**Serotonin 5-HT₄ receptors and forebrain cholinergic system: receptor expression
in identified cell populations.**

Raúl Peñas-Cazorla¹ and M.Teresa Vilaró^{1,2}

**1: Department of Neurochemistry and Neuropharmacology, Institut
d'Investigacions Biomèdiques de Barcelona, (CSIC-IDIBAPS), 08036
Barcelona, Spain**

**2: Centro de Investigación Biomédica en Red de Enfermedades
Neurodegenerativas (CIBERNED), Spain**

Corresponding author: M. Teresa Vilaró, Institut d' Investigacions Biomèdiques de
Barcelona (IIBB-CSIC, IDIBAPS), Rosselló, 161 6th floor, 08036 Barcelona, Spain.

Phone: 34-93 363 83 20; FAX: 34-93 363 83 01; e-mail: teresa.vilaro@iibb.csic.es

ABSTRACT

Activation of serotonin 5-HT₄ receptors has pro-cognitive effects on memory performance. The proposed underlying neurochemical mechanism is the enhancement of acetylcholine release in frontal cortex and hippocampus elicited by 5-HT₄ agonists. Although 5-HT₄ receptors are present in brain areas related to cognition, e.g. hippocampus and cortex, the cellular localization of the receptors that might modulate acetylcholine release is unknown at present. We have analysed, using dual-label in situ hybridization, the cellular localization of 5-HT₄ receptor mRNA in identified neuronal populations of the rat basal forebrain, which is the source of the cholinergic innervation to cortex and hippocampus. 5-HT₄ receptor mRNA was visualized with isotopically-labeled oligonucleotide probes, whereas cholinergic, glutamatergic, GABAergic and parvalbumin-synthesizing neurons were identified with digoxigenin-labeled oligonucleotide probes. 5-HT₄ receptor mRNA was not detected in the basal forebrain cholinergic cell population. In contrast, basal forebrain GABAergic, parvalbumin-synthesizing, and glutamatergic cells contained 5-HT₄ receptor mRNA. Hippocampal and cortical glutamatergic neurons also express this receptor. These results indicate that 5-HT₄ receptors are not synthesized by cholinergic cells and thus would be absent from cholinergic terminals. In contrast, several non-cholinergic cell populations within the basal forebrain and its target hippocampal and cortical areas express these receptors and are thus likely to mediate the enhancement of acetylcholine release elicited by 5-HT₄ agonists.

Keywords: acetylcholine release, septal nuclei, nucleus basalis of Meynert, basal forebrain GABAergic neurons, basal forebrain glutamatergic neurons, hippocampus

1. Introduction

The existence of the serotonin 5-HT₄ receptor family was first proposed in 1988, when a serotonin receptor was observed in cultures of mouse colliculi which was able to stimulate adenylate cyclase and was insensitive to antagonists of the three serotonin receptor families described until then, i.e. 5-HT₁, 5-HT₂, and 5-HT₃ (Dumuis et al., 1988). This and several other lines of evidence from different laboratories (see Ford and Clarke, 1993 for a review) resulted in the unequivocal establishment of the presence of 5-HT₄ receptors in many other CNS regions and peripheral organs (reviewed in Eglen et al., 1995; Hegde and Eglen, 1996; Eglen and Hegde, 1996).

Serotonin 5-HT₄ receptors were first cloned in rat, where two splice variants were described, differing after Leu358 in the length and sequence of the carboxy terminus, and termed 5-HT_{4S} and 5-HT_{4L} for short and long, respectively (Gerald et al., 1995). Subsequent cloning efforts in other species (Blondel et al., 1998; Claeysen et al., 1998; Claeysen et al., 1999) showed the existence of additional C-terminal splice variants, and this prompted the adoption of the alphabetical nomenclature, by which 5-HT_{4S} and 5-HT_{4L} became 5-HT_{4(a)} and 5-HT_{4(b)}, respectively. At present, and including all the isoforms cloned in three mammalian species (human, rat and mouse), nine different C-terminal splice variants and one internal splice variant have been cloned (Bender et al., 2000; Vilaró et al., 2002; Bockaert et al., 2004; Brattelid et al., 2004; Ray et al., 2009).

Notable among the diverse behavioural effects elicited by pharmacological interventions on 5-HT₄ receptors is the pro-cognitive effect of 5-HT₄ agonists, which seem to be able to modulate several aspects of memory performance (acquisition, consolidation, reversal of induced deficits) (see Bockaert et al., 2004; Perez-Garcia and Meneses, 2008 and references therein). A putative neuroanatomical substrate for this

modulation is provided by the presence of 5-HT₄ receptors in several brain regions involved in learning and memory, i.e. hippocampus, cortex, basal forebrain (Waeber et al., 1994; Patel et al., 1995; Reynolds et al., 1995; Compan et al., 1996; Bonaventure et al., 2000; Varnas et al., 2003; Vilaró et al., 2005; Marner et al., 2009). On the other hand, the underlying neurochemical mechanism for these pro-cognitive effects has been proposed to be the enhancement of acetylcholine release in cortical and hippocampal regions by 5-HT₄ receptor stimulation (Consolo et al., 1994; Yamaguchi et al., 1997a; Yamaguchi et al., 1997b; Siniscalchi et al., 1999; Matsumoto et al., 2001). This has prompted the suggestion that 5-HT₄ receptor agonists, alone or in combination with acetylcholinesterase inhibitors, might be valuable therapeutic tools for the treatment of cognitive deficits associated with impairment of the cholinergic system due to normal aging or pathological situations such as Alzheimer's disease (Moser et al., 2002; Lamirault et al., 2003; Bockaert et al., 2004). However, the precise cellular localization of the 5-HT₄ receptors that may mediate the effects on the cholinergic system is unknown. Therefore, in the present study we have analysed the sites of synthesis of 5-HT₄ receptors that may be related with cholinergic function and cognitive performance by studying the cellular localization of their mRNA in different identified neuronal populations in the basal forebrain (BF) and its hippocampal target areas.

2. Experimental procedures

2.1. Tissue preparation

Adult male Wistar rats weighting 200-300g (n=6) 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 the European regulations (European Communities Council directive 86/609/EEC of November 24

1986 [O.J. of E.C. L358, 18/12/1986]). Experimental procedures were approved by the required ethical committees and local authorities.

Animals were killed by decapitation. The brains were quickly removed, frozen on dry ice and kept at -20°C. Tissue sections, 20 µm thick, were cut on a microtome-cryostat (Microm HM500 OM, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane; Sigma, St Louis, MO) coated slides, and kept at -20°C until used for in situ hybridization or for acetylcholinesterase staining. The latter was done as previously described (Vilaró et al., 1992). The anatomical levels included in the hybridization studies spanned all the rostrocaudal extent of the basal forebrain where cholinergic cells are located.

2.2. Hybridization probes

For the detection of 5-HT₄ receptor mRNA, 6 different oligodeoxyribonucleotides (50 nucleotides long) were used simultaneously. They were complementary to the following bases of the rat 5-HT₄ receptor mRNA (Gerald et al., 1995) (base numbering corresponds to the sequence of the 5-HT_{4(a)} splice variant, GenBank accession number U20906): r5HT4N, bases 21-70; r5HT4SL, bases 960-1009; r5-HT4SL/2, bases 683-732; r5-HT4SL/3, bases 741-790; r5HT4SL/4, bases 1029-1078; r5HT4SL/5, bases 258-307. All these regions of the mRNA are common to all four splice variants (r5-HT_{4(a)}, r5-HT_{4(b)}, r5-HT_{4(c1)}, and r5-HT_{4(e)}) cloned to date in the rat (Gerald et al., 1995; Claeysen et al., 1999; Ray et al., 2009)

Cholinergic cells were visualized by the presence of the mRNA for the enzyme choline acetyltransferase (ChAT). Two oligonucleotides complementary to bases 571-618 and 1321-1368 of the rat ChAT cDNA sequence (Ishii et al., 1990) were used. GABAergic cells were identified by the presence of the mRNA for the GABA-synthesizing enzyme GAD65 (glutamic acid decarboxylase, isoform of molecular

weight 65 kDa). Two oligonucleotides complementary to rat GAD65 mRNA were made, corresponding to bases 159-213 and 514-558 (GenBank accession number NM_012563). Neurons containing parvalbumin mRNA were identified with two oligonucleotides complementary to bases 175-215 and 264-308 (GenBank accession number M12725) of rat parvalbumin mRNA. Finally, cells containing mRNA for the vesicular glutamate transporter vGLUT2 (putatively glutamatergic) were also identified with six oligonucleotides complementary to bases 311-355, 466-510, 530-574, 2072-2116, 2156-2200, and 2356-2400 (GenBank accession number AF271235).

The oligonucleotides were all synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands). Comparison of the oligonucleotide sequences with EMBL and GenBank databases with Basic Local Alignment Search Tool (BLAST) indicated that the probes do not show any significant similarity with mRNAs other than their corresponding targets in the rat.

Oligonucleotides for 5-HT₄ receptor mRNA were labeled at their 3'-end using [³³P] α -dATP (3000 Ci/mmol, New England Nuclear, Boston, MA, USA) and terminal deoxynucleotidyltransferase (TdT) (Oncogene Research Products, San Diego, CA, USA). Oligonucleotides for ChAT, GAD65, vGLUT2 and parvalbumin (100 pmol) were non-radioactively labeled at their 3'-end with TdT (recombinant, Roche Applied Science, Penzberg, Germany) and Digoxigenin-labeled nucleotides (Dig-11-dUTP, Roche Applied Science, Mannheim, Germany) according to a previously described procedure (Schmitz et al., 1991). Labeled probes were purified from non-incorporated nucleotides with ProbeQuant G-50 micro columns (GE Healthcare, Little Chalfont, UK).

2.3. *In situ* hybridization histochemistry procedure

The protocols for single- and double-label *in situ* hybridization histochemistry were based on previously described procedures (Vilaró et al., 1996; Landry et al.,

2000; Serrats et al., 2003). Frozen tissue sections were brought to room temperature, fixed for 20 min at 4°C in 4% paraformaldehyde in phosphate-buffered saline (1xPBS: 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3xPBS, twice for 5 min each in 1xPBS at room temperature, and incubated for 2 min at 21°C in a freshly prepared solution of predigested 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. Proteolytic activity was stopped by immersion for 30 sec in 2 mg/ml glycine in 1xPBS. Tissues were finally rinsed in 1xPBS and dehydrated through 70% and 100% ethanol. For hybridization, radioactively- and non-radioactively labeled probes were diluted 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 (approximately 1.5 - 2 nM each probe). Tissue sections were covered with 60-70 µl of hybridization solution containing the labeled probes, overlaid with Nescofilm coverslips (Bando Chemical Ind, Kobe, Japan) and incubated overnight at 42°C in humid boxes. Sections were washed four times (45 min each) in 0.6M NaCl, 10mM Tris-HCl pH 7.5 at 60°C. Sections hybridized with ³³P- and Dig-labeled probes simultaneously were washed in the same buffer at room temperature for 10 min, whereas sections hybridized with radioactive probes only were dehydrated through 70% and 100% ethanol and allowed to air dry.

