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Expression of serotonin 5-HT_{2C} receptors in GABAergic cells of the anterior raphe nuclei.

Jordi Serrats, Guadalupe Mengod, Roser Cortés*

Department of Neurochemistry,
Institut d'Investigacions Biomèdiques de Barcelona (IIBB) – CSIC (IDIBAPS),
Barcelona, Spain.

*Corresponding Author:

Roser Cortés
Dept. of Neurochemistry
IIBB – CSIC (IDIBAPS)
Rosselló 161, 6th floor
E-08036-Barcelona
Spain

tel. 34 93 363 83 22
Fax. 34 93 363 83 01
e-mail: rccngr@iibb.csic.es

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Abstract

We have used double *in situ* hybridization to examine the cellular localization of 5-HT_{2C} receptor mRNA in relation to serotonergic and GABAergic neurons in the anterior raphe nuclei of the rat. In the dorsal and median raphe nuclei 5-HT_{2C} receptor mRNA was not detected in serotonergic cells identified as those expressing serotonin (5-HT) transporter mRNA. In contrast, 5-HT_{2C} receptor mRNA was found in most GABAergic cells, recognized by the presence of glutamic acid decarboxylase mRNA. Such 5-HT_{2C} receptor-positive GABAergic neurons were mainly located in the intermediolateral and lateral portions of the dorsal raphe and lateral part of the median raphe. The present data give anatomical support to a previous hypothesis that proposed a negative-feedback loop involving reciprocal connections between GABAergic interneurons bearing 5-$HT_{2A/2C}$ receptors and 5-HT neurons in the dorsal raphe and surrounding areas. According to this model, the excitation of GABAergic interneurons through these 5-HT_{2C} (and also 5-HT_{2A}) receptors would result in the suppression of 5-HT cell firing.

Keywords: dorsal raphe – median raphe – GAD – 5-HT transporter – serotonergic lesions – 5,7-DHT – *in situ* hybridization –

Running title: 5-HT_{2C} receptors in the raphe nuclei

Abbreviations used in text:

5-HT	5-hydroxytryptamine, serotonin
5-HTT	serotonin transporter
5,7-DHT	5,7-dihydroxytryptamine
Dig	digoxigenin
DOI	1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane)
DR	dorsal raphe nucleus
GAD	glutamic acid decarboxylase
IPSCs	inhibitory postsynaptic currents
MnR	median raphe nucleus

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a neurotransmitter involved in the control of a variety of functions, such as mood, appetite or sleep-wake cycle (Leibowitz and Alexander, 1998; Barnes and Sharp, 1999). Alterations in serotonergic neurotransmission have been related to several neurodegenerative and psychiatric disorders such as Alzheimer's disease (Dewar et al., 1990), depression (Gross-Isseroff et al., 1990b) and schizophrenia (Burnet et al., 1996) as well as to normal aging (Gross-Isseroff et al., 1990a). The physiological and pharmacological effects of 5-HT and serotonergic drugs are mediated by multiple receptor subtypes that have been classified into seven subfamilies (5-HT_1-5-HT_7) (Hoyer et al., 1994) and which are differentially distributed in the CNS (Palacios et al., 1990; Radja et al., 1991; Mengod et al., 1996). The 5-HT_2 receptor family comprises three subtypes: 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. They share similarities in their molecular structure, pharmacology and signal transduction pathways (Hoyer et al., 1994; Barnes and Sharp, 1999). The 5-HT_{2C} receptor (formerly named 5-HT_{1C}) was initially described in porcine choroid plexus (Pazos et al., 1984) and was soon cloned thereafter (Julius et al., 1988). Its anatomical distribution has been studied in rat brain using radioligand binding autoradiography, *in situ* hybridization, and immunohistochemistry (Pazos and Palacios, 1985; Hoffman and Mezey, 1989; Molineaux et al., 1989; Mengod et al., 1990; Pompeiano et al., 1994; Abramowski et al., 1995; Wright et al., 1995; Eberle-Wang et al., 1997; Clemett et al., 2000). These studies have shown the presence of 5-HT_{2C} receptors in the raphe nuclei and surrounding areas.

The finding of 5-HT_{2C}-immunoreactive cells in the raphe nuclei has let to the proposal that some 5-HT neurons might express these receptors (Clemett et al., 2000). In contrast, electrophysiological data rather suggest that 5-HT_{2C} receptors are located on local GABAergic neurons, inside or close to the dorsal raphe (DR) (Liu et al., 2000), being part of a local negative-feedback circuit which would involve reciprocal connections between GABAergic and 5-HT neurons. This model has been proposed to explain the increases in the frequency of inhibitory postsynaptic currents (IPSCs) induced by 5-HT and the 5-$HT_{2A/2C}$ agonist DOI (1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane) when applied to rat brain slices containing the DR.

The purpose of the present study was to determine whether 5-HT_{2C} receptors in the dorsal raphe (DR) and median raphe (MnR) nuclei are expressed by serotonergic, GABAergic neurons, or both. Thus, we have used double-label *in situ* hybridization histochemistry in order to simultaneously visualize 5-HT_{2C} receptor mRNA and serotonergic and GABAergic cell markers, 5-HT transporter (5-HTT) mRNA and glutamic acid decarboxylase (GAD) mRNA, respectively.

2. Materials and methods

2.1. Specimens

Adult male Wistar rats (n=25) (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-hours light-dark circle 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 -20ºC. Tissue sections, 14 µm thick, were cut using a microtome-cryostat (HM500 OM, Microm, Walldorf, Germany), thaw-mounted onto slides coated with APTS (3-aminopropyltriethoxysilane, Sigma, St Louis, USA) and kept at –20ºC until use.

2.2. 5,7-Dihydroxytryptamine lesions

Forty-five minutes before 5,7-dihydroxytryptamine (5,7-DHT) administration, rats (n=4) were pretreated with desipramine (25 mg kg^{-1}, i.p.) (ICN Biomedicals Inc., Aurora, Ohio, USA) in order to prevent uptake of the toxin by noradrenaline neurons. The animals were then anesthetized with chloral hydrate (350 mg kg^{-1}) and placed in a stereotaxic frame. Each rat received an injection of freshly prepared solution of 420 µg 5,7-DHT creatinine sulphate (equivalent to 200 µg of 5,7-DHT base) (ICN Biomedicals Inc.) in 20 µl of 0.1% ascorbic acid in saline into the lateral ventricle at coordinates: AP -0.8, ML -1.4, DV –4.2 mm using a Hamilton syringe. Sham operated animals (n=4) were also pretreated with desipramine and received an i.c.v. injection of vehicle (20 µl of 0.1% ascorbic acid in saline) at the same stereotaxic coordinates. The animals were allowed to survive for 14 days, and then they were killed and the brains processed as described above.

