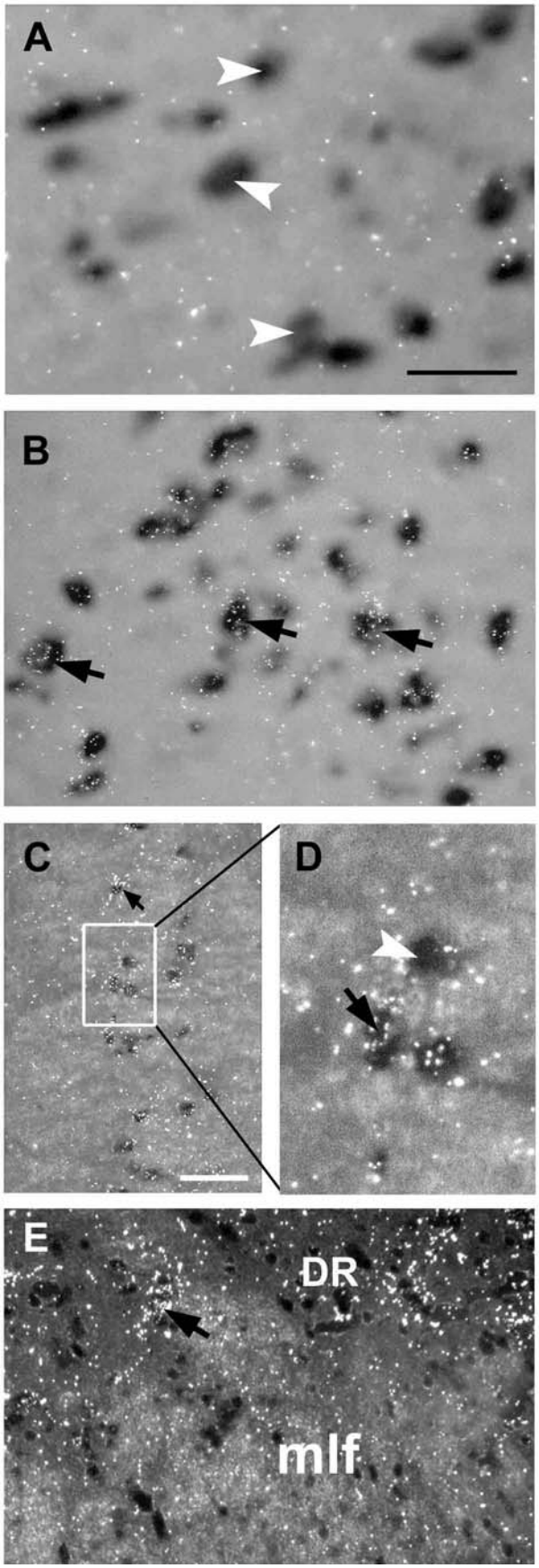

Fig. 4. High-magnification dark-field photomicrographs show the simultaneous detection of two species of mRNAs using a ^{33}P-labeled oligonucleotide probe for 5-ht$_{5B}$ receptor mRNA (silver grains) and digoxigenin-labeled probes for 5-HTT mRNA (dark precipitate), in dorsal raphe lateral wings (**A**), medial portion of dorsal raphe (**B**), central superior nucleus (**C** and detail enlarged in **D**) and medial longitudinal fasciculus (mlf) (**E**). Black arrows point to double-labeled cells and white arrowheads point to single 5-HTT mRNA labeled cells. In the lateral wings of the dorsal raphe, 5-HTT mRNA is expressed in cells which do not display 5-ht$_{5B}$ receptor mRNA signal (**A**), whereas in the medial portion of the dorsal raphe (**B**), as well as in the central superior nucleus (**C**,**D**), most cells containing 5-HTT mRNA co-express 5-ht$_{5B}$ receptor mRNA. Note that photomicrographs were taken in the plane of the autoradiographic grains and therefore cellular staining is slightly out of focus. **E:** the absence of labeling in white matter (mlf) in comparison with the labeled cells of the DR (arrow). Scale bar = 50 µm (**A,** for **B,D; C** for **E**).

