Immunohistochemical localisation of the serotonin 5-HT2B receptor in mouse gut, cardiovascular system, and brain

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Abstract **We recently reported the cloning of a new member of the serotonin 5-HT2 family, the 5-HT2B receptor. We now report the production and characterisation of a specific antiserum directed against the C-terminal portion of the mouse 5-HT2B receptor. After affinity purification, this polyclonal antibody recognises specifically the mouse 5-HT2B receptor. Immunohistochemical analysis of cryosections from various adult mouse tissues reveals a major 5-HT2B receptor expression in stomach, intestine and pulmonary smooth muscles as well as in myocardium. Furthermore, the antiserum recognises specific areas of the mouse brain, including cerebellar Purkinje cells and their projection areas.**

Key words: Antibodies; Cerebellum; Heart; Smooth muscle

1. Introduction

Over 95% of the total body serotonin (5-hydroxytryptamine, 5-HT) is synthesised in the gastrointestinal tract and stored in blood platelets, the brain containing only a minor proportion. Historically, the discovery of 5-HT in the brain shifted the major focus of attention to the central nervous system (CNS). However, interest continues to develop concerning its peripheral action on smooth muscle cells (SMC) in particular. Gaddum and Picarelli [1] suggested the presence of at least two distinct types of 5-HT receptor in the peripheral organs, since 5-HT contracts guinea pig ileum by (i) a direct action on SMC (D type) and (ii) an indirect action mediated by release of acetylcholine from parasympathetic nerves (M types). The peripheral D type has since been assimilated to the 'central 5-HT2 receptor'.

The wide variety of behavioural and physiological functions mediated by 5-HT is reflected in the numerous receptor subtypes. These receptors have been classified depending on their transduction mechanisms: the 5-HT1 and 5-HT5 subtype as adenylyl cyclase inhibitors, 5-HT2 as phospholipase C stimulators, and 5-HT4, 5-HT6, and 5-HT7 as adenylyl cyclase activators, are all members of the G protein-coupled receptors. The 5-HT3 subtype is a 5-HT gated channel [2].

5-HT2 receptors mediate many of the central and peripheral physiological functions of 5-HT. Cardiovascular effects include contraction of blood vessels and shape change in platelets; central nervous system effects are implicated in obsessive compulsive disorders, sleep, learning, memory, anxiety, appetite, depression, pain, sexual behaviour and mediation of hallucinogenic effects of lysergic acid diethylamide and related hallucinogens. Recently, this family has expanded to include 5-HT2A, 2B, and 2C subtypes.

The 5-HT2B receptor subtype is a potential target for therapeutic compounds, especially for the treatment of migraine [3] and cardiovascular diseases, and is active in the CNS (see [4] for review of potential action of the 5-HT2B receptor on the CNS). At the pharmacological level, the antagonists ritanserin and pizotifen have high affinity for the 5-HT2B receptor but are not selective. Some pharmacological properties of antagonistic compounds such as yohimbine relate this pharmacology to that of the 5-HT1B-1D subfamily. The group of T. Blackburn reported the synthesis of a first generation of selective antagonist, the SB200646A compound which possess high affinity for 5-HT2B and 5-HT2C but not 5-HT2A nor any other receptor tested [5]. This compound acts on hypolocomotion, hypophagia and anxiogenic paradigms [6]. A second generation of specific antagonists has recently been reported [7,8].

In the mouse, the major sites of expression for 5-HT2B are known, by RT-PCR experiment, to be present in adult stomach, intestine and heart, with detectable expression also in the brain and kidney [9]. In addition, expression of the 5-HT2B receptor is observed by in situ hybridisation, from day 8 of mouse embryonic development, in the neural fold and in the heart rudiment [10], and pharmacologically active during serotonergic differentiation of the mouse teratocarcinoma derived 1C11* cell lines [11]. Similarly, the human 5-HT2B receptor is widely expressed in the CNS as well as in the periphery [5,12,13]. Transcripts are detected by Northern blot in human liver and in lung, heart and kidney mRNA, with faint signals in the brain and placenta. Interestingly, we have also isolated the human 5-HT2B cDNA from spinal cord, foetal brain, aorta, internal mammary artery, placenta, neuroblastoma cell line SHSY-5Y, and CSH carcinoid tumour cDNA libraries [13]. The rat 5-HT2B receptor expression was shown to be restricted to rat stomach fundus [14,15]. However, recent reports indicate that this receptor is also expressed in rat blood vessels endothelium by pharmacological [16] and molecular studies [17].