2.3. Hybridization probes

The oligodeoxyribonucleotide probes used were complementary to the following bases: 1100-1147 of the rat 5-HT_{2C} receptor cDNA (Genbank acc. no. U35315); 305-352 and 865-912 of the rat 5-HTT cDNA (Genbank acc. no. M79450); 191-235 of the rat GAD_{67} cDNA (Genbank acc. no. M34445) were synthesized on a 380 Applied Biosystems DNA synthesizer (Foster City Biosystem, Foster City, USA) and purified on a 20% polyacrylamide / 8 M urea preparative sequencing gel.

Oligonucleotides were labeled either with ^{33}P or with digoxigenin (Dig). For the radioactive method, the oligonucleotides (2 pmol) were 3'-end-labeled with [^{33}P]α-dATP (>2500 Ci/mmol; DuPont-NEN, Boston, USA) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany), purified by centrifugation using QIAquick Nucleotide Removal Kit (QIAGEN GmbH, Hilden, Germany). Alternatively, for the non-radioactive 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.

2.4. 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., 2003a). 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 rt, twice for 5 min 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 albumine), 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-labeled probes in the hybridization buffer were in the same range (approximately 1.5 nM). Tissue sections were covered with hybridization buffer containing the labeled probe(s), overlaid with Nescofilm coverslips (Bando Chemical Ind, 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 (pH 7.5) at 60ºC.

2.5. Development of radioactive and non-radioactive hybridization signal

The slides that had been hybridized only with ^{33}P-labeled probes were directly dehydrated through ethanol and allowed to air-dry. In parallel, sections hybridized with Dig-labeled 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, 2mM $MgCl_2$ 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 overnight with 330 µg/ml nitroblue tetrazolium and 165 µg/ml bromochloroindolyl phosphate (Gibco BRL, Gaithersburg, USA) in alkaline buffer. The enzymatic reaction was stopped by extensive rinsings 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-labeled oligonucleotides) were dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, UK) diluted 1:1 with distilled water. The sections were exposed in the dark at 4°C for 1 week in the case of GAD mRNA or 6 weeks in the case of 5-HT_{2C} receptor mRNA, and finally developed in Kodak D19 (Kodak, Rochester, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford).

For film autoradiography, some hybridized sections were exposed to Biomax-MR (Kodak) films for 2-4 weeks at –70ºC with intensifying screens. Consecutive sections were stained with thionin for anatomical reference.

2.6. Specificity of the probes

The specificity of the probes has been previously established and published elsewhere (Pompeiano et al., 1994; Serrats et al., 2003a). The control experiments performed included: 1) determination of the thermo-stability of hybrids obtained with every probe, 2) blockade of the hybridization signal by competition of the labeled probe with 50-fold excess of the same unlabeled oligonucleotide, 3) for each mRNA species at least two different oligonucleotide probes were assayed separately, and hybridization signal was considered specific only when the regional and cellular labeling obtained with both probes independently was identical, 4) to assure the specificity of the non-radioactive hybridization signal it was compared to that obtained with the same probes radioactively labeled.

2.7. Analysis of the results

Tissue sections were examined and photographed under bright- and dark-field illumination in a Wild 420 macroscope (Leica, Heerbrugg, Germany), in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) and in an Axioplan Zeiss microscope (Carl Zeiss, Oberkochen, Germany). Hybridization signal obtained with non-radioactive oligonucleotides (5-HTT mRNA and GAD mRNA probes) was identified as a dark staining (alkaline phosphatase reaction product) surrounding or covering cellular nuclei. Radioactive hybridization signal (GAD mRNA and 5-HT_{2C} receptor mRNA) was considered positive when accumulations of silver grains over cell profiles were at least three-fold higher than average background.

In double in situ hybridization experiments, single- and double-labeled cells were counted in emulsion-dipped tissue sections from 11 animals (3 control rats and 8 rats from lesion studies -4 lesioned and 4 sham animals-) taken at four anteroposterior levels of the DR and MnR. Cell counting was performed manually at the microscope with the help of analySIS Software. 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). The figures were prepared for publication using Adobe Photoshop software (Adobe, San Jose, USA). Data were statistically analyzed using one-way ANOVA analysis (GraphPad Software Inc., San Diego, USA). Significance level was considered at p-value (p)<0.05.

3. Results

The presence of 5-HT_{2C} receptor mRNA in cells of the rat raphe nuclei has been examined at four different coronal planes of the DR and MnR, corresponding to -7.1, -7.9, -8.6 and -9.1 mm from bregma according to Paxinos and Watson (Paxinos and Watson, 1998). A macroscopic view of the localization of 5-HT_{2C} receptor mRNA positive cells in the DR and superior portion of MnR at level 44 of atlas of Swanson (Swanson, 1998) is illustrated in Fig.1.

5-HT_{2C} receptor mRNA hybridization signal could be clearly seen as autoradiographic grains concentrated over cell profiles in the DR and MnR, as well as in other structures of the rat upper pons including the periaqueductal gray, superior and inferior colliculi, tegmental nuclei and reticular formation, as shown in Fig. 1. Within the DR, 5-HT_{2C} receptor mRNA positive cells were mainly detected in the intermediolateral portion and lateral wings (see Figs. 1 and 2A), and only few cells were found in the central aspects of the nucleus (including the dorsomedial, ventromedial, interfascicular and caudal subnuclei). Two types of cell profiles could be distinguished depending on the density of 5-HT_{2C} receptor mRNA labeling: strongly labeled and weakly labeled cells, which were intermingled in the DR. In the MnR, 5-HT_{2C} receptor mRNA-containing cells were mainly located in the lateral zone (Fig. 2B). The majority of them showed a weak hybridization signal, but some strongly labeled cells were also seen, confined to the dorsal portion of the MnR.

5-HTT and GAD mRNAs were used as markers of serotonergic and GABAergic cells respectively and were detected in tissue sections using either non-radioactive or radioactive probes. A large number of 5-HTT mRNA-positive cells were visualized in the DR and MnR. Numerous GAD mRNA-containing cells could also be identified in these

nuclei, mainly located in the ventrolateral and intermediolateral portions of the DR and in the lateral aspects of the MnR as we have recently reported (Serrats et al., 2003a).

In order to analyze the cellular coexistence of the different mRNAs, we performed double-label in situ hybridization experiments on tissue sections from 3 rats taken at four anteroposterior anatomical levels of through the DR and MnR. We used ^{33}P-labeled oligonucleotides to identify 5-HT$_{2C}$ receptor mRNA and Dig-labeled oligonucleotides to detect 5-HTT or GAD mRNAs (Fig. 2C,D). We observed that in the raphe nuclei 5-HT$_{2C}$ receptor mRNA and 5-HTT mRNA were expressed in different cell populations. Thus, we did not visualize any serotonergic cell expressing detectable levels of 5-HT$_{2C}$ receptor mRNA in the anterior raphe nuclei (Fig. 2C).