2.4. Development of radioactive and non-radioactive hybridization signal

Sections hybridized with ³³P- and Dig-labeled probes 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 MgCl₂ and 0.5% bovine

serum albumin (Fraction V, Sigma-Aldrich) and incubated overnight at 4°C in the same solution containing an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Fab fragments, 1:5000 dilution; Roche Applied Science, Penzberg, Germany). Afterwards, they were washed three times (10 min each) in the same buffer, and twice (10 min each) in an alkaline buffer containing 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 5 mM MgCl₂. Alkaline phosphatase activity was developed by incubating the sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Applied Science, Penzberg, Germany) diluted in 10 ml of alkaline buffer overnight at room temperature in the dark. The enzymatic reaction was stopped by washing three times, 10 min each, in the alkaline buffer containing 1 mM EDTA. The sections were then briefly dipped in water, 70% and 100% ethanol, and air-dried. Sections incubated with Dig-labeled probes were dipped in a solution of 2% collodion in amyl acetate (Electron Microscopy Sciences, Hatfield, PA, USA) and allowed to dry. All sections were dipped into Ilford K5 nuclear emulsion (Harman Technology Ltd, Mobberley, Cheshire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4°C for 6 weeks, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min, and fixed in Ilford Hypam fixer (Harman Technology Ltd, Mobberley, Cheshire, UK).

2.5. Controls for specificity of hybridization signals.

Several routine control experiments are performed with all the oligonucleotide probes used for in situ hybridization in our laboratory. In brief, the following controls were performed (not shown): a) When two or more probes aimed at the same mRNA species were used independently as hybridization probes in consecutive sections, identical patterns of hybridization signal were observed. b) For any given labeled probe, inclusion during hybridization of an excess of the same unlabeled oligonucleotide resulted in absence of specific hybridization signals. The

autoradiographic signal remaining under these conditions was taken as nonspecific tissue signal. c) Inclusion during hybridization of an excess of an unrelated unlabeled oligonucleotide left specific hybridization signals unaffected. d) The thermal stability of the hybrids was studied by washing a series of consecutive sections at increasing temperatures. The intensity of specific signals suffered a very sharp decrease as washing temperature increased whereas no such sharp decrease was observed in background levels of signal.

2.6. Analysis of the results.

The anatomical levels included in the hybridization studies spanned all the rostrocaudal extent of the basal forebrain where cholinergic cells are located, from the very anterior pole of the nucleus of the vertical limb of the diagonal band (approximate Bregma level +1.6 mm) to the more caudal extent of the basal nucleus of Meynert (approximate Bregma -2.8 mm) (Paxinos and Watson, 1998).

Tissue sections were examined under bright- and dark-field illumination in a Wild 420 macroscope (Leica, Heerbrugg, Germany), and in a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) or in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with bright- and dark-field condensers for transmitted light. A Darklite illuminator (Micro Video Instruments, Avon, MA, USA) was used in some instances to improve the visualization of autoradiographic silver grains and to capture bright- and dark field images simultaneously.

Cholinergic, GABAergic, glutamatergic, and parvalbumin-containing neurons were identified as cellular profiles exhibiting a dark precipitate (alkaline phosphatase reaction product). 5-HT₄ receptor mRNA hybridization signal was considered positive when visually identified accumulation of silver grains over the stained cellular profiles, or unstained areas of similar surface, was estimated by microscopic visual inspection

to be approximately 3-fold higher than the background levels of signal in regions known to be devoid of 5-HT₄ receptor mRNA. In all the experiments, control sections were included that were incubated with the labeled probes, both isotopically and digoxigenin-labeled, plus a large excess of the same unlabeled oligonucleotides. These sections served as control for specificity of positive signals.

For qualitative analysis, and for any given combination of probes, microscopic inspection was performed on multiple sections spanning all the rostrocaudal extent of the basal forebrain where cholinergic cells are located. For quantitative analysis, two anatomical coronal levels were chosen therein for cell counting, one at an approximate distance from Bregma of +0.20mm (according to Paxinos and Watson, 1998) which includes the medial septum (MS), the vertical limb of the diagonal band (VDB) and the rostral part of the horizontal limb of the diagonal band (HDB) (Fig 1A) and a second one at an approximate distance from Bregma of -0.30mm, where the caudal portion of the HDB coexists with the more laterally located magnocellular preoptic nucleus (MCPO) (Fig 1B). Cell counting was performed in these regions because they contain a complex mixture of different neuronal phenotypes and it was of interest to have a quantitative estimate of the magnitude of 5-HT₄ receptor expression in the different cell populations. Numbers of counted cells (Table 1) in the MS and VDB are presented as aggregate numbers, due to the diffuse boundaries between these two areas. Counted cells in the HDB include cells counted at both rostro-caudal levels of this structure. Cell counting was performed as follows. Dark- and bright-field digital photomicrographs were taken with the 10x objective of a Zeiss Axioplan microscope equipped with a digital camera (DXM1200 F, Nikon) and ACT-1 Nikon Software. For any given tissue section, photographs were taken from as many contiguous microscopic fields as necessary to encompass all the extent of the region of interest. The borders of the different subregions to be analyzed (MS, VDB, HDB) were defined as follows. For a given anatomical level, a photomontage was obtained with all the contiguous bright field

photomicrographs of the ChAT-hybridized (or sometimes the GAD65-hybridized) tissue section. It was printed at low magnification and the borders were drawn on these prints with the help of the anatomical atlas (Paxinos and Watson, 1998) and considering the known distribution of cholinergic and/or GABAergic cells in the basal forebrain. The boundaries drawn were considered later for cell counting in the individual photomicrographs viewed at higher magnification. Labeled cells were counted with the help of the program AnalySIS (Soft Imaging Systems GmbH, Germany), which allowed the superimposition of the bright and dark field images of the same microscopic field. Visual inspection of the superimposed image on the computer screen allowed manual cell counting. For each neurochemical marker and anatomical level, 2-3 animals (1-2 sections/animal) were counted. Three types of cells were considered: a) doubly-labeled cells showing dark precipitate, indicative of positive signal for the mRNA of the different neurochemical markers, plus accumulation of silver grains, indicating positive signal for 5-HT₄ receptor mRNA; b) cells showing signal for 5-HT₄ receptor mRNA only, i.e. accumulation of silver grains over precipitate-free tissue areas similar in size to nearby stained cellular profiles; c) cells showing signal for the neurochemical marker only.

2.7. Preparation of figures

Microphotography was performed as described above. Figures were prepared for publication using Adobe Photoshop software (Adobe Software, San Jose, CA, USA). Contrast and brightness of the images were adjusted digitally if necessary. Some of the illustrations of labeled cells at high magnification were prepared with the help of some features of the Photoshop software as follows. Dark and bright field photomicrographs (20x objective) were taken from the area to be illustrated. The dark field image was inverted with the Photoshop software, so that silver grains were seen dark on a white background. The inverted image was superimposed on the bright field image showing the digoxigenin-labeled cells and both images were blended with the

Multiply option selected. The area to be illustrated in more detail was then selected from this blended image and scaled up as convenient.

3. Results

The regional distribution of 5-HT₄ receptor mRNA observed in the present study was in agreement with our previous studies (Vilaró et al., 1996; Vilaró et al., 2005). Thus the highest levels of hybridization signal were observed in the olfactory tubercle, hippocampus and caudate-putamen. In comparison with these regions, the intensity of the hybridization signal observed in the basal forebrain cholinergic projection system, i.e. in the medial septum/diagonal band (MS/DB) complex (Fig.1) and nucleus basalis of Meynert (NbM), was of much lower intensity. Given this lower signal intensity in these regions of interest, some considerations on the sensitivity of our method are warranted here. Since we were aware from our previous studies that the levels of 5-HT₄ receptor mRNA expression were low in the basal forebrain (Vilaró et al., 2005), we chose to increase the sensitivity of our method by increasing the number of different oligonucleotide probes complementary to 5-HT₄ receptor mRNA. Thus, six different probes directed to different regions of the mRNA were used simultaneously in the present study as compared to our previous studies where one (Vilaró et al., 1996) or two (Vilaró et al., 2005) probes had been used. With this approach, sensitivity was improved, as evidenced by the higher number of cells positive for 5-HT₄ mRNA as compared to our previous study. This was, however, at expenses of generating increased levels of tissue background due to the use of higher concentrations of radioactively labeled probe during tissue hybridization. Nevertheless, the signal to noise ratio was still good enough and labeled cells were easily discernible from background levels of signal.

3.1. 5-HT₄ receptor mRNA-containing cells and cholinergic cells in the MS/DB complex and NbM.

In the MS/DB complex 5-HT₄ receptor mRNA-containing cells displayed a more widespread and homogeneous distribution when compared to ChAT mRNA positive cells. In the MS and in the vertical limb of the diagonal band (VDB), cholinergic cells were distributed in a band-like fashion, i.e. concentrated in the midline region and in a band located laterally to these midline neurons, with a region in-between where cholinergic cells were more scarce. In contrast, 5-HT₄ receptor mRNA positive cells were distributed more homogeneously and were present in regions more lateral than the laterally located cholinergic cells. A similar situation was observed in the horizontal limb of the diagonal band (HDB) at rostral levels, where ChAT mRNA positive cells were often seen in a ring-shaped distribution, whereas 5-HT₄ receptor mRNA positive cells were more homogeneously distributed, also in the inner regions of the ring formed by cholinergic cells. At more caudal levels, where the HDB coexists with the contiguous, more laterally located magnocellular preoptic nucleus (MCPO), 5-HT₄ receptor mRNA positive cells were observed scattered in both nuclei, whereas cholinergic cells were particularly concentrated in the HDB. Cell counting in tissue sections doubly-labeled for 5-HT₄ receptor mRNA and ChAT mRNA (Table 1) indicated that 5-HT₄ receptor mRNA positive cells (a total of 794 cells of this type were counted) were much more numerous than ChAT mRNA positive cells (a total of 348 cells of this type were counted) in all the regions analyzed (MS/VDB and HDB). However, we did not observe any population of cells labeled simultaneously for both mRNAs in these areas. In other words, 5-HT₄ receptor mRNA was not detected in ChAT mRNA positive, i.e. cholinergic, cells (Fig. 2).