We present here the characterisation of a subtype-specific antiserum directed against the 5-HT2B receptor. This antiserum reveals, for the first time, expression of the 5-HT2B receptor not only in several mouse peripheral tissues including gut and lung SMC, and myocardium, but also in the adult brain.

2. Materials and methods

2.1. Peptide synthesis, production and characterisation of antibodies

The peptide sequence was selected from the protein sequence, and

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corresponds to the C-terminal part of mouse 5-HT2B receptor. The sequence has the following 31 amino acid sequence: CSTIQSSSIILLDTLLTENDGDKAEEQVSYI. For immunisation, the peptide (2 mg) was coupled through cystein to ovalbumin (10 mg) as a carrier protein, using *m*-maleimidobenzoyl-*N*-hyroxysuccinimde (MBS) as the linking agent [18].

Male New Zealand White rabbits were inoculated with ovalbumin conjugated peptides following the published procedure [19]. After coupling of the peptide with the SulfoLink Coupling Gel (Pierce), antibodies were affinity-purified. After serum loading, the column was washed and antibodies were eluted with 0. 1 M glycine (pH 2. 8), and neutralised in the presence of 50 mM Tris-HCl (pH 9. 5). After protein quantitation using the Bradford method, the IgG were pooled, dialysed against PBS, and stored at −80°C until use.

2.2. COS cells transfection and immunocytohistochemistry

Cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and antibiotics (100 U/ml penicillin; 100 U/ml streptomycin). Cells were transfected for 3 days, using a modified calcium-phosphate method [20] with pSG-5 expression vector [21] containing the entire coding region of the mouse 5-HT2B receptor cDNA. Untransfected cells were used as a control. Cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and permeabilised with 4% PFA containing 0.1% Triton X-100 and 0.1% NP 40 for 10 min at RT. After overnight incubation with primary affinity-purified antibody (1:200), biotinylated anti-rabbit antibody (1:200, Vector laboratories) was added for 1 h, followed by Avidin-Biotin Complex (ABC; Vector laboratories) for 30 min at RT, and revealed by diaminobenzidine (DAB) colour reaction.

2.3. SDS-PAGE and Western blot analysis

For Western blot analysis, the membrane fraction was obtained by the following procedure: COS-1 transfected cells were homogenised in 1 ml TC (50 mM Tris-HCl, pH 7. 5, 4 mM $CaCl_2$) buffer and centrifuged for 15 min at 4°C, 12000 rpm. The pellet was resuspended in 0.5 ml of TC buffer; for tissue membrane fraction, frozen organs were homogenised in 10 volumes of 0.25 M sucrose at 4°C and centrifuged at $500\times g$ for 10 min. The supernatant was then centrifuged at $100000\times g$ for 30 min at 4°C. After quantitation by Bradford assay (Bio-Rad), the proteins were resolved by 12% SDS-PAGE [22] after denaturation with urea sample buffer (0.1 M Tris-HCl, pH 6. 8, 14 M urea, 1% SDS, 1% β-mercaptoethanol, 0.02% bromophenolblue). Proteins were transferred into nitrocellulose membranes [23]. After blocking in 5% non-fat dry milk in TBST (Tris-buffered saline; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h at RT with agitation, the membranes were incubated with affinity-purified antibody (1:500) overnight at 4°C. After incubation with peroxidase-conjugated donkey anti-rabbit antibody (1:5000, Jackson ImmunoRes. Lab.) for 1 h at RT, visualisation was achieved with the Enhanced Chemi-Luminescence (ECL; Amersham) method.