In contrast, 5-HT$_{2C}$ receptor mRNA was often found in GAD mRNA-containing cells in the DR and MnR (Fig. 2D). In the DR the amount of cells per section that co-expressed both mRNAs varied from 33.4 ± 8.5 to 49.3 ± 9.0 (mean ± SEM, n=3), depending on the anteroposterior level of nucleus. Still, a substantial number of cells expressing 5-HT$_{2C}$ receptor mRNA was devoid of the GABAergic marker (17.3 ± 1.5 to 23.3 ± 0.9 cells per section). Although most GABAergic cells in the DR were found to express 5-HT$_{2C}$ receptor mRNA (84.32 ± 0.97%), we also detected GAD mRNA-positive cells without apparent 5-HT$_{2C}$ receptor mRNA labeling (5.1 ± 2 .4 to 10.1 ± 2.2 cells per section). A similar situation was found in the MnR, although the number of cells that co-expressed both 5-HT$_{2C}$ receptor and GAD mRNA was lower (23.1 ± 3.5 to 26.8 ± 2.4 cells per section, accounting for the 79.00 ± 1.69% of the total number of GAD mRNA positive cells). The MnR also contained 5-HT$_{2C}$ receptor mRNA-positive cells devoid of GAD mRNA (13.4 ± 3.6 to 24.5 ± 1.5 cells per section) and GAD mRNA cells devoid of 5-HT$_{2C}$ receptor mRNA signal (2.1 ± 1.2 to 11 ± 2.9 cells per section, depending on the anteroposterior level of the MnR).

Effect of 5,7-DHT lesions

Intracerebroventricular (i.c.v.) injection of 5,7-DHT induced a large depletion of 5-HTT mRNA labeling in the DR and MnR (Fig. 3A,A'). Thus, 5-HTT mRNA hybridization signal was completely absent in the DR of the lesioned rats, and only a few labeled cells could be observed in the most ventral part of the MnR. In contrast, GAD mRNA signal in lesioned animals did not differ from sham-operated rats (Fig. 3B,B'). 5-HT$_{2C}$ receptor

mRNA-positive cells were still present in the DR and MnR after the lesion (Fig. 3C,C'). In the DR of treated rats, particularly in intermediate levels of the nucleus, we observed a slight increment in the number of 5-HT_{2C} receptor mRNA positive cells. Thus, the average total number of 5-HT_{2C} receptor mRNA-containing cells counted in 4 sections was 170.0 ± 5.7 in lesioned rats compared to 141.5 ± 10.6 in sham operated rats. However, there were no statistically significant differences between these data.

4. Discussion

In this study we have visualized the presence of 5-HT_{2C} receptor mRNA in cells of the anterior raphe nuclei. Our data basically demonstrate that 5-HT_{2C} receptor mRNA is not expressed by serotonergic cells but, in contrast, it is found in the majority of GABAergic neurons located within the DR and MnR. Although the existence of 5-HT_{2C} receptor mRNA in the anterior raphe nuclei has been described before (Hoffman and Mezey, 1989; Molineaux et al., 1989; Pompeiano et al., 1994; Wright et al., 1995), the phenotype of the cells that express this receptor remained to be determined.

Cells showing 5-HT_{2C} receptor mRNA hybridization signal were heterogeneously distributed within the DR and MnR, and were mainly located in their lateral and intermediolateral parts. In contrast, only few 5-HT_{2C} positive cells were found in the central portions of the DR, where numerous 5-HT neurons are tightly packed together (Steinbusch, 1981) (see also Fig. 3A). Our results are in very good agreement with the reported histochemical detection of 5-HT_{2C} receptor–like immunoreactivity in the rat DR (Clemett et al., 2000) and are compatible with data from autoradiographic studies using [^{3}H]5-HT or [^{3}H]mesulergine, which have shown the presence of moderate labeling of 5-HT_{2C} binding sites in the raphe region (Pazos and Palacios, 1985). Similarly to the raphe nuclei, in most areas of the rat brain there is a good correlation between the anatomical distribution of 5-HT_{2C} receptor mRNA, 5-HT_{2C} receptor immunoreactivity and 5-HT_{2C} receptor binding sites (Pazos and Palacios, 1985; Hoffman and Mezey, 1989; Molineaux et al., 1989; Mengod et al., 1990; Pompeiano et al., 1994; Abramowski et al., 1995; Wright et al., 1995; Clemett et al., 2000), suggesting that 5-HT_{2C} receptors are located at the level of cell body and/or dendrites.

In order to determine whether 5-HT_{2C} receptor-bearing cells in the raphe are serotonergic or GABAergic, we have examined the cellular localization of 5-HT and

GABAergic markers. Since it has been shown that 5-HTT is exclusively expressed by 5-HT neurons (Rattray et al., 1999), we have taken 5-HTT mRNA as indicator of serotonergic phenotype. GABAergic cells were identified as those containing GAD_{67} mRNA. Our data on the cellular localization of 5-HTT and GAD mRNAs in the rat raphe nuclei are in agreement with published studies. Thus, 5-HTT mRNA hybridization signal was very strong in the raphe nuclei, as it has been shown in different species (Fujita et al., 1993; Rattray et al., 1994; Charnay et al., 1996; McLaughlin et al., 1996; Bengel et al., 1997; Rattray et al., 1999; Serrats et al., 2003a), and in agreement with studies using ligand binding autoradiography (Dawson and Wamsley, 1983; D'Amato et al., 1987), and immunohistochemistry (Sur et al., 1996). The distribution of 5-HTT mRNA-positive cells observed here is fully comparable to the known location of serotonergic cell bodies (Dahlström and Fuxe, 1964; Steinbusch, 1981). On the other hand, we found GAD_{67} mRNA-labeled cell profiles in the ventrolateral and intermediolateral aspects of the DR, in the MnR and other cell groups, as we have already reported (Serrats et al., 2003a). This organization of GAD mRNA labeled cells is identical to that described in former studies using *in situ* hybridization and immunohistochemistry (Nanopoulos et al., 1982; Mugnaini and Oertel, 1985; Julien et al., 1987; Wang et al., 1992; Feldblum et al., 1993; Ford et al., 1995). The hybridization signal obtained for GAD and 5-HTT mRNA was much stronger than that of $5\text{-}HT_{2C}$ receptor mRNA.