All along the rostrocaudal extent of the nucleus basalis of Meynert (NbM), 5-HT₄ receptor mRNA positive cells were observed intermingled with, and often in close proximity to, ChAT mRNA positive cells, but no doubly-labeled cells were found (Fig. 3

A-D). In contrast, in sections consecutive or very close to the ones hybridized with probes for ChAT mRNA, double labeling indicated that these 5-HT₄ receptor mRNA positive cells were GABAergic as shown by their expression of GAD65 mRNA (Fig. 3 E-H). No attempt of cell counting was performed in the NbM due to the diffuse limits of this nucleus.

3.2. 5-HT₄ receptor mRNA-containing cells and GABAergic cells in the MS/DB complex and NbM.

In the MS/DB complex, GABAergic cells were identified by their expression of mRNA for the GABA-synthesizing enzyme GAD65 (Fig. 4). They were more numerous and displayed a more homogeneous distribution when compared with cholinergic cells. Cell counting in tissue sections doubly-labeled for 5-HT₄ receptor mRNA and GAD65 mRNA indicated that approximately one half of the cells expressing 5-HT₄ receptor mRNA in the MS/VDB also expressed mRNA for GAD65, i.e. were GABAergic. Thus, of a total of 743 5-HT₄ receptor mRNA positive cells that were counted in the MS/VDB, 352 of these cells were also labeled by GAD65 mRNA probes. In the HDB, both at rostral and more caudal levels where it coexists with the MCPO, approximately two thirds of the 5-HT₄ receptor mRNA positive cells were GABAergic (836 cells positive for 5-HT₄ receptor mRNA were counted, of which 578 were also labeled by GAD65 mRNA probes). In all the regions, around 50% of the GABAergic cells contained 5-HT₄ receptor mRNA (Table 1).

As stated above, 5-HT₄ receptor mRNA positive cells in the region of the NbM were shown to express GAD65 mRNA (Fig. 3 E-H).

3.3. 5-HT₄ receptor mRNA-containing cells and cells expressing parvalbumin mRNA in the MS/DB complex.

A subpopulation of the GABAergic cells present in the MS/DB complex expresses the calcium binding protein parvalbumin (PV) and are thought to represent septo-hippocampal and septo-cortical GABAergic projection neurons (Freund, 1989; Gritti et al., 2003). Probes for PV mRNA were thus used in the present study to identify this population of GABAergic projection neurons (Fig. 5). PV mRNA positive cells were much less numerous than GAD65 mRNA containing neurons. In sections doubly-labeled for 5-HT₄ receptor mRNA and PV mRNA, of the cells positive for 5-HT₄ receptor mRNA in MS/VDB and HDB (936 and 293 counted cells, respectively), around 10-15% were also positive for PV mRNA (74 and 43 cells, respectively). Of the cells positive for PV mRNA in the MS/VDB, around 20-30 % were also positive for 5-HT₄ receptor mRNA, whereas at more caudal levels of the HDB, the percentage was close to 50% (Table 1).

3.4. 5-HT₄ receptor mRNA-containing cells and glutamatergic cells in the MS/DB complex.

The MS/DB complex contains a population of glutamatergic cells that express the vesicular glutamate transporter vGLUT2 (Lin et al., 2003; Hur and Zaborszky, 2005). We have identified this population with probes for vGLUT2 mRNA (Fig. 6). Cell counting in sections doubly-labeled for vGLUT2 mRNA and 5-HT₄ receptor mRNA indicated that in the MS/VDB and HDB, around 20 % of the cells positive for 5-HT₄ receptor mRNA were also positive for vGLUT2 mRNA (a total of 889 cells positive for 5-HT₄ receptor mRNA were counted, of which 188 were also labeled by vGLUT2 mRNA probes). Of the cells positive for vGLUT2 mRNA, around 40% and 70% were also positive for 5-HT₄ receptor mRNA in the MS/VDB and HDB, respectively (Table 1).

3.5. 5-HT₄ receptor mRNA-containing cells in the hippocampus.

The hippocampus is, together with the olfactory tubercle and the caudate-putamen, the region of the rat brain presenting the strongest hybridization signal for 5-HT₄ receptor mRNA (Vilaró et al., 2005), with very strong signals detected over the pyramidal cell layer of the CA subfields and the granule cell layer of the dentate gyrus. Nissl staining of sections hybridized with radioactive probes for 5-HT₄ receptor mRNA confirmed that the vast majority of the cellular profiles observed in these cell layers were strongly labeled (Fig. 7), thus indicating that most of the pyramidal and granule cells express this receptor.

Hippocampal GABAergic neurons, that include interneurons and projection neurons (Freund and Buzsaki, 1996), were identified by their expression of GAD65 mRNA. In tissue sections doubly-labeled for 5-HT₄ receptor mRNA and GAD65 mRNA, 5-HT₄ receptor mRNA was never observed to be present in GABAergic hippocampal neurons of the CA subfields and dentate gyrus (Fig.8).

3.6. 5-HT₄ receptor mRNA-containing cells in the lateral septum.

The lateral septum, which is a component of the septo-hippocampal-septal loop was also analysed. 5-HT₄ receptor mRNA was strongly expressed in most of the GABAergic neurons of the dorsal part of the lateral septal nucleus (Fig. 9).

4. Discussion

The main finding of the present in situ hybridization study is that cholinergic neurons of the MS/DB complex and NbM do not express detectable levels of serotonin 5-HT₄ receptor mRNA. In contrast, 5-HT₄ receptor mRNA is readily detectable in GABAergic, glutamatergic and parvalbumin-containing neurons in the MS/DB complex and in GABAergic neurons within the region of the NbM.

4.1. Technical aspects

Some technical considerations regarding the sensitivity of the technique are warranted before proceeding to further anatomical and functional considerations. When reporting negative results from in situ hybridization experiments, an issue to be considered is the sensitivity of the probes used. A priori, oligonucleotide probes are less sensitive than, for example, riboprobes (i.e. RNA probes obtained by transcription of cloned fragments of the receptor cDNA). However, one way of overcoming this limitation and increasing the sensitivity of oligonucleotide-based in situ hybridization is by the simultaneous use of multiple oligonucleotide probes complementary to different regions of the same receptor mRNA. In our experience, we have observed this fact by simply increasing the number of oligonucleotide probes for a given mRNA from one to two. This was the case, for example, for ChAT mRNA (Vilaró et al., 1992; Vilaró et al., 1994) and for 5-HT₄ receptor mRNA in our two previous studies (compare for example figure 2A in (Vilaró et al., 1996) with figure 1F in (Vilaró et al., 2005)). In the present study we have further increased to six the number of probes used simultaneously for the detection of 5-HT₄ receptor mRNA, and this has resulted in an increased sensitivity when compared to our previous study (Vilaró et al., 2005), especially in the regions of relatively low receptor expression such as, for example, the MS/DB complex. To our knowledge, there is no study available reporting the distribution of 5-HT₄ receptor mRNA in rat brain detected with riboprobes, thus precluding a comparison of the

sensitivities of both approaches. However, data in the literature indicate that a multi-oligoprobe approach can be as sensitive as a riboprobe to detect mRNA expression in brain (Broide et al., 2004).

4.2. Anatomical considerations

Basal forebrain (BF) neurons, with their projections to hippocampal and cortical areas, are thought to play important roles in cognitive processes (Baxter and Chiba, 1999). The cholinergic component of the BF projection system was the first to be acknowledged as playing an important role (see Fibiger, 1991; Wenk, 1997; Gold, 2003 for reviews), but subsequently other neurochemically distinct components were described in the basal forebrain, such as the GABAergic (reviewed in Sarter and Bruno, 2002) and, more recently, the glutamatergic cell populations (Manns et al., 2001; Lin et al., 2003). These non-cholinergic components may also be important for cognitive processes as illustrated by the fact that selective cholinergic lesions of the BF in rats produce less severe cognitive deficits than do more generalized, non-selective lesions of the same areas (Dunnett et al., 1991; Lee et al., 1994; Parent and Baxter, 2004).

Much is known about the interconnections between the BF and its different cortical and hippocampal targets. A summary is provided in the following paragraphs and in Fig 10. A major projection component in the BF is accounted for by the cholinergic projection neurons, which form a continuous cell column extending from the MS/DB complex rostrally, through the magnocellular preoptic area/substantia innominata region and including the NbM more caudally (Schwaber et al., 1987). The projection patterns of these cholinergic neurons have been extensively studied. Thus, the MS/VDB complex (the cholinergic cell groups Ch1 and Ch2 according to (Mesulam et al., 1983) project mainly to hippocampal areas, the HDB (Ch3 group) projects mainly

to the olfactory bulb, and more caudally located cholinergic cells in the substantia innominata/NbM region (Ch4 group) provide projections to the different cortical areas (Mesulam et al., 1983; Woolf et al., 1984). A second major neuronal component of BF is composed of GABAergic neurons, which greatly outnumber cholinergic cells in rat BF (Zaborszky et al., 1999). A fraction of the BF GABAergic cells are projection neurons and express the calcium binding protein Parvalbumin (PV) and others are GABAergic interneurons (Freund, 1989; Brauer et al., 1991; Gritti et al., 1993). PV-containing GABAergic projection neurons innervate both hippocampal (Freund, 1989; Kiss et al., 1990) and cortical targets (Gritti et al., 2003). A population of putative glutamatergic cells has been described in rat BF on the basis of the presence of different glutamatergic markers (Manns et al., 2001; Colom et al., 2005) and in particular of vesicular glutamate transporter 2 (vGLUT2) (Lin et al., 2003; Hur and Zaborszky, 2005). Some of these glutamatergic cells are projection neurons that project to hippocampus (Colom et al., 2005) and cortical areas (Gritti et al., 1997; Manns et al., 2001; Hur and Zaborszky, 2005; Henny and Jones, 2008) whereas a major proportion may represent local circuit neurons (Hajszan et al., 2004; Manseau et al., 2005).

In addition to their projections to cortical and hippocampal areas, all three classes of neurons, i.e. cholinergic, GABAergic and glutamatergic BF neurons participate in local axonal projections in the BF. Thus, MS/DB complex cholinergic neurons send local axon collaterals that contact septal GABAergic (Leranth and Frotscher, 1989; Brauer et al., 1998) or Parvalbumin-containing neurons (Brauer et al., 1998) and, similarly, cholinergic BF neurons are contacted by GABAergic terminals (Leranth and Frotscher, 1989; Gritti et al., 1993) which may, at least in part, originate from local GABAergic interneurons (Gritti et al., 1993; Pang et al., 1998; Zaborszky and Duque, 2000). In addition, glutamatergic (i.e. vGLUT2-expressing) terminals of a predominant intrinsic origin have been described to make synaptic contacts with

parvalbumin-containing (Wu et al., 2003; Hajszan et al., 2004) and cholinergic septohippocampal neurons (Wu et al., 2004).