2.4. 5-HT2B immunohistochemistry

For preparation of frozen sections, 7 week old C57BL/6 mouse tissue was incubated in PBS for 20 min, transferred to plastic wells and embedded with OCT compound (Miles, Inc.), except the brain, which was first incubated in 2-methyl butane solution on dry ice for 15 min. All the preparations were stored at −80°C until sectioning. 10 μm thick frozen sections were cut on a cryostat, collected on gelatinised slides and kept at −80°C until use.

For immunohistochemistry, sections were air-dried, fixed with acetone (or 4% PFA) for 10 min at 4°C and transferred into PBST (phosphate-buffered saline and 0.1% Triton X-100). After incubation with 1% hydrogen peroxide in PBST for 30 min at RT and blocking with 1.5% NGS (normal goat serum) in PBST for 30 min at RT, the affinity-purified primary antibodies diluted 1/200 with PBST containing 10% ovalbumin, 1.5% NGS was added for 1 h at RT. For the control the same solution was pre-absorbed by addition of 10 μM synthetic peptides. After incubation with diluted biotinylated secondary antibody solution (1/200) for 1 h, and with ABC, the immunoreaction product was visualised with DAB or VIP substrate kit (Vector laboratories) for 5–10 min. Slides were counterstained with haematoxylin, dehydrated in ethanol, cleared with LMR solution (Labo-Moderne), cover-slipped with Eukitt (Mounting medium, Labonard), and examined with a Zeiss light microscope.

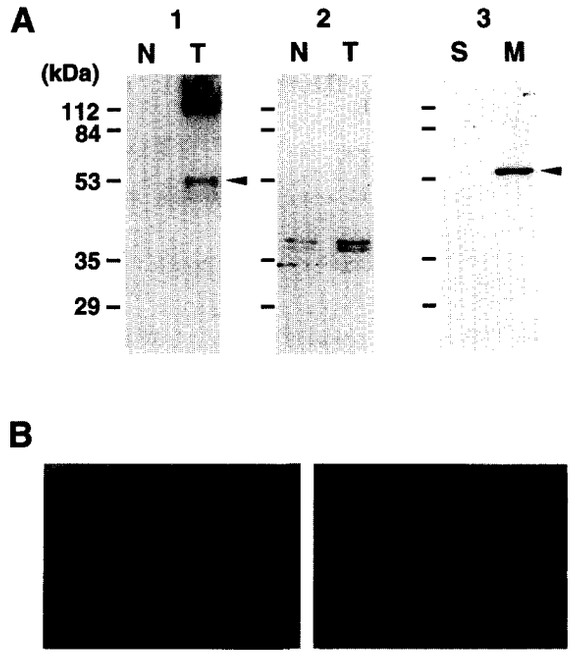

Fig. 1. Characterisation of the 5-HT2B receptor antiserum. A: Western blot analysis. Protein extracts from non-transfected (N) Cos-1 cells and transfected (T) with a 5-HT2B cDNA containing expression vector were used for blotting analysis. Panel 1 is the result of incubation of the membrane with 5-HT2B-specific antiserum at 1/500, whereas in panel 2 the same serum has been incubated in presence of the immunising peptide (10 μM), removing the specific band. The apparent molecular weight of the protein markers is indicated on the left and the specific protein band (53.6 kDa) is indicated by the arrow. The upper band (112 kDa) migrates with an apparent molecular weight of a dimer and is resistant to treatment by tunicamycin, *N*-glycosidase or alkylating agent (iodoacetamide). This band is specific to the 5-HT2B-transfected cells and probably represents receptor aggregates due to the high receptor expression level in Cos cells since it never appears with protein tissue samples (see below). Panel 3 shows the result obtained with protein extracts from adult mouse stomach, the soluble fraction (S) and the membrane fraction (M) after incubation with the 5-HT2B-specific antiserum, revealing one single immunoreactive band of the correct apparent molecular weight in the membrane fraction, which can be blocked by excess of the the immunising peptide (not shown). B: Immunocytochemistry of Cos-1 cells. Non-transfected (N) Cos-1 cells and transfected (T) with a 5-HT2B cDNA containing expression vector were used for immunocytochemistry. After fixation in 4% PFA, the cells were incubated with affinity-purified antibody (1/200), followed by ABC and revealed by DAB colour reaction. Only the transfected cells present immunoreactive signal.