Our double-label experiments indicate that $5\text{-}HT_{2C}$ receptor mRNA is expressed by most GABAergic cells of the anterior raphe nuclei, but not by 5-HTT mRNA positive cells. The non-serotonergic nature of $5\text{-}HT_{2C}$ receptor-containing cells is further confirmed by the results obtained after i.c.v. administration of 5,7-DHT. Thus, as expected, after 14-day survival 5-HT neurons underwent degeneration and 5-HTT mRNA was depleted, whereas neither GAD mRNA nor $5\text{-}HT_{2C}$ receptor mRNAs were significantly affected, indicating that cells expressing these mRNAs were spared after the lesion. Still, we cannot completely rule out the possibility that $5\text{-}HT_{2C}$ receptor mRNA might exist in serotonergic cells that lack 5-HTT. It has been reported that some populations of 5-HT neurons do not bear the transporter in their axons (Brown and Molliver, 2000) but, according to Rattray et al. (1999), putative serotonergic neurons devoid of 5-HTT would represent a minor population, accounting for less than 2% of the

total. The absence of 5-HTT would render these neurons insensitive to the neurotoxin 5,7-DHT, and thus no changes would be expected after the lesion in these cells either.

The presence of 5-HT_{2C} receptor mRNA in GABAergic cells in the raphe nuclei is in accordance with electrophysiological data from Liu et al. (2000). By means of intracellular recordings, these authors have observed that the application of 5-HT to DR slices induces an increase in the frequency of IPSCs in 5-HT neurons. This effect is blocked by the $GABA_A$ antagonist bicuculline, suggesting an involvement of GABAergic neurons, and is partially inhibited by 5-HT_{2A} and 5-HT_{2C} antagonists. Moreover, the effect of the 5-$HT_{2A/2C}$ agonist DOI in this experimental model is markedly greater than that of 5-HT, which might be explained by a dual action of 5-HT with opposing effects at 5-$HT_{2A/2C}$ (excitatory) and 5-HT_{1A} (inhibitory) receptors. Thus, taking into account that GABA is among the neurotransmitters that control the activity of serotonergic neurons of the raphe (Gallager and Aghajanian, 1976), and that there is histochemical evidence showing the presence of GABAergic interneurons in the DR and of GABAergic axon terminals that establish synaptic contacts with serotonergic neurons (Wang et al., 1992), Liu et al. (2000) have proposed the existence of a negative-feedback loop in the raphe in which 5-HT collaterals would innervate GABAergic neurons bearing 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors. These GABAergic neurons in turn would synapse upon 5-HT neurons. In addition to the presence of 5-HT_{2C} receptors on DR GABAergic cells described here, we have also shown the expression of 5-HT_{1A} mRNA in these cells (Serrats et al., 2003b). It still remains to be determined whether these GABAergic neurons also express 5-HT_{2A} receptors.

5-HT_{2C} receptor expression in GABAergic neurons has already been described in other brain structures such as the substantia nigra and ventral tegmental area (Eberle-Wang et al., 1997). It is noteworthy that the situation in these dopaminergic nuclei is similar to our observations in the raphe, since 5-HT_{2C} receptor is found in a subpopulation of GAD mRNA positive neurons but never in dopamine cells. In line with this, it has been observed that 5-HT_{2C} receptor agonists exert inhibitory effects on mesocorticolimbic dopaminergic neurons, which are presumably mediated by GABAergic interneurons (Di Giovanni et al., 2001). It has been suggested that, thanks to their ability to disinhibit the mesolimbic dopaminergic function, 5-HT_{2C} antagonists might be useful as antidepressants and in the treatment of negative symptoms of

schizophrenia (Di Matteo et al., 2001). In view of the present data, we can also speculate that the blockade of 5-HT_{2C} receptors on DR GABA interneurons might be used to decrease the GABAergic tone and indirectly increase the firing of 5-HT cells. This mechanism might open further possibilities for the use of 5-HT_{2C} antagonists in the treatment of depression and can be –at least in part– responsible for the actions of the non-selective 5-$HT_{2A/2C}$ antagonist mianserin as a clinically effective antidepressant.

After 5,7-DHT lesions the number of 5-HT_{2C} receptor mRNA-positive cells in the DR did not significantly vary. This is in contrast with data from other authors that reported an up-regulation of 5-HT_{2C} receptors in projection areas following different types of 5,7-DHT lesions, including increases in 5-HT_{2C} receptor binding in the striatum (Compan et al., 1998) and cerebral cortex (Blurton and Wood, 1986), increases in 5-HT_{2C} receptor immunoreactivity (Sharma et al., 1997) and 5-HT_{2C} receptor-mediated phosphoinositide hydrolysis (Conn et al., 1987) in the choroid plexus. It is known that 5-HT_{2C} receptors are expressed in all these regions (Pompeiano et al., 1994), and are located postsynaptically to 5-HT terminals. These up-regulation phenomena are interpreted as a response of 5-HT_{2C} receptors to the depletion of the neurotransmitter subsequent to the degeneration of these nerve terminals. Further studies would be necessary to clarify whether the lack of 5-HT also induces a process of hypersensitivity of 5-HT_{2C} receptors in the DR.

We have also visualized here 5-HT_{2C} receptor mRNA in cells apparently devoid of GAD mRNA. These GAD mRNA-negative, 5-HTT mRNA-negative neurons that contain 5-HT_{2C} receptor might belong to other cellular types which have been described in the raphe nuclei, such as glutamatergic neurons (Gras et al., 2002), neurons containing tyrosine hydroxylase (Ochi and Shimizu, 1978; Björklund and Lindvall, 1984; Stratford and Wirtshafter, 1990; Kirifides et al., 2001), or enkephalin (Petrusz et al., 1985; Wang and Nakai, 1993) but, as discussed above, they might be also, at least in part, serotonergic cells that do not express the transporter. It remains to be determined whether these 5-HT_{2C} receptor-containing cells are interneurons or projection neurons.

In summary, this report provides an anatomical evidence to support previous electrophysiological results that suggested the localization of 5-HT_{2C} receptor in GABAergic neurons of the raphe nuclei (Liu et al., 2000). Thus, we have observed that 5-HT_{2C} receptor mRNA is expressed in GABAergic neurons of the raphe nuclei and

surrounding areas. Moreover, we have detected 5-HT_{2C} receptor mRNA in other types of neurons in the DR and MnR, pointing out to a complex regulation of the activity of serotonergic neurons through the activation of 5-HT_{2C} receptors of the raphe nuclei.

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Statement of interest

The authors declare that they have no competing financial interests.

Reference list

Abramowski, D., Rigo, M., Duc, D., Hoyer, D., Staufenbiel, M., 1995. Localization of the 5-hydroxytryptamine2C receptor protein in human and rat brain using specific antisera. Neuropharmacol. 34**,** 1635-1645.

Barnes, N.M., Sharp, T., 1999. A review of central 5-HT receptors and their function. Neuropharmacol. 38**,** 1083-1152.