In turn, the cortical and hippocampal targets of the basal forebrain project back to the basal forebrain. Thus, the hippocampus projects directly to the MS/DB complex (Alonso and Kohler, 1982; Gaykema et al., 1991a) by way of connections from GABAergic non-pyramidal projection neurons in CA fields (Toth and Freund, 1992). The targets in the MS/DB complex of these hippocamposeptal fibers are mainly parvalbumin positive, GABAergic projection neurons (Toth et al., 1993) and to a lesser extent cholinergic cells (Gaykema et al., 1991a; Toth et al., 1993). These hippocamposeptal fibers make synaptic contacts with septohippocampal neurons (Toth et al., 1993). In addition to this direct projection, the hippocampus may influence the MS/DB complex by way of a glutamatergic, excitatory projection from the hippocampal pyramidal neurons to the lateral septal nucleus (Zaczek et al., 1979; Alonso and Kohler, 1982; Leranth and Frotscher, 1989; Gaykema et al., 1991a) which in turn projects to the MS/DB complex. This lateral septum to MS/DB projection has a restricted but detectable extent (Witter et al., 1992). Similarly, projections from cortical areas to basal forebrain have been described (Zaborszky et al., 1997). The prefrontal cortex constitutes the major source of cortical afferents to BF regions (Jaskiw et al., 1991; Vertes, 2004), and although initial studies suggested that this prefrontal input contacted cholinergic cells in the BF (Gaykema et al., 1991b), later electron microscopic studies (Zaborszky et al., 1997) showed that the glutamatergic prefrontocortical afferents to BF closely apposed cholinergic cells but could not unequivocally be shown to make synaptic contacts with them. In contrast, prefrontocortical afferents were shown to make synaptic contacts with parvalbumin positive, GABAergic neurons and also with parvalbumin negative and probably non-cholinergic neuronal elements (Zaborszky et al., 1997). This latter component might include prefrontal glutamatergic input onto local or projection glutamatergic neurons in

BF, which, to our knowledge, has not yet been proven experimentally but has recently been hypothesized (Gyengesi et al., 2008).

The present results indicate that 5-HT₄ receptors are synthesized by several of the above-mentioned neuronal populations. The main association of 5-HT₄ receptor synthesis in the BF seems to be with GABAergic cells, since approximately one half of the GABAergic cells in MS/DB complex contains 5-HT₄ receptor mRNA and, conversely, a majority (between one half and two thirds) of 5-HT₄ receptor-synthesizing cells are GABAergic. Moreover, in the NbM, all the 5-HT₄ receptor-synthesizing cells were found to be GABAergic. In an attempt to further identify the nature of the GABAergic cells that synthesize 5-HT₄ receptors, parvalbumin was used as a marker of the GABAergic projection cell population (Freund, 1989; Gritti et al., 2003). Only a minor portion, between 10 and 15%, of the 5-HT₄ receptor mRNA-positive neurons in the MS/DB complex were positive for the marker parvalbumin. Thus, the combined results obtained from experiments with probes for parvalbumin and GAD65 mRNAs indicate that only a minor fraction of basal forebrain 5-HT₄ receptors located in GABAergic cells are on projection neurons whereas a major fraction is located on GABAergic interneurons. However, when one considers the population of Parvalbumin-containing GABAergic projection cells, a significant fraction (from approximately 25% in the MS/VDB to almost 50% in the HDB area) of these cells expresses 5-HT₄ receptors, thus suggesting that these receptors may also have a prominent and direct role in the modulation of the BF GABAergic projection system.

An association of 5-HT₄ receptors was also observed with glutamatergic cells, as defined by their expression of the vesicular glutamate transporter vGLUT2 (Lin et al., 2003; Hur and Zaborszky, 2005). Thus, about 20-25% of the cells that synthesize 5-HT₄ receptors are glutamatergic in the MS/VDB complex and HDB, whereas when one considers the population of glutamatergic cells, around 30% and 65% of these cells express 5-HT₄ receptor mRNA in these two areas respectively. It remains to be

established whether glutamatergic cells expressing 5-HT₄ receptors correspond to projection neurons, local circuit neurons or both. Nevertheless, since a majority of glutamatergic cells in the MS/DB complex are in fact local circuit neurons (Hajszan et al., 2004; Manseau et al., 2005), and given the high fraction (30-65%) of glutamatergic cells that express 5-HT₄ receptors, it can be presumed that at least a fraction of the local BF glutamatergic neurons express 5-HT₄ receptors.

In contrast, BF cholinergic cells were never seen to contain detectable levels of mRNA for 5-HT₄ receptors. Thus, the present results suggest that any effect that 5-HT₄ receptor activation may have on acetylcholine release is likely to be mediated by non-cholinergic cell populations that do express this receptor.

As mentioned above, hippocampus and cortex (namely prefrontal cortex) also project to basal forebrain. 5-HT₄ receptors are expressed in some of the populations that provide these afferents to BF. Thus, in the indirect hippocampo-lateral septum-MS/DB projection, 5-HT₄ receptors are present at two different levels, i.e. in the glutamatergic, thus excitatory, hippocampal pyramidal neurons and in the GABAergic, thus inhibitory, cells of the dorsal part of the lateral septum, which have been shown to project to the VDB (Witter et al., 1992). In contrast, the direct hippocampo-MS/DB projection arising from non-pyramidal GABAergic neurons is devoid of 5-HT₄ receptors, as indicated by the fact that 5-HT₄ receptor mRNA was not observed in hippocampal neurons that express the GABAergic marker GAD65. In the case of cortical afferents to basal forebrain, 5-HT₄ receptors are expressed by a fraction of glutamatergic projection neurons in the prefrontal cortex (Feng et al., 2001 and our own unpublished observations), although it remains to be established experimentally whether they are present specifically in the corticobasal neurons.

4.3. Functional considerations

Several studies have shown an involvement of 5-HT₄ receptors in the control of acetylcholine release. In the following paragraphs, an attempt will be made to provide an anatomical substrate for the observed functional effects of stimulation of 5-HT₄ receptors on ACh release in the light of the data presented herein. Many of the studies that have addressed the effects of activation of 5-HT₄ receptors on the release of acetylcholine have concentrated in the frontal or prefrontal cortex (Consolo et al., 1994; Yamaguchi et al., 1997a; Yamaguchi et al., 1997b; Siniscalchi et al., 1999; Johnson et al., 2012). Some of these studies demonstrate that activation of 5-HT₄ receptors enhances the release of ACh in these cortical areas but the fact that drugs are applied systemically or intracerebroventricularly precludes the assignment of the receptors involved to particular cellular populations (Consolo et al., 1994; Nair and Gudelsky, 2005; Johnson et al., 2012). Another series of in vivo microdialysis experiments have shown that enhancement of serotonergic transmission either by serotonin reuptake inhibitors or by serotonin releasing agents results in increased release of ACh in frontal cortex which is blocked by 5-HT₄ receptor antagonists applied locally in the frontal cortex through the dialysis probe (Yamaguchi et al., 1997a; Yamaguchi et al., 1997b; Nair and Gudelsky, 2005). Given the presence of 5-HT₄ receptor mRNA in frontocortical glutamatergic projection neurons (Feng et al., 2001 and our own unpublished observations) and its absence from cholinergic BF neurons described in the present study, it is possible that the 5-HT₄ receptors in frontal cortex that control the release of ACh correspond to the receptors located in glutamatergic projection neurons. Activation of these receptors could result in excitation of these glutamatergic neurons, which could in turn excite neuronal elements in the basal forebrain. One possible target of this frontocortical excitatory input to BF could be the cholinergic neurons themselves, although, as mentioned in preceding paragraphs, direct and unequivocal ultrastructural evidence of glutamatergic prefrontocortical input to BF

cholinergic neurons does not exist. Another possible target would be other BF glutamatergic interneurons which could then contact and excite cholinergic neurons. In support of this, it has been shown that vGLUT2 positive puncta exist in close proximity to cholinergic, GABAergic and glutamatergic MS/DB neurons and that strong glutamatergic responses are detected in identified cholinergic, GABAergic and glutamatergic neurons in MS/DB (Manseau et al., 2005). In addition, vGLUT1 and vGLUT2 synapses have been described on BF cholinergic cells, and these latter have been proposed to arise, at least in part, from glutamatergic vGLUT2-expressing neurons in the BF itself (Hur et al., 2009). Furthermore, several indications support a role for glutamate in the facilitation of ACh release in the cortex. Thus, it has been shown that excitation of the prefrontal cortex by local perfusion of the glutamatergic agonist AMPA dose-dependently increases ACh efflux in parietal cortex (Nelson et al., 2005). Also, it has been shown that microinjection of glutamate into NbM results in increased activity of NbM cholinergic neurons as measured by high affinity choline uptake in prefrontal cortex (Wenk, 1984) and increased cortical ACh release (Kurosawa et al., 1989). Thus, the anatomical and neurochemical bases exist to support an involvement of 5-HT₄ receptors present in glutamatergic projection neurons of the frontal cortex in the control of cortical ACh release.

Activation of 5-HT₄ receptors present in the NbM by local application of a 5-HT₄ receptor agonist has been shown to enhance the acquisition and consolidation of spatial memory, an effect allegedly due to a potentiation of the activity of the cholinergic NbM-cortical pathways (Orsetti et al., 2003). However, our present findings showing a lack of expression of 5-HT₄ receptors in cholinergic cells in the NbM together with their expression in GABAergic cells located in this brain area, suggest that the NbM cholinergic system is not the direct recipient of this 5-HT₄ influence. This is supported by the fact that local application into NbM of indeloxazine, an inhibitor of 5-HT and noradrenaline reuptake with a facilitatory effect on 5-HT release, is unable to

alter ACh release in frontal cortex (Yamaguchi et al., 1997b). Thus, it seems possible that the promnesic effect observed after 5-HT₄ receptor agonist administration into the NbM (Orsetti et al., 2003) is mediated by corticopetal GABAergic neurons which have been suggested to participate in the cognitive functions of the basal forebrain-cortical circuitry (Baxter and Chiba, 1999; Semba, 2000; Sarter and Bruno, 2002). Furthermore, it has been shown that a major proportion of the serotonergic innervation from the dorsal raphe nucleus to the substantia innominata and NbM seems to establish contacts with non-cholinergic cells (Gasbarri et al., 1999), thus suggesting that the 5-HT₄-endowed GABAergic neurons in NbM may indeed receive serotonergic input in normal physiological conditions.