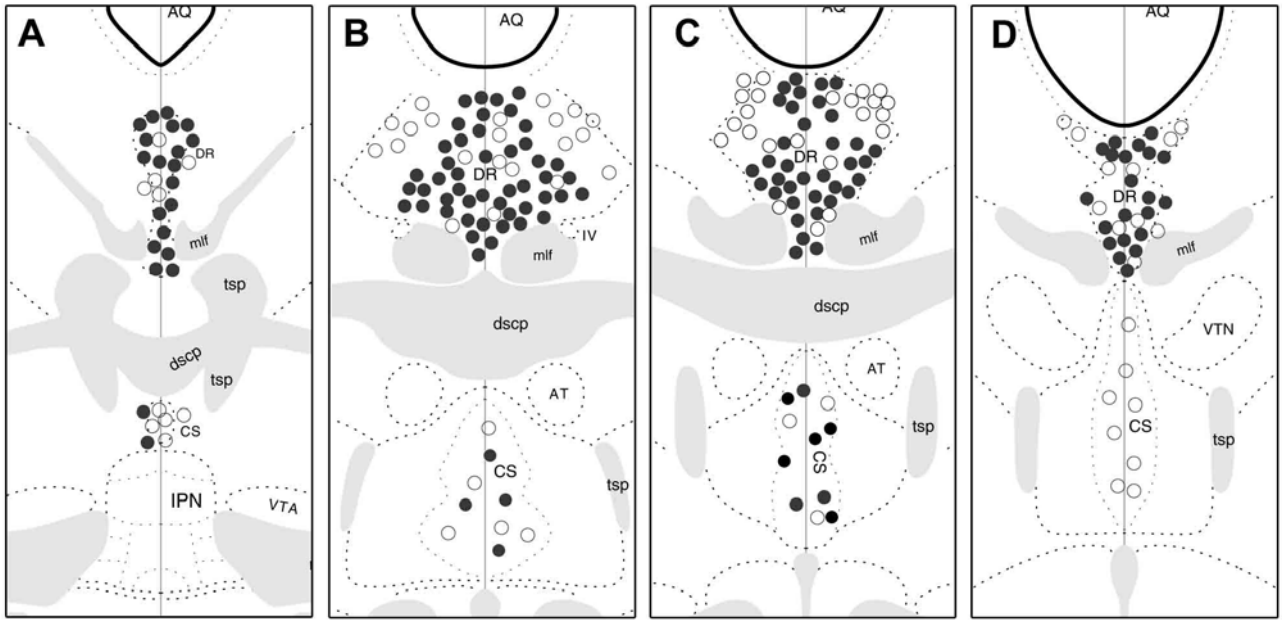

Fig. 5. Schematic representations of the rat lower midbrain and upper pons showing the subregional location of cells coexpressing 5-ht_{5B} receptor mRNA and 5-HTT mRNA (filled circles) and cells expressing only 5-HTT mRNA (empty circles) in the DR and CS. Each circle represents 3 labeled cell profiles. The drawings were modified from the atlas of Swanson (1998) representing levels from –7.10 mm to 8.3 mm from bregma. AT: anterior tegmental nucleus; AQ: cerebral aqueduct; CS: central superior nucleus; DR: dorsal raphe; dscp: decussation of the superior cerebellar peduncle; IPN: interpeduncular nucleus; mlf: medial longitudinal fasciculus; tsp: tectospinal pathway; VTA: ventral tegmental area; VTN: ventral tegmental nucleus.

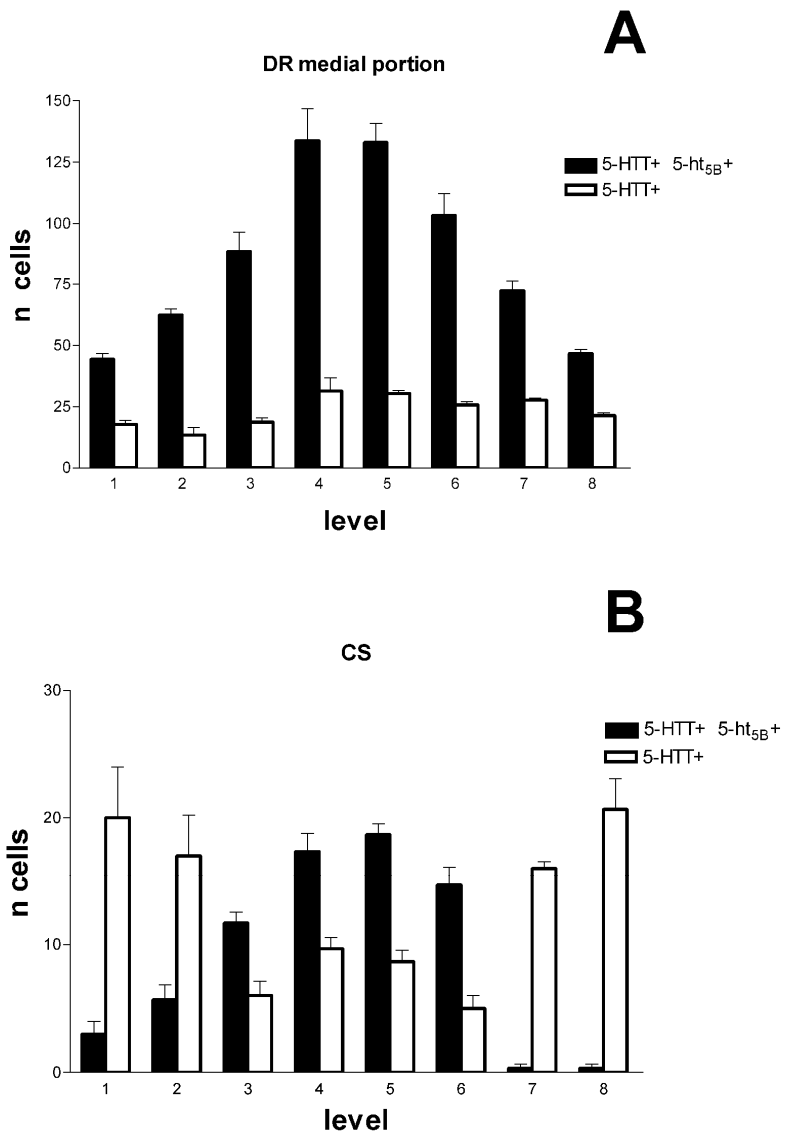

Fig. 6. Degree of co-localization of 5-ht$_{5B}$ receptor mRNA with 5-HTT mRNA in the medial portion of the DR (**A**) and in the CS (**B**). Bars indicate the total number of cell profiles co-expressing 5-HTT mRNA and 5-ht$_{5B}$ receptor mRNA (black bars) or single 5-HTT mRNA-labeled cell profiles (white bars) per section in these nuclei. In the DR, only the medial portion was considered, including the ventromedial, dorsomedial and interfascicular portions of the nucleus, but not the lateral wings. Cells were counted in 14 µm-thick coronal sections taken at eight different anteroposterior levels (1-8) comprised between planes –7.0 to –9.0 mm from bregma. Data are cell counts (mean ± SD) and were obtained from three animals.