Bengel, D., Jöhren, O., Andrews, A.M., Heils, A., Mössner, R., Sanvitto, G.L., Saavedra, J.M., Lesch, K.-P., Murphy, D.L., 1997. Cellular localization and expression of the serotonin transporter in mouse brain. Brain Res. 778**,** 338-345.

Björklund A. and Lindvall O., 1984. Dopamine-containing systems in the CNS. In: Björklund A., Hökfelt T. (Eds.), Handbook of Chemical Neuroanatomy. Vol 2, Part 1: Classical Transmitters in the brain. Elsevier, Amsterdam, pp. 55-122.

Blurton, P.A., Wood, M.D., 1986. Identification of multiple binding sites for [^{3}H]5-hydroxytryptamine in the rat CNS. J. Neurochem. 46**,** 1392-1398.

Brown, P., Molliver, M.E., 2000. Dual serotonin (5-HT) projections to the nucleus accumbens core and shell: Relation of the 5-HT transporter to amphetamine-induced neurotoxicity. J. Neurosci. 20**,** 1952-1963.

Burnet, P.W., Eastwood, S.L., Harrison, P.J., 1996. 5-HT_{1A} and 5-HT_{2A} receptor mRNAs and binding site densities are differentially altered in schizophrenia. Neuropsychopharmacology 15**,** 442-455.

Charnay, Y., Leger, L., Vallet, P.G., Greggio, B., Hof, P.R., Jouvet, M., Bouras, C., 1996. Mapping of serotonin transporter messenger RNA-containing nerve cell populations in the cat brainstem. J. Chem. Neuroanat. 10**,** 93-100.

Clemett, D.A., Punhani, T., Duxon, M.S., Blackburn, T.P., Fone, K.C.F., 2000. Immunohistochemical localisation of the 5-HT_{2C} receptor protein in the rat CNS. Neuropharmacol. 39**,** 123-132.

Compan, V., Segu, L., Buhot, M.C., Daszuta, A., 1998. Selective increases in serotonin 5-HT1B/1D and 5-HT2A/2C binding sites in adult rat basal ganglia following lesions of serotonergic neurons. Brain Res. 793**,** 103-111.

Conn, P.J., Janowsky, A., Sanders-Bush, E., 1987. Denervation supersensitivity of 5-HT-1c receptors in rat choroid plexus. Brain Res. 400**,** 396-398.

D'Amato, R.J., Largent, B.L., Snowman, A.M., Snyder, S.H., 1987. Selective labeling of serotonin uptake sites in rat brain by [^{3}H]citalopram contrasted to labeling of multiple sites by [^{3}H]imipramine. J. Pharmacol. Exp. Ther. 242**,** 364-371.

Dahlström, A., Fuxe, K., 1964. Evidence for the existence of monoamine-containing neurons in the central nervous system. Acta Physiol. Scand. 62, Supp 232**,** 1-55.

Dawson, T.M., Wamsley, J.K., 1983. Autoradiographic localization of [3H]-imipramine binding sites: Association with serotonergic neurons. Brain Res. Bull. 11**,** 325-334.

Dewar, D., Graham, D.I., McCulloch, J., 1990. 5 HT_2 receptors in dementia of Alzheimer type: a quantitative autoradiographic study of frontal cortex and hippocampus. J. Neural Transm. Park. Dis. Dement. Sect. 2**,** 129-137.

Di Giovanni, G., Di Matteo, V., La Grutta, V., Esposito, E., 2001. m-Chlorophenylpiperazine excites non-dopaminergic neurons in the rat substantia nigra and ventral tegmental area by activating serotonin-2C receptors. Neuroscience 103**,** 111-116.

Di Matteo, V., De Blasi, A., Di Giulio, C., Esposito, E., 2001. Role of 5-HT_{2C} receptors in the control of central dopamine function. Trends Pharmacol. Sci. 22**,** 229-232.

Eberle-Wang, K., Mikeladze, Z., Uryu, K., Chesselet, M.F., 1997. Pattern of expression of the serotonin2C receptor messenger RNA in the basal ganglia of adult rats. J. Comp. Neurol. 384**,** 233-247.

Feldblum, S., Erlander, M.G., Tobin, A.J., 1993. Different distributions of GAD_{65} and GAD_{67} mRNAs suggest that the two glutamate decarboxylases play distinctive functional roles. J. Neurosci. Res. 34**,** 689-706.

Ford, B., Holmes, C.J., Mainville, L., Jones, B.E., 1995. GABAergic neurons in the rat pontomesencephalic tegmentum: codistribution with cholinergic and other tegmental neurons projecting to the posterior lateral hypothalamus. J. Comp Neurol. 363**,** 177-196.

Fujita, M., Shimada, S., Maeno, H., Nishimura, T., Tohyama, M., 1993. Cellular localization of serotonin transporter mRNA in the rat brain. Neurosci. Lett. 162**,** 59-62.

Gallager, D.W., Aghajanian, G.K., 1976. Effect of antipsychotic drugs on the firing of dorsal raphe cells. I. Role of adrenergic system. Eur. J. Pharmacol. 39**,** 341-355.

Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M., Gasnier, B., Giros, B., el Mestikawy, S., 2002. A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. J. Neurosci. 22**,** 5442-5451.

Gross-Isseroff, R., Salama, D., Israeli, M., Biegon, A., 1990a. Autoradiographic analysis of age-dependent changes in serotonin $5\text{-}HT_2$ receptors of the human brain postmortem. Brain Res. 519**,** 223-227.

Gross-Isseroff, R., Salama, D., Israeli, M., Biegon, A., 1990b. Autoradiographic analysis of [^{3}H]ketanserin binding in the human brain postmortem: effect of suicide. Brain Res. 507**,** 208-215.

Hoffman, B.J., Mezey, E., 1989. Distribution of serotonin $5\text{-}HT_{1C}$ receptor mRNA in adult rat brain. FEBS Lett. 247**,** 453-462.

Hoyer, D., Clarke, D.E., Fozard, J.R., Hartig, P.R., Martin, G.R., Mylecharane, E.J., Saxena, P.R., Humphrey, P.P., 1994. VII. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). Pharmacol. Rev. 46**,** 157-203.

Julien, J.F., Legay, F., Dumas, S., Tappaz, M., Mallet, J., 1987. Molecular cloning, expression and in situ hybridization of rat brain glutamic acid decarboxylase messenger RNA. Neurosci. Lett. 73**,** 173-180.

Julius, D., MacDermott, A.B., Axel, R., Jessel, T.M., 1988. Molecular characterisation of a functional cDNA encoding the serotonin1C receptor. Science 214**,** 558-564.

Kirifides, M.L., Simpson, K.L., Lin, R.C., Waterhouse, B.D., 2001. Topographic organization and neurochemical identity of dorsal raphe neurons that project to the trigeminal somatosensory pathway in the rat. J. Comp. Neurol. 435**,** 325-340.