Several other studies have addressed the effects of activation of 5-HT₄ receptors on the release of acetylcholine in the hippocampus. An *in vivo* microdialysis study has shown that local application of a 5-HT₄ receptor agonist in the hippocampus results in increased levels of hippocampal ACh (Matsumoto et al., 2001). A direct effect of the agonist on cholinergic terminals to facilitate ACh release is not supported by the present results of a lack of expression of 5-HT₄ receptor mRNA in cholinergic neurons of MS/VDB. Given the high levels of expression of 5-HT₄ receptors in the hippocampus itself (Waeber et al., 1994; Vilaró et al., 2005 and present results), it is likely that these are the receptors that mediate the facilitating effect on ACh release. We have shown that the expression of 5-HT₄ receptor mRNA in the hippocampus is restricted to the populations of principal cells, i.e. the glutamatergic pyramidal cells of Cornu Ammonis and granule cells of the dentate gyrus, whereas hippocampal GABAergic neurons, that include interneurons and long range projection neurons (Freund and Buzsaki, 1996), are devoid of 5-HT₄ receptor mRNA. The question arises as to which is the neuronal circuitry responsible for the ultimate activation of MS/DB cholinergic neurons that results in enhancement of ACh release in the hippocampus in response to hippocampal 5-HT₄ receptor activation. A direct glutamatergic projection from

hippocampal pyramidal neurons to MS/DB complex was postulated in earlier studies (Walaas and Fonnum, 1980; Alonso and Kohler, 1982) but it has not been further substantiated, therefore lending little support to its involvement in the observed effects on ACh release. On the other hand, it has been shown that pyramidal cells of CA1 project to the prefrontal cortex, contacting both pyramidal projection neurons and interneurons (Carr and Sesack, 1996; Gabbott et al., 2002), and that hippocampal stimulation results in excitation of most of the recorded pyramidal neurons in the prefrontal cortex (Degenetais et al., 2003). Thus, it is conceivable that 5-HT₄-mediated excitation of hippocampal projection neurons results in excitation of prefrontocortical pyramidal neurons which, in turn, may project to the basal forebrain (Jaskiw et al., 1991; Vertes, 2004) and ultimately elicit excitation of cholinergic septo-hippocampal cells possibly by involving local circuit glutamatergic excitatory MS/DB neurons (Manseau et al., 2005). A further possible circuit might involve the GABAergic hippocampal projection neurons. Thus, 5-HT₄ receptor-mediated excitation of pyramidal neurons would result in excitation of non-principal GABAergic neurons with which they are connected (Freund and Buzsaki, 1996; Somogyi et al., 1998). Some of these GABAergic cells are local circuit neurons but others project to the MS/DB region (Toth and Freund, 1992; Freund and Buzsaki, 1996), where they mainly contact, and likely inhibit, parvalbumin-containing GABAergic neurons (Toth et al., 1993). Inhibition of these inhibitory MS/DB neurons, which make local recurrent contacts with cholinergic septohippocampal cells (Leranth and Frotscher, 1989) could eventually result in disinhibition of cholinergic neurons and thus in increases of ACh output in the hippocampus.

Finally, other studies have used slices from different brain regions to address the effects of 5-HT₄ receptor stimulation on ACh release. Thus, stimulation of 5-HT₄ receptors increased electrically-stimulated ³H-choline efflux from slices from guinea pig fronto-parietal cortex and hippocampus and from rat hippocampus (Siniscalchi et al.,

1999). In contrast, in hippocampal synaptosomal preparations also tested in the study, superfusion with a 5-HT₄ receptor agonist did not modify ³H-choline efflux (Siniscalchi et al., 1999). The lack of effect on synaptosomes would be in agreement with the lack of expression of 5-HT₄ receptors in cholinergic neurons reported herein. In the slice preparations, the observed effects cannot be mediated by circuitry involving distant brain regions, thus local events must be involved. In the rat hippocampal slice preparation, 5-HT₄ receptors are located on glutamatergic neurons, as indicated by the present results. Similarly, it can be assumed, by analogy with the rat (Vilaró et al., 2005), that in the guinea pig cortical and hippocampal slice preparations 5-HT₄ receptors are also located on glutamatergic pyramidal neurons. Thus, an activation of these hippocampal or cortical 5-HT₄ receptors may result in excitation of these neurons and in increased glutamate release within the slice. This glutamate might in turn be able to modulate ACh release provided that glutamate receptors exist on cholinergic terminals. Support for this localisation comes from the fact that, in cortical synaptosomal preparations, activation of AMPA type glutamate receptors enhances the release of [³H]ACh (Gherzi et al., 2003), thus indicating the presence of AMPA receptors on cholinergic terminals. Furthermore, it has been shown that cholinergic basal forebrain neurons express several of the glutamate receptor subunits (GluR1-4) that form AMPA receptors (Martin et al., 1993; Page and Everitt, 1995), and AMPA receptors have been detected in presynaptic locations in the hippocampus (Fabian-Fine et al., 2000). Interestingly, in this latter study, AMPA receptors have been described on presynaptic axon terminals that in turn receive axo-axonic synapses, of a putative excitatory nature.

In conclusion, the present results lend support to the absence of 5-HT₄ receptors from cholinergic BF neurons and point to the involvement of GABAergic and/or glutamatergic cell populations within the BF itself or in cortical and hippocampal regions in the enhancement of acetylcholine release elicited by 5-HT₄ receptor

agonists. These neuroanatomical data provide the basis for more detailed microdialysis and/or tract tracing studies that should result in deeper knowledge of the circuits involved in the observed pro-cognitive effects elicited by activation of 5-HT₄ receptors.

Acknowledgements: Grant information: This research was funded by Plan Nacional I+D+I (Grant sponsor: Ministerio de Ciencia y Tecnología (Spain); Grant sponsor: European Union/European Regional Development Funds; Grant number: SAF2002-03408). Support from the Generalitat de Catalunya (Grup de Recerca Consolidat 2009SGR220) is also acknowledged. R. P-C was recipient of a predoctoral fellowship from Ministerio de Ciencia y Tecnología).

References

- Alonso A and Kohler C (1982) Evidence for separate projections of hippocampal pyramidal and non-pyramidal neurons to different parts of the septum in the rat brain. *Neurosci Lett* 31:209-214.
- Baxter MG and Chiba AA (1999) Cognitive functions of the basal forebrain. *Curr Opin Neurobiol* 9:178-183.
- Bender E, Pindon A, van O, I, Zhang YB, Gommeren W, Verhasselt P, Jurzak M, Leysen J, and Luyten W (2000) Structure of the human serotonin 5-HT₄ receptor gene and cloning of a novel 5-HT₄ splice variant. *J Neurochem* 74:478-489.
- Blondel O, Gastineau M, Dahmoune Y, Langlois M, and Fischmeister R (1998) Cloning, expression, and pharmacology of four human 5-hydroxytryptamine 4 receptor isoforms produced by alternative splicing in the carboxyl terminus. *J Neurochem* 70:2252-2261.
- Bockaert J, Claeysen S, Compan V, and Dumuis A (2004) 5-HT₄ receptors. *Curr Drug Targets CNS Neurol Disord* 3:39-51.
- Bonaventure P, Hall H, Gommeren W, Cras P, Langlois X, Jurzak M, and Leysen JE (2000) Mapping of serotonin 5-HT₄ receptor mRNA and ligand binding sites in the post-mortem human brain. *Synapse* 36:35-46.
- Brattelid T, Kvingedal AM, Krobert KA, Andressen KW, Bach T, Hystad ME, Kaumann AJ, and Levy FO (2004) Cloning, pharmacological characterisation and tissue distribution of a novel 5-HT₄ receptor splice variant, 5-HT₄(i). *Naunyn Schmiedeberg's Arch Pharmacol* 369:616-628.
- Brauer K, Schober A, Wolff JR, Winkelmann E, Lupp H, Luth HJ, and Bottcher H (1991) Morphology of neurons in the rat basal forebrain nuclei: comparison between NADPH-diaphorase histochemistry and immunohistochemistry of glutamic acid decarboxylase, choline acetyltransferase, somatostatin and parvalbumin. *J Hirnforsch* 32:1-17.

- Brauer K, Seeger G, Hartig W, Rossner S, Poethke R, Kacza J, Schliebs R, Bruckner G, and Bigl V (1998) Electron microscopic evidence for a cholinergic innervation of GABAergic parvalbumin-immunoreactive neurons in the rat medial septum. *J Neurosci Res* 54:248-253.
- Broide RS, Trembleau A, Ellison JA, Cooper J, Lo D, Young WG, Morrison JH, and Bloom FE (2004) Standardized quantitative in situ hybridization using radioactive oligonucleotide probes for detecting relative levels of mRNA transcripts verified by real-time PCR. *Brain Res* 1000:211-222.
- Carr DB and Sesack SR (1996) Hippocampal afferents to the rat prefrontal cortex: synaptic targets and relation to dopamine terminals. *J Comp Neurol* 369:1-15.
- Claeysen S, Faye P, Sebben M, Taviaux S, Bockaert J, and Dumuis A (1998) 5-HT₄ receptors: cloning and expression of new splice variants. *Ann N Y Acad Sci* 861:49-56.
- Claeysen S, Sebben M, Becamel C, Bockaert J, and Dumuis A (1999) Novel brain-specific 5-HT₄ receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol Pharmacol* 55:910-920.
- Colom LV, Castaneda MT, Reyna T, Hernandez S, and Garrido-Sanabria E (2005) Characterization of medial septal glutamatergic neurons and their projection to the hippocampus. *Synapse* 58:151-164.
- Compan V, Daszuta A, Salin P, Sebben M, Bockaert J, and Dumuis A (1996) Lesion study of the distribution of serotonin 5-HT₄ receptors in rat basal ganglia and hippocampus. *Eur J Neurosci* 8:2591-2598.
- Consolo S, Arnaboldi S, Giorgi S, Russi G, and Ladinsky H (1994) 5-HT₄ receptor stimulation facilitates acetylcholine release in rat frontal cortex. *Neuroreport* 5:1230-1232.
- Degenetais E, Thierry AM, Glowinski J, and Gioanni Y (2003) Synaptic influence of hippocampus on pyramidal cells of the rat prefrontal cortex: an in vivo intracellular recording study. *Cereb Cortex* 13:782-792.