3. Results

3.1. Production and characterisation of antisera to peptide sequence of the 5-HT2B C-terminus

Polyclonal antiserum was raised to ovalbumin conjugates of the synthetic peptide CSTIQSSSIILLDTLLTENDGKAEEQVSYI. This sequence corresponds to the C-terminal part of the mouse 5-HT2B receptor. The rationale for choosing this particular sequence was its specificity for this receptor subfamily since a screen of the protein data bank does not reveal any significant homology to known proteins except the human and rat 5-HT2B receptor sequences, scoring 77% and 83% of homology respectively. The reactivity of the antiserum to the peptide sequence was confirmed by immunobinding dot assays. We, then, characterised this antiserum using Cos-1

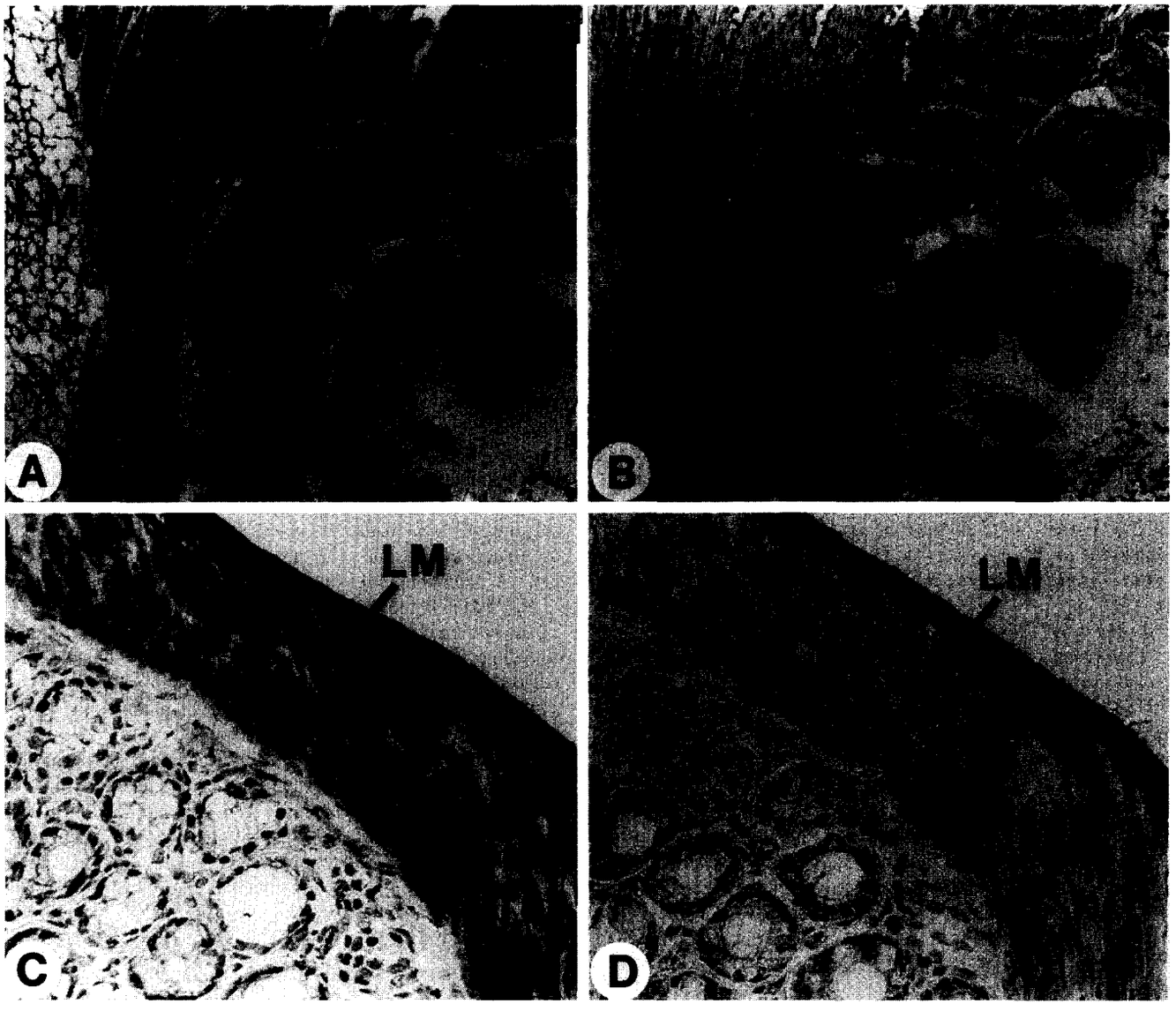

Fig. 2. Gastrointestinal distribution of the 5-HT2B receptor. Sections of mouse stomach (A, B) and intestine (C, D) were incubated with 5-HT2B antibodies alone (A, C) or in presence of an excess of immunising peptide (B, D) and revealed by VIP staining (A, B) or DAB (C, D), and counterstained with haematoxylin. The staining observed over the squamous epithelium in the control is not specific (B). The different smooth muscle layers are labelled, the muscularis mucosae (MM), the muscularis externa composed of the inner circular layer (CM) and the outer longitudinal layer (LM). All these different smooth muscle cell layers appear to express the 5-HT2B receptor. The original magnification is 100× (A, B) and 400× (C, D).

cells transiently transfected by receptor cDNA-containing pSG5 plasmid. Membrane fraction from 5-HT2B transfected Cos-1 (not from non-transfected cells or transfected by 5-HT1A, 1B, 2A, 2C receptor cDNAs [24]) are specifically recognised by this antiserum after immunoblotting (Fig. 1A) and revealed a 53 kDa band which fits with the expected 53.6 kDa size of the receptor. A higher molecular