Landry, M., Holmberg, K., Zhang, X., Hökfelt, T., 2000. Effect of axotomy on expression of NPY, galanin, and NPY Y1 and Y2 receptors in dorsal root ganglia and the superior cervical ganglion studied with double-labeling in situ hybridization and immunohistochemistry. Exp. Neurol. 162**,** 361-384.

Leibowitz, S.F., Alexander, J.T., 1998. Hypothalamic serotonin in control of eating behavior, meal size, and body weight. Biol. Psychiatry 44**,** 851-864.

Liu, R., Jolas, T., Aghajanian, G., 2000. Serotonin 5-HT_2 receptors activate local GABA inhibitory inputs to serotonergic neurons of the dorsal raphe nucleus. Brain Res. 873**,** 34-45.

McLaughlin, D.P., Little, K.Y., López, J.F., Watson, S.J., 1996. Expression of serotonin transporter mRNA in human brainstem raphe nuclei. Neuropsychopharmacol. 15**,** 523-529.

Mengod, G., Nguyen, H., Le, H., Waeber, C., Lubbert, H., Palacios, J.M., 1990. The distribution and cellular localization of the serotonin 1C receptor mRNA in the rodent brain examined by in situ hybridization histochemistry. Comparison with receptor binding distribution. Neuroscience 35**,** 577-591.

Mengod, G., Vilaró, M.T., Raurich, A., López-Giménez, J.F., Cortés, R., Palacios, J.M., 1996. 5-HT receptors in mammalian brain: receptor autoradiography and in situ hybridization studies of new ligands and newly identified receptors. Histochem. J. 28**,** 747-758.

Molineaux, S.M., Jessell, T.M., Axel, R., Julius, D., 1989. 5-HT1c receptor is a prominent serotonin receptor subtype in the central nervous system. Proc. Natl. Acad. Sci. USA 86**,** 6793-6797.

Mugnaini E. and Oertel W., 1985. An atlas of the distribution of GABAergic neurons and terminals in the rat CNS as revealed by GAD immunohistochemistry. In: Björklund A., Hökfelt T. (Eds.), Handbook of Chemical Neuroanatomy. Vol. 4, Part 1: GABA and neuropeptides in the CNS. Elsevier, Amsterdam, pp. 436-595.

Nanopoulos, D., Belin, M.F., Maitre, M., Vincendon, G., Pujol, J.F., 1982. Immunocytochemical evidence for the existence of GABAergic neurons in the nucleus raphe dorsalis. Possible existence of neurons containing serotonin and GABA. Brain Res. 232**,** 375-389.

Ochi, J., Shimizu, K., 1978. Occurrence of dopamine-containing neurons in the midbrain raphe nuclei of the rat. Neurosci. Lett. 8**,** 317-320.

Palacios, J.M., Waeber, C., Hoyer, D., Mengod, G., 1990. Distribution of serotonin receptors. Ann. NY Acad. Sci. 600**,** 36-52.

Paxinos G. and Watson C. (1998) The Rat Brain In Stereotaxic Coordinates, Academic Press, San Diego.

Pazos, A., Hoyer, D., Palacios, J.M., 1984. The binding of serotonergic ligands to the porcine choroid plexus: characterization of a new type of serotonin recognition site. Eur. J. Pharmacol. 106**,** 539-546.

Pazos, A., Palacios, J.M., 1985. Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Res. 346**,** 205-230.

Petrusz P., Merchantelier I., and Maderdrut J. L., 1985. Distribution of enkephalin-containing neurons in the central nervous system. In: Björklund A., Hökfelt T. (Eds.), Handbook of Chemical Neuroanatomy. Vol. 4, Part 1 :GABA and neurpeptidesin the CNS. Elsevier, Amsterdam, pp. 273-334.

Pompeiano, M., Palacios, J.M., Mengod, G., 1994. Distribution of the serotonin 5-HT_2 receptor family mRNAs: comparison between 5-HT_2 and 5-HT_{2C} receptors. Mol. Brain Res. 23**,** 163-178.

Radja, F., Laporte, A.M., Daval, G., Vergé, D., Gozlan, H., Hamon, M., 1991. Autoradiography of serotonin receptor subtypes in the central nervous system. Neurochem. Int. 18**,** 1-15.

Rattray, M., Michael, G.J., Lee, J., Wotherspoon, G., Bendotti, C., Priestley, J.V., 1999. Intraregional variation in expression of serotonin transporter messenger RNA by 5-hydroxytryptamine neurons. Neurosci. 88**,** 169-183.

Rattray, M., Wotherspoon, G., Savery, D., Baldessari, S., Marden, C., Priestley, J.V., Bendotti, C., 1994. Chronic D-fenfluramine decreases serotonin transporter messenger RNA expression in dorsal raphe nucleus. Eur. J. Pharmacol. 268**,** 439-442.

Schmitz, G.G., Walter, T., Seibl, R., Kessler, C., 1991. Nonradioactive labeling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. Anal. Biochem. 192**,** 222-231.

Serrats, J., Artigas, F., Mengod, G., Cortés, R., 2003a. $GABA_B$ receptor mRNA in the raphe nuclei: co-expression with serotonin transporter and glutamic acid decarboxylase. J. Neurochem. 84**,** 743-752.

Serrats, J., Mengod, G., Cortés, R., 2003b. Cellular localization of 5-HT_{1A}, 5-HT_{1B} and α_{1B} receptor mRNAs in the raphe nuclei. A double in situ hybridization study throughout the raphe nuclei. Eur. Neuropsychopharmacol. 13 (Suppl. 4)**,** S104.

Sharma, A., Punhani, T., Fone, K.C., 1997. Distribution of the 5-hydroxytryptamine$_{2C}$ receptor protein in adult rat brain and spinal cord determined using a receptor-directed antibody: Effect of 5,7-dihydroxytryptamine. Synapse 27**,** 45-56.

Steinbusch, H.W.M., 1981. Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. Neurosci. 6**,** 557-618.

Stratford, T.R., Wirtshafter, D., 1990. Ascending dopaminergic projections from the dorsal raphe nucleus in the rat. Brain Res. 511**,** 173-176.

Sur, C., Betz, H., Schloss, P., 1996. Immunocytochemical detection of the serotonin transporter in rat brain. Neurosci. 73**,** 217-231.

Swanson L. W. (1998) Brain Maps: Structure of the Rat Brain, Elsevier, Amsterdam.

Tomiyama, M., Palacios, J.M., Cortés, R., Vilaró, M.T., Mengod, G., 1997. Distribution of AMPA receptor subunit mRNAs in the human basal ganglia: an in situ hybridization study. Mol. Brain Res. 46**,** 281-289.