- Dumuis A, Bouhelal R, Sebben M, Cory R, and Bockaert J (1988) A nonclassical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. *Mol Pharmacol* 34:880-887.
- Dunnett SB, Everitt BJ, and Robbins TW (1991) The basal forebrain-cortical cholinergic system: interpreting the functional consequences of excitotoxic lesions. *Trends Neurosci* 14:494-501.
- Eglen RM and Hegde SS (1996) 5-Hydroxytryptamine (5-HT)₄ receptors: physiology, pharmacology and therapeutic potential. *Exp Opin Invest Drugs* 5:373-388.
- Eglen RM, Wong EH, Dumuis A, and Bockaert J (1995) Central 5-HT₄ receptors. *Trends Pharmacol Sci* 16:391-398.
- Fabian-Fine R, Volkhardt W, Fine A, and Stewart MG (2000) Age-dependent pre- and postsynaptic distribution of AMPA receptors at synapses in CA3 stratum radiatum of hippocampal slice cultures compared with intact brain. *Eur J Neurosci* 12:3687-3700.
- Feng J, Cai X, Zhao J, and Yan Z (2001) Serotonin receptors modulate GABA(A) receptor channels through activation of anchored protein kinase C in prefrontal cortical neurons. *J Neurosci* 21:6502-6511.
- Fibiger HC (1991) Cholinergic mechanisms in learning, memory and dementia: a review of recent evidence. *Trends Neurosci* 14:220-223.
- Ford AP and Clarke DE (1993) The 5-HT₄ receptor. *Med Res Rev* 13:633-662.
- Freund TF (1989) GABAergic septohippocampal neurons contain parvalbumin. *Brain Res* 478:375-381.
- Freund TF and Buzsaki G (1996) Interneurons of the hippocampus. *Hippocampus* 6:347-470.
- Gabbott P, Headlam A, and Busby S (2002) Morphological evidence that CA1 hippocampal afferents monosynaptically innervate PV-containing neurons and NADPH-diaphorase reactive cells in the medial prefrontal cortex (Areas 25/32) of the rat. *Brain Res* 946:314-322.

- Gasbarri A, Sulli A, Pacitti C, and McGaugh JL (1999) Serotonergic input to cholinergic neurons in the substantia innominata and nucleus basalis magnocellularis in the rat. *Neuroscience* 91:1129-1142.
- Gaykema RP, van der KJ, Hersh LB, and Luiten PG (1991a) Patterns of direct projections from the hippocampus to the medial septum-diagonal band complex: anterograde tracing with Phaseolus vulgaris leucoagglutinin combined with immunohistochemistry of choline acetyltransferase. *Neuroscience* 43:349-360.
- Gaykema RP, van Weeghel R, Hersh LB, and Luiten PG (1991b) Prefrontal cortical projections to the cholinergic neurons in the basal forebrain. *J Comp Neurol* 303:563-583.
- Gerald C, Adham N, Kao HT, Olsen MA, Laz TM, Schechter LE, Bard JA, Vaysse PJ, Hartig PR, and Branchek TA (1995) The 5-HT₄ receptor: molecular cloning and pharmacological characterization of two splice variants. *EMBO J* 14:2806-2815.
- Gherzi C, Bonfanti A, Manzari B, Feligioni M, Raiteri M, and Pittaluga A (2003) Pharmacological heterogeneity of release-regulating presynaptic AMPA/kainate receptors in the rat brain: study with receptor antagonists. *Neurochem Int* 42:283-292.
- Gold PE (2003) Acetylcholine modulation of neural systems involved in learning and memory. *Neurobiol Learn Mem* 80:194-210.
- Gritti I, Mainville L, and Jones BE (1993) Codistribution of GABA- with acetylcholine-synthesizing neurons in the basal forebrain of the rat. *J Comp Neurol* 329:438-457.
- Gritti I, Mainville L, Mancina M, and Jones BE (1997) GABAergic and other noncholinergic basal forebrain neurons, together with cholinergic neurons, project to the mesocortex and isocortex in the rat. *J Comp Neurol* 383:163-177.
- Gritti I, Manns ID, Mainville L, and Jones BE (2003) Parvalbumin, calbindin, or calretinin in cortically projecting and GABAergic, cholinergic, or glutamatergic basal forebrain neurons of the rat. *J Comp Neurol* 458:11-31.

- Gyengesi E, Zaborszky L, and Detari L (2008) The effect of prefrontal stimulation on the firing of basal forebrain neurons in urethane anesthetized rat. *Brain Res Bull* 75:570-580.
- Hajszan T, Alreja M, and Leranth C (2004) Intrinsic vesicular glutamate transporter 2-immunoreactive input to septohippocampal parvalbumin-containing neurons: novel glutamatergic local circuit cells. *Hippocampus* 14:499-509.
- Hegde SS and Eglen RM (1996) Peripheral 5-HT₄ receptors. *FASEB J* 10:1398-1407.
- Henny P and Jones BE (2008) Projections from basal forebrain to prefrontal cortex comprise cholinergic, GABAergic and glutamatergic inputs to pyramidal cells or interneurons. *Eur J Neurosci* 27:654-670.
- Hur EE, Edwards RH, Rommer E, and Zaborszky L (2009) Vesicular glutamate transporter 1 and vesicular glutamate transporter 2 synapses on cholinergic neurons in the sublentiform gray of the rat basal forebrain: a double-label electron microscopic study. *Neuroscience* 164:1721-1731.
- Hur EE and Zaborszky L (2005) Vglut2 afferents to the medial prefrontal and primary somatosensory cortices: a combined retrograde tracing in situ hybridization. *J Comp Neurol* 483:351-373.
- Ishii K, Oda Y, Ichikawa T, and Deguchi T (1990) Complementary DNAs for choline acetyltransferase from spinal cords of rat and mouse: nucleotide sequences, expression in mammalian cells, and in situ hybridization. *Brain Res Mol Brain Res* 7:151-159.
- Jaskiw GE, Tizabi Y, Lipska BK, Kolachana BS, Wyatt RJ, and Gilad GM (1991) Evidence for a frontocortical-septal glutamatergic pathway and compensatory changes in septal glutamate uptake after cortical and fornix lesions in the rat. *Brain Res* 550:7-10.
- Johnson DE, Drummond E, Grimwood S, Sawant-Basak A, Miller E, Tseng E, McDowell LL, Vanase-Frawley MA, Fisher KE, Rubitski DM, Stutzman-Engwall KJ, Nelson RT, Horner WE, Gorczyca RR, Hajos M, and Siok CJ (2012) The 5-hydroxytryptamine₄ receptor agonists prucalopride and PRX-03140 increase acetylcholine and histamine levels in the rat

prefrontal cortex and the power of stimulated hippocampal theta oscillations. *J Pharmacol Exp Ther* 341:681-691.

Kiss J, Patel AJ, and Freund TF (1990) Distribution of septohippocampal neurons containing parvalbumin or choline acetyltransferase in the rat brain. *J Comp Neurol* 298:362-372.

Kurosawa M, Sato A, and Sato Y (1989) Stimulation of the nucleus basalis of Meynert increases acetylcholine release in the cerebral cortex in rats. *Neurosci Lett* 98:45-50.

Lamirault L, Guillou C, Thal C, and Simon H (2003) Combined treatment with galanthaminium bromide, a new cholinesterase inhibitor, and RS 67333, a partial agonist of 5-HT₄ receptors, enhances place and object recognition in young adult and old rats. *Prog Neuropsychopharmacol Biol Psychiatry* 27:185-195.

Landry M, Holmberg K, Zhang X, and Hokfelt 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.

Lee MG, Chrobak JJ, Sik A, Wiley RG, and Buzsaki G (1994) Hippocampal theta activity following selective lesion of the septal cholinergic system. *Neuroscience* 62:1033-1047.

Leranth C and Frotscher M (1989) Organization of the septal region in the rat brain: cholinergic- GABAergic interconnections and the termination of hippocampo-septal fibers [published erratum appears in *J Comp Neurol* 1990 Feb 15;292(3):495]. *J Comp Neurol* 289:304-314.

Lin W, McKinney K, Liu L, Lakhani S, and Jennes L (2003) Distribution of vesicular glutamate transporter-2 messenger ribonucleic Acid and protein in the septum-hypothalamus of the rat. *Endocrinology* 144:662-670.

Manns ID, Mainville L, and Jones BE (2001) Evidence for glutamate, in addition to acetylcholine and GABA, neurotransmitter synthesis in basal forebrain neurons projecting to the entorhinal cortex. *Neuroscience* 107:249-263.

- Manseau F, Danik M, and Williams S (2005) A functional glutamatergic neurone network in the medial septum and diagonal band area. J Physiol 566:865-884.**
- Marner L, Gillings N, Comley RA, Baare WF, Rabiner EA, Wilson AA, Houle S, Hasselbalch SG, Svarer C, Gunn RN, Laruelle M, and Knudsen GM (2009) Kinetic modeling of ¹¹C-SB207145 binding to 5-HT₄ receptors in the human brain in vivo. J Nucl Med 50:900-908.**
- Martin LJ, Blackstone CD, Levey AI, Huganir RL, and Price DL (1993) Cellular localizations of AMPA glutamate receptors within the basal forebrain magnocellular complex of rat and monkey. J Neurosci 13:2249-2263.**
- Matsumoto M, Togashi H, Mori K, Ueno K, Ohashi S, Kojima T, and Yoshioka M (2001) Evidence for involvement of central 5-HT₄ receptors in cholinergic function associated with cognitive processes: behavioral, electrophysiological, and neurochemical studies. J Pharmacol Exp Ther 296:676-682.**
- Mesulam MM, Mufson EJ, Wainer BH, and Levey AI (1983) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience 10:1185-1201.**
- Moser PC, Bergis OE, Jegham S, Lochead A, Duconseille E, Terranova JP, Caille D, Berque-Bestel I, Lezoualc'h F, Fischmeister R, Dumuis A, Bockaert J, George P, Soubrie P, and Scatton B (2002) SL65.0155, a novel 5-hydroxytryptamine₄ receptor partial agonist with potent cognition-enhancing properties. J Pharmacol Exp Ther 302:731-741.**
- Nair SG and Gudelsky GA (2005) 3,4-Methylenedioxymethamphetamine (MDMA) enhances the release of acetylcholine by 5-HT₄ and D₁ receptor mechanisms in the rat prefrontal cortex. Synapse 58:229-235.**
- Nelson CL, Sarter M, and Bruno JP (2005) Prefrontal cortical modulation of acetylcholine release in posterior parietal cortex. Neuroscience 132:347-359.**
- Orsetti M, Dellarole A, Ferri S, and Ghi P (2003) Acquisition, retention, and recall of memory after injection of RS67333, a 5-HT₄ receptor agonist, into the nucleus basalis magnocellularis of the rat. Learn Mem 10:420-426.**