weight band (112 kDa) is resistant to treatment by tunicamycin or *N*-glycosidase. We therefore have no evidence for *N*-glycosylation of the mouse 5-HT2B receptor and the rat 5-HT2B receptor has no consensus sequence for *N*-glycosylation in its amino-terminus. This band is specific to the 5-HT2B-transfected cells and therefore probably represents receptor aggregates due to the high receptor expression level in Cos cells since it never appears with protein tissue samples (see below). Fixed 5-HT2B-transfected Cos-1 cells (not non-transfected cells) can be specifically recognised by the antiserum (Fig. 1B) and immunoreactivity is abolished by antiserum pre-absorption with homologous peptide prior to immunocytochemical procedures. On tissue sample, stomach membrane fraction presents a single strong immuno-reactive band of similar size (Fig. 1A-3) which can also be blocked by an excess of the immunising peptide (not shown).

The previous analysis of mouse 5-HT2B expression sites performed by RT-PCR showed a major site of expression for the mRNA in the gastrointestinal tissue [9]. We therefore used the 5-HT2B antiserum to refine this information and to investigate the exact sites of expression of the 5-HT2B receptor.

3.2. The 5-HT2B receptor is expressed in gut SMC

Immunohistochemistry performed on cryosections stained

by VIP or DAB reveals strong specific expression of the receptor in external cell layers of the stomach. This corresponds to SMC of the stomach and includes the muscularis externa with the outer longitudinal (LM) and the inner circular layer (CM) as well as the muscularis mucosae (MM) (Fig. 2A,B). Similarly, the large intestine expresses the 5-HT2B receptor in longitudinal and circular cell layers which again represents the different SMC layers (Fig. 2C,D). Strong expression can also be detected in oesophagus, duodenum, and ileum smooth muscles (Table 1).