Wang, Q.P., Ochiai, H., Nakai, Y., 1992. GABAergic innervation of serotonergic neurons in the dorsal raphe nucleus of the rat studied by electron microscopy double immunostaining. Brain Res. Bull. 29**,** 943-948.

Wang, Q.-P., Nakai, Y., 1993. Enkephalinergic innervation of GABAergic neurons in the dorsal raphe nucleus of the rat. Brain Res. Bull. 32**,** 315-320.

Wright, D.E., Seroogy, K.B., Lundgren, K.H., Davis, B.M., Jennes, L., 1995. Comparative localization of serotonin1A, 1C, and 2 receptor subtype mRNAs in rat brain. J. Comp Neurol. 351**,** 357-373.

Figures

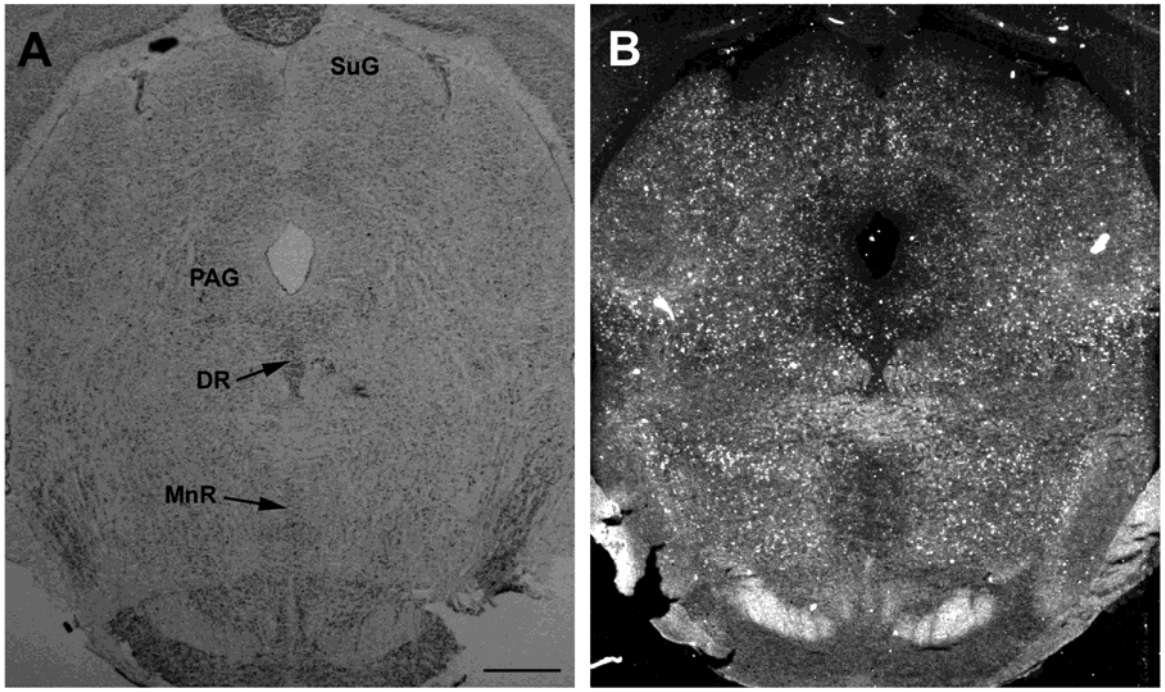

Fig. 1. Macroscopic visualization of 5-HT_{2C} receptor mRNA in the rat raphe nuclei. **A** is a consecutive section to **B** stained with cresyl violet for anatomical reference. **B** is a dark-field photomicrograph from an emulsion dipped-section from the upper brainstem, where numerous cell profiles expressing 5-HT_{2C} receptor mRNA are visualized, in areas such as periaqueductal gray (PAG), superior colliculus (SuG), and dorsal (DR) and median (MnR) raphe nuclei. Note that in the DR 5-HT_{2C} receptor mRNA signal is restricted to lateral aspects of the nucleus, whereas only few positive cells are found in its medial parts. Bar = 1 mm.