- Page KJ and Everitt BJ (1995) The distribution of neurons coexpressing immunoreactivity to AMPA-sensitive glutamate receptor subtypes (GluR1-4) and nerve growth factor receptor in the rat basal forebrain. Eur J Neurosci 7:1022-1033.**
- Pang K, Tepper JM, and Zaborszky L (1998) Morphological and electrophysiological characteristics of noncholinergic basal forebrain neurons. J Comp Neurol 394:186-204.**
- Parent MB and Baxter MG (2004) Septohippocampal acetylcholine: involved in but not necessary for learning and memory? Learn Mem 11:9-20.**
- Patel S, Roberts J, Moorman J, and Reavill C (1995) Localization of serotonin-4 receptors in the striatonigral pathway in rat brain. Neuroscience 69:1159-1167.**
- Paxinos,G. and Watson,C., (1997). The rat brain in stereotaxic coordinates, Compact 3rd ed., Academic Press, San Diego.**
- Paxinos,G. and Watson,C., (1998). The rat brain in stereotaxic coordinates, 4th ed., Academic Press, San Diego.**
- Perez-Garcia G and Meneses A (2008) Memory formation, amnesia, improved memory and reversed amnesia: 5-HT role. Behav Brain Res 195:17-29.**
- Ray AM, Kelsell RE, Houp JA, Kelly FM, Medhurst AD, Cox HM, and Calver AR (2009) Identification of a novel 5-HT(4) receptor splice variant (r5-HT(4c1)) and preliminary characterisation of specific 5-HT(4a) and 5-HT(4b) receptor antibodies. Eur J Pharmacol 604:1-11.**
- Reynolds GP, Mason SL, Meldrum A, De Keczer S, Parnes H, Eglen RM, and Wong EH (1995) 5-Hydroxytryptamine (5-HT)₄ receptors in post mortem human brain tissue: distribution, pharmacology and effects of neurodegenerative diseases. Br J Pharmacol 114:993-998.**
- Sarter M and Bruno JP (2002) The neglected constituent of the basal forebrain corticopetal projection system: GABAergic projections. Eur J Neurosci 15:1867-1873.**

- Schmitz GG, Walter T, Seibl R, and Kessler C (1991) Nonradioactive labeling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. *Anal Biochem* 192:222-231.
- Schwaber JS, Rogers WT, Satoh K, and Fibiger HC (1987) Distribution and organization of cholinergic neurons in the rat forebrain demonstrated by computer-aided data acquisition and three-dimensional reconstruction. *J Comp Neurol* 263:309-325.
- Semba K (2000) Multiple output pathways of the basal forebrain: organization, chemical heterogeneity, and roles in vigilance. *Behav Brain Res* 115:117-141.
- Serrats J, Artigas F, Mengod G, and Cortes R (2003) GABAB receptor mRNA in the raphe nuclei: co-expression with serotonin transporter and glutamic acid decarboxylase. *J Neurochem* 84:743-752.
- Siniscalchi A, Badini I, Beani L, and Bianchi C (1999) 5-HT₄ receptor modulation of acetylcholine outflow in guinea pig brain slices. *Neuroreport* 10:547-551.
- Somogyi P, Tamas G, Lujan R, and Buhl EH (1998) Salient features of synaptic organisation in the cerebral cortex. *Brain Res Brain Res Rev* 26:113-135.
- Toth K, Borhegyi Z, and Freund TF (1993) Postsynaptic targets of GABAergic hippocampal neurons in the medial septum-diagonal band of Broca complex. *J Neurosci* 13:3712-3724.
- Toth K and Freund TF (1992) Calbindin D28k-containing nonpyramidal cells in the rat hippocampus: their immunoreactivity for GABA and projection to the medial septum. *Neuroscience* 49:793-805.
- Varnas K, Halldin C, Pike VW, and Hall H (2003) Distribution of 5-HT₄ receptors in the postmortem human brain--an autoradiographic study using [¹²⁵I]SB 207710. *Eur Neuropsychopharmacol* 13:228-234.
- Vertes RP (2004) Differential projections of the infralimbic and prelimbic cortex in the rat. *Synapse* 51:32-58.

- Vilaró MT, Cortés R, Gerald C, Branchek TA, Palacios JM, and Mengod G (1996) Localization of 5-HT₄ receptor mRNA in rat brain by in situ hybridization histochemistry. *Brain Res Mol Brain Res* 43:356-360.
- Vilaró MT, Cortes R, and Mengod G (2005) Serotonin 5-HT₄ receptors and their mRNAs in rat and guinea pig brain: distribution and effects of neurotoxic lesions. *J Comp Neurol* 484:418-439.
- Vilaró MT, Doménech T, Palacios JM, and Mengod G (2002) Cloning and characterization of a novel human 5-HT(4) receptor variant that lacks the alternatively spliced carboxy terminal exon. RT-PCR distribution in human brain and periphery of multiple 5-HT(4) receptor variants. *Neuropharmacology* 42:60-73.
- Vilaró MT, Palacios JM, and Mengod G (1994) Multiplicity of muscarinic autoreceptor subtypes? Comparison of the distribution of cholinergic cells and cells containing mRNA for five subtypes of muscarinic receptors in the rat brain. *Brain Res Mol Brain Res* 21:30-46.
- Vilaró MT, Wiederhold KH, Palacios JM, and Mengod G (1992) Muscarinic M2 receptor mRNA expression and receptor binding in cholinergic and non-cholinergic cells in the rat brain: a correlative study using in situ hybridization histochemistry and receptor autoradiography. *Neuroscience* 47:367-393.
- Waeber C, Sebben M, Nieoullon A, Bockaert J, and Dumuis A (1994) Regional distribution and ontogeny of 5-HT₄ binding sites in rodent brain. *Neuropharmacology* 33:527-541.
- Walaas I and Fonnum F (1980) Biochemical evidence for glutamate as a transmitter in hippocampal efferents to the basal forebrain and hypothalamus in the rat brain. *Neuroscience* 5:1691-1698.
- Wenk GL (1984) Pharmacological manipulations of the substantia innominata-cortical cholinergic pathway. *Neurosci Lett* 51:99-103.
- Wenk GL (1997) The nucleus basalis magnocellularis cholinergic system: one hundred years of progress. *Neurobiol Learn Mem* 67:85-95.

- Witter MP, Daelmans HE, Jorritsma-Byham B, Staiger JF, and Wouterlood FG (1992) Restricted origin and distribution of projections from the lateral to the medial septal complex in rat and guinea pig. *Neurosci Lett* 148:164-168.
- Woolf NJ, Eckenstein F, and Butcher LL (1984) Cholinergic systems in the rat brain: I. projections to the limbic telencephalon. *Brain Res Bull* 13:751-784.
- Wu M, Hajszan T, Leranthy C, and Alreja M (2003) Nicotine recruits a local glutamatergic circuit to excite septohippocampal GABAergic neurons. *Eur J Neurosci* 18:1155-1168.
- Wu M, Hajszan T, Xu C, Leranthy C, and Alreja M (2004) Group I metabotropic glutamate receptor activation produces a direct excitation of identified septohippocampal cholinergic neurons. *J Neurophysiol* 92:1216-1225.
- Yamaguchi T, Suzuki M, and Yamamoto M (1997a) Evidence for 5-HT₄ receptor involvement in the enhancement of acetylcholine release by p-chloroamphetamine in rat frontal cortex. *Brain Res* 772:95-101.
- Yamaguchi T, Suzuki M, and Yamamoto M (1997b) Facilitation of acetylcholine release in rat frontal cortex by indeloxazine hydrochloride: involvement of endogenous serotonin and 5-HT₄ receptors. *Naunyn Schmiedeberg's Arch Pharmacol* 356:712-720.
- Zaborszky L and Duque A (2000) Local synaptic connections of basal forebrain neurons. *Behav Brain Res* 115:143-158.
- Zaborszky L, Gaykema RP, Swanson DJ, and Cullinan WE (1997) Cortical input to the basal forebrain. *Neuroscience* 79:1051-1078.
- Zaborszky L, Pang K, Somogyi J, Nadasdy Z, and Kallo I (1999) The basal forebrain corticopetal system revisited. *Ann N Y Acad Sci* 877:339-367.
- Zaczek R, Hedreen JC, and Coyle JT (1979) Evidence for a hippocampal-septal glutamatergic pathway in the rat. *Exp Neurol* 65:145-156.

Figure Legends

Fig. 1

Distribution of 5-HT₄ receptor mRNA in the region of the medial septum and diagonal band of the rat. A, B: darkfield photomicrographs of emulsion-dipped sections hybridized with a mixture of six different ³³P-labeled oligonucleotide probes complementary to regions of 5-HT₄ receptor mRNA common to all C-terminal splice variants. A', B': staining for acetylcholinesterase (performed as described in (Vilaró et al., 1992) of the corresponding anatomical levels. Frames in A' and B' indicate the regions shown in A and B respectively. Scale bars: 0.5 mm (in A, B), 2mm (in A', B'). Abbreviations: CPu: caudate putamen; HDB: nucleus of the horizontal limb of the diagonal band, MCPO: magnocellular preoptic nucleus, MS: medial septal nucleus, OTu: olfactory tubercle, VDB: nucleus of the vertical limb of the diagonal band.

Fig. 2

Cellular localization of 5-HT₄ receptor mRNA in sections of the basal forebrain hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for choline acetyltransferase (ChAT) mRNA. Upper row: medial septal nucleus; middle row: nucleus of the vertical limb of the diagonal band; lower row: nucleus of the horizontal limb of the diagonal band. A,D,G: Dark field photomicrographs showing the localization of cells containing 5-HT₄ receptor mRNA (clusters of bright silver grains). B,E,H: Bright field photomicrographs of the same fields showing the localization of cells containing ChAT mRNA, i.e. cholinergic cells (dark precipitate). C,F,I: Higher magnification images (obtained by blending the corresponding dark and bright field images as reported in Experimental procedures) of the areas indicated by the frames, showing cells positive for ChAT mRNA (dark precipitate, arrowheads) and cells positive for 5-HT₄ receptor mRNA (accumulations of dark silver grains, arrows). Note the lack of co-localization of both mRNAs. Scale bars: 50 µm in A (valid for left and central columns), 10 µm in C (valid for right column).