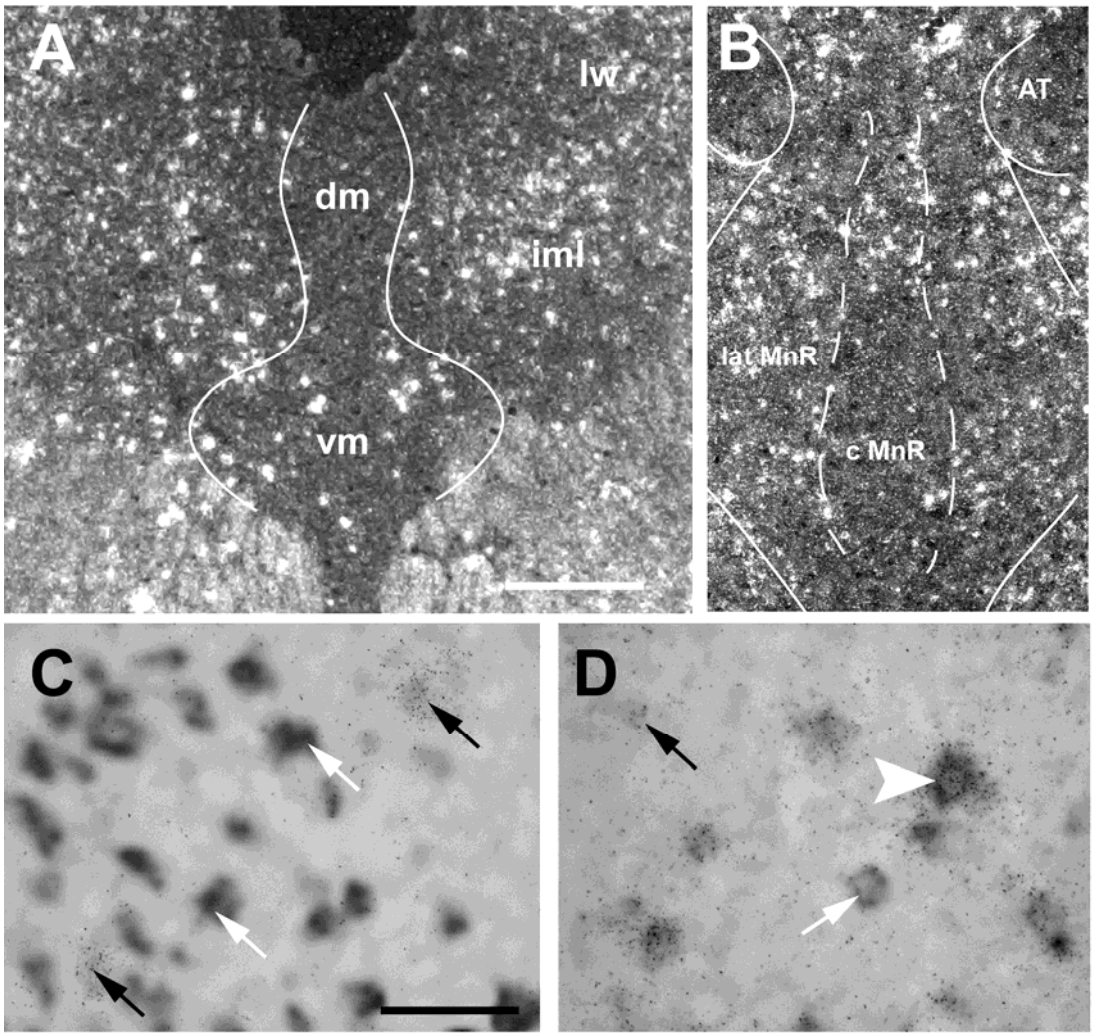

Fig. 2. Cellular visualization of 5-HT_{2C} receptor mRNA in the rat raphe nuclei and co-localization with phenotype markers. **A** and **B** are dark-field photomicrographs showing the 5-HT_{2C} receptor mRNA signal in the DR (**A**) and MnR (**B**) where the approximate limits of the different subnuclear divisions are depicted. Note the absence of labeling in the dorsomedial DR (dm) and ventral portion of the central part of the MnR (c MnR). In contrast, abundant 5-HT_{2C} receptor mRNA signal can be observed in the intermediolateral (iml) aspects and lateral wings (lw) of the DR, lateral part of the MnR (lat MnR) and dorsal pole of the central MnR, whereas only relatively sparse labeled cells are found in the ventromedial DR (vm). **C** and **D** are high-magnification photomicrographs from the DR showing the simultaneous detection of two species of mRNAs using a ^{33}P-labeled oligonucleotide probe for 5-HT_{2C} receptor mRNA (silver grains) and Dig-labeled probes for 5-HTT mRNA (dark precipitate in **C**) or GAD mRNA (dark precipitate in **D**). White arrowheads point to double labeled cells. White arrows point to single Dig-labeled cells. Black arrows point to single 5-HT_{2C} receptor mRNA-labeled cells. Bar in A = B = 500 µm. Bar in C = D = 50 µm.

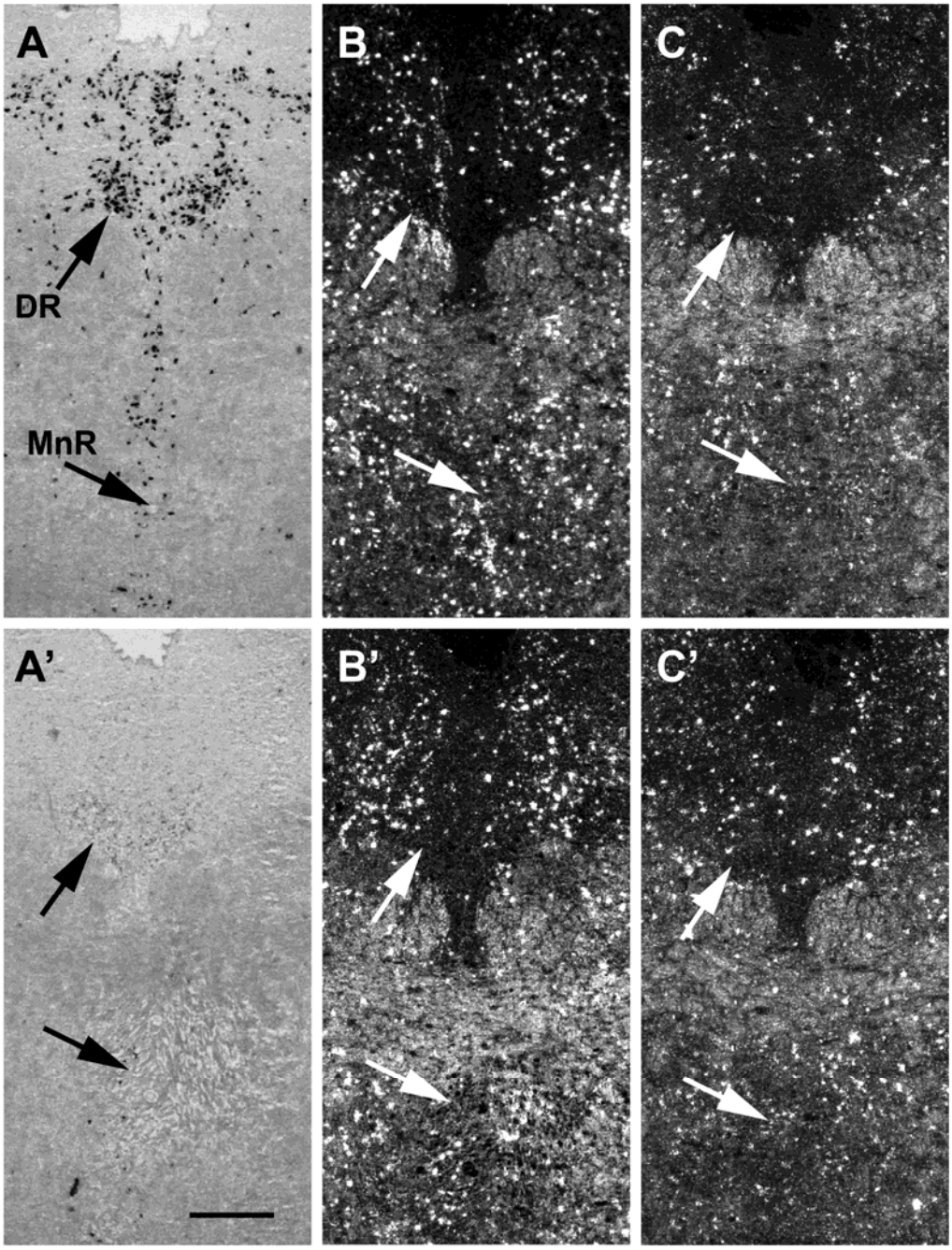

Fig. 3. Effect of 5,7-dihydroxytryptamine lesions on the expression of 5-HTT, GAD and 5-HT_{2C} receptor mRNAs in the raphe nuclei. 5-HTT (**A**), GAD (**B**) and 5-HT_{2C} receptor (**C**) mRNAs were expressed in sham (vehicle treated) rats. In contrast, 5-HTT mRNA expression is completely abolished in DR and only few labeled cells remain in the MnR of 5,7-DHT treated rats (**A'**), while GAD (**B'**) and 5-HT_{2C} receptor (**C'**) mRNAs hybridization signal is still present in the DR, MnR and surrounding structures. Bar = 500 µm.