Fig. 3

Cellular localization of 5-HT₄ receptor mRNA in sections of the nucleus basalis of Meynert hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for choline acetyltransferase (ChAT) mRNA (A-D) or for glutamic acid decarboxylase GAD65 (E-H). A and E: Dark field photomicrographs showing the localization of cells containing 5-HT₄ receptor mRNA (clusters of bright silver grains). B and F: Bright field photomicrographs of the same fields showing (dark precipitate) the localization of cells containing ChAT mRNA, i.e. cholinergic cells (B) or GAD65 mRNA, i.e. GABAergic cells (F). The cells shown in the frames in A-B and E-F are shown at higher magnification in C-D and G-H respectively, which were obtained by blending the corresponding dark and bright field images. Arrows: cells positive for 5-HT₄ receptor mRNA; arrowheads: cells positive for ChAT mRNA; double arrowheads: cells doubly labeled for 5-HT₄ receptor mRNA and for GAD65 mRNA. Scale bars: 50 µm in A (valid for left and central columns), 10 µm in C (valid for right column).

Fig. 4

Cellular localization of 5-HT₄ receptor mRNA in sections of the basal forebrain hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for glutamic acid decarboxylase (GAD65) mRNA. Upper row: medial septal nucleus; middle row: nucleus of the vertical limb of the diagonal band; lower row: nucleus of the horizontal limb of the diagonal band. A,D,G: Dark field photomicrographs showing the localization of cells containing 5-HT₄ receptor mRNA (clusters of bright silver grains). B,E,H: Bright field photomicrographs of the same fields showing the localization of cells containing GAD65 mRNA, i.e. GABAergic cells (dark precipitate). C,F,I: Higher magnification images (obtained by blending the corresponding dark and bright field images) of the areas indicated by the frames, showing cells positive for GAD65 mRNA (dark precipitate) and cells positive for 5-HT₄ receptor mRNA (dark silver grains). Arrows: cells positive for 5-HT₄ receptor mRNA; arrowheads: cells positive for GAD65 mRNA; double arrowheads: doubly labeled cells. Scale bars: 50 µm in A (valid for left and central columns), 10 µm in C (valid for right column).

Fig. 5

Cellular localization of 5-HT₄ receptor mRNA in sections of the basal forebrain hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for parvalbumin mRNA. Upper row: medial septal nucleus; middle row: nucleus of the vertical limb of the diagonal band; lower row: nucleus of the horizontal limb of the diagonal band. A,D,G: Dark field photomicrographs showing the localization of cells containing 5-HT₄ receptor mRNA (clusters of bright silver grains). B,E,H: Bright field photomicrographs of the same fields showing the localization of cells containing parvalbumin mRNA (dark precipitate). C,F,I: Higher magnification images (obtained by blending the corresponding dark and bright field images) of the areas indicated by the frames, showing cells positive for parvalbumin mRNA (dark precipitate) and cells positive for 5-HT₄ receptor mRNA (dark silver grains). Arrows: cells positive for 5-HT₄ receptor mRNA; arrowheads: cells positive for parvalbumin mRNA; double arrowheads: doubly labeled cells. Scale bars: 50 µm in A (valid for left and central columns), 20 µm in C (valid for right column).

Fig. 6

Cellular localization of 5-HT₄ receptor mRNA in sections of the basal forebrain hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for the vesicular glutamate transporter vGLUT2 mRNA. Upper row: medial septal nucleus; middle row: nucleus of the vertical limb of the diagonal band; lower row: nucleus of the horizontal limb of the diagonal band. A,E,I: Dark field photomicrographs showing the localization of cells containing 5-HT₄ receptor mRNA (clusters of bright silver grains). B,F,J: Bright field photomicrographs of the same fields showing the localization of cells containing vGLUT2 mRNA, i.e. glutamatergic cells (dark precipitate). C,D,G,H,K,L: Higher magnification images (obtained by blending the corresponding dark and bright field images) of the areas indicated by the frames showing cells positive for vGLUT2 mRNA (dark precipitate) and cells positive for 5-HT₄ receptor mRNA (dark silver grains). Arrows: cells positive for 5-HT₄ receptor mRNA; arrowheads: cells positive for vGLUT2 mRNA; double arrowheads: doubly labeled cells. Scale bars: 50 µm in A (valid for left and central columns), 20 µm in C (valid for right column).

Fig. 7

Cellular localization of 5-HT₄ receptor mRNA in the hippocampus. A: low-power dark field photomicrograph of an emulsion dipped section hybridized with ³³P-labeled probes for 5-HT₄ receptor mRNA. B: Nissl (cresyl violet) staining of a close section. C,D: Dark field (C) and bright field (D, cresyl violet staining) higher magnification of a region equivalent to the one shown by the arrow in A. E: High-magnification bright-field photomicrograph of the frame shown in D. Note how the majority of the cells in the pyramidal cell layer contain mRNA for 5-HT₄ receptors. Scale bars: 1 mm in B, 50 μm in D, 20 μm in E. Abbreviations: CA1: CA1 subfield of Ammon's horn; CA3: CA3 subfield of Ammon's horn; DG: dentate gyrus.

Fig. 8

Cellular localization of 5-HT₄ receptor mRNA in sections of the hippocampus hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for glutamic acid decarboxylase (GAD65) mRNA. Upper row: CA1 region; middle row: CA3 region; lower row: dentate gyrus. A,C,E: Images obtained after superimposing dark- and bright field photomicrographs with the AnalySIS software. B,D,F: High magnification photomicrographs of the frames in A,C,E taken with a Darklite illuminator showing dark- and bright field simultaneously. Note how the cells that contain GAD65 mRNA (dark precipitate) do not contain 5-HT₄ receptor mRNA (silver grains). Scale bars: 100 μm in A, 25 μm in B.

Fig. 9

Cellular localization of 5-HT₄ receptor mRNA in sections of the lateral septum hybridized simultaneously with ³³P-labeled probes for 5-HT₄ receptor mRNA and with digoxigenin-labeled probes for glutamic acid decarboxylase (GAD65) mRNA. A: Low-power, bilateral dark field photomicrograph of an emulsion-dipped section hybridized with ³³P-labeled probes for 5-HT₄ receptor mRNA. B,C: Bright- (B) and dark- (C) field photomicrographs of the lateral septal nucleus where cells containing GAD65 mRNA (B, dark precipitate) or 5-HT₄ receptor mRNA (C, silver grains) are shown. D: Higher magnification image (obtained by blending the corresponding dark and bright field images) of the frame shown in B,C. Arrowhead: cell positive only for GAD65 mRNA; double arrowheads: doubly labeled cells. Scale bars: 0.2 mm in A, 100 μm in B, 20 μm in D.

in D. Abbreviations: cc: corpus callosum; CPu: caudate putamen; LSD: lateral septal nucleus, dorsal part; LV: lateral ventricle.

Fig. 10

Schematic drawing depicting the expression of 5-HT₄ receptor mRNA in the different neuronal populations present in the basal forebrain and its hippocampal and cortical targets that have been analysed in the present study. Known projections between the different cell populations have also been depicted. Colours indicate the different neurotransmitter phenotypes known to exist in these areas and identified in our study by in situ hybridization. Numbers close to the cell type or to the axonal projections indicate literature references. White dots within neuronal cell bodies indicate expression of 5-HT₄ receptor mRNA. Outlines of coronal brain sections were taken from the the atlas of Paxinos and Watson (1997). Abbreviations: Gra: granule cell layer of dentate gyrus; LS: lateral septal nucleus; MS-DB complex: medial septum-diagonal band complex; NbM: nucleus basalis of Meynert; PrFCx: prefrontal cortex; Pyr: pyramidal cell layer of hippocampus. References: 1: Mesulam et al 1983; 2: Woolf et al 1984; 3: Freund 1989; 4: Kiss et al 1990; 5: Gritti et al 2003; 6: Freund 1989; 7: Brauer et al 1991; 8: Gritti et al 1993; 9: Colom et al 2005; 10: Gritti et al 1997; 11: Manns et al 2001; 12: Hur and Zaborsky 2005; 13: Henny and Jones 2008; 14: Hajszan et al 2004; 15: Manseau et al 2005; 16: Leranth and Frotscher 1989; 17: Brauer et al 1998; 18: Pang et al 1998; 19: Zaborszky and Duque 2000; 20: Wu et al 2003; 21: Hajszan et al 2004; 22: Wu et al 2004; 23: Toth and Freund 1992; 24: Toth et al 1993; 25: Gaykema et al 1991a; 26: Zaczek et al 1979; 27: Alonso and Kohler 1982; 28: Leranth and Frotscher 1989; 29: Witter et al 1992; 30: Jaskiw et al 1991; 31: Vertes 2004; 32: Zaborsky et al 1997; 33: Gyengesi et al 2008; 34: Carr and Sesack, 1996

	ChAT		GAD65		Parvalbumin		vGLUT2	
	MS/VDB	HDB	MS/VDB	HDB	MS/VDB	HDB	MS/VDB	HDB
5-HT ₄ +/marker -	339	455	391	258	862	250	479	222
5-HT ₄ -/marker +	89	259	332	588	229	49	217	38
5-HT ₄ +/marker +	0	0	352	578	74	43	120	68

Table I. Total number of cells counted for each different neurochemical marker. For any given region and marker, numbers represent total number of cells that express the mRNAs indicated in 2-3 different tissue sections. Presence or absence of the corresponding mRNA is indicated by + and – respectively.

Figure 1
[Click here to download high resolution image](#)

Figure 1

Figure 2
[Click here to download high resolution image](#)

Figure 2

Figure 3
[Click here to download high resolution image](#)

Figure 3

Figure 4
[Click here to download high resolution image](#)

Figure 4

Figure 5
[Click here to download high resolution image](#)

Figure 5

Figure 6
[Click here to download high resolution image](#)

Figure 6

Figure 7
[Click here to download high resolution image](#)

Figure 7

Figure 8
[Click here to download high resolution image](#)

Figure 8

Figure 9
[Click here to download high resolution image](#)

Figure 9

Figure 10

Table 1

	ChAT		GAD65		Parvalbumin		vGLUT2	
	MS/VDB	HDB	MS/VDB	HDB	MS/VDB	HDB	MS/VDB	HDB
5-HT ₄ +/marker -	339	455	391	258	862	250	479	222
5-HT ₄ -/marker +	89	259	332	588	229	49	217	38
5-HT ₄ +/marker +	0	0	352	578	74	43	120	68

Table I. Total number of cells counted for each diferent neurochemical marker. For any given region and marker, numbers represent total number of cells that express the mRNAs indicated in 2-3 different tissue sections. Presence or absence of the corresponding mRNA is indicated by + and – respectively.