3.3. The 5-HT2B receptor is expressed in lung and myocardium

The previous RT-PCR analysis of mouse 5-HT2B mRNA showed the other major site of expression in adult and embryonic lung and heart. When we used the 5-HT2B antiserum on sections of the lung region, we observed a strong staining of the bronchial SMC (Fig. 3A,B). When staining was performed on myocardium sections, the immunoreactivity was located in striated fibres similar to myocardial fibres, whereas the surrounding connective tissue was free from staining (Fig. 3C,D). Protein extracts from heart or lung tissue cannot be evidenced on Western blot probably due to a low expression level as suggested by the RNA amount [9].

3.4. The 5-HT2B receptor is expressed in the mouse brain

Expression of the 5-HT2B receptor mRNA was also detected in the mouse brain [9]. We therefore used the 5-HT2B antiserum on mouse brain sections. The strongest expression of the 5-HT2B receptor is in cerebellum. Closer examination of this expression indicates that Purkinje cells (Pu), granular cell layer (Gc), as well as interpositus cell bodies (Ip) are positive (Fig. 4). Therefore, the Purkinje cells, their axonal projections through the granular cells to the interpositus nuclei, and the interpositus nuclei (Fig. 4A,C) are positive for the receptor. This is in good agreement with the expression of the mRNA in the cerebellum, more precisely over the Purkinje cell layer seen by in situ hybridisation (unpublished).

4. Discussion

One important step in understanding the physiological roles of a specific subtype of receptor is a precise knowledge of the sites of expression. G-protein-coupled receptors are poorly antigenic and therefore their sites of expression are most frequently deduced from mRNA localisation (in situ hybridisation) or binding data on tissue fractions with specific radioligand. The 5-HT2 subfamily of receptors remain poorly understood in terms of expression since specific pharmacological compounds are not yet available for the different subtypes. We report here the characterisation of an antiserum specific for the 5-HT2B receptor and the localisation of its expression.

We developed a serum against a 5-HT2B-specific peptide corresponding to the C-terminus of the mouse 5-HT2B receptor. This serum appears monospecific to the 5-HT2B receptor protein on Western blot analysis in both Cos-transfected cells and stomach tissue membrane fraction (Fig. 1) which express a high level of 5-HT2B receptor mRNA [9]. When we stained sections of mouse tissue, we could reveal immunoreactivity on different organs and this expression corresponds to the mRNA detection (Table 1).

The 5-HT2B receptor expression is detected in most SMC of the gut from oesophagus, duodenum and stomach to intestine (Fig. 2, Table 1). This expression suggests that 5-HT2B receptors may participate in 5-HT-dependent gut contraction. The rat fundic strip has been known for a long time to be extremely sensitive to 5-HT [25]. However, the receptor which mediates the fundic SMC contraction has not been easy to characterise pharmacologically. The rat fundus receptor was first classified as a 5-HT1-like receptor and later as an orphan receptor. Only recently, the new subtype of 5-HT2 receptors, 5-HT2B, has been cloned from human, mouse and rat species [9,13–15]. It has been shown to mediate the contraction of the rat stomach fundus [26] and human intestine [27]. Therefore, this report confirms the 5-HT2B localisation and the probable

Table 1
Localization of serotonin 5-HT2B receptor in mouse

Organ	Tissue distribution	Immunoreactivity[a]	mRNA level[b]
Stomach	smooth muscle	++++	++++
Oesophagus	smooth muscle	+++	?
Duodenum	smooth muscle	+++	?
Intestine			
small intestine	smooth muscle	++	+++
large intestine	smooth muscle	++++	+++
Heart	myocardium	++	++
Lung			++
bronchiae	smooth muscle	++++	?
artery	smooth muscle	+++	?
	endothelium	++	?
Kidney	cortex	+	++
Adrenal gland	cortex	+	?
Liver		−	−
Spleen		−	−
Brain			+
cerebellum			++
	Purkinje cell	+	?
	granular cell layer	++	?
	interposed cellular nuclei	++	?
cerebrum	hippocampus	±	+

[a]Relative immunoreactivity was estimated based on colour intensity of staining.
[b]Relative mRNA expresssion level was determined by quantitative RT-PCR [9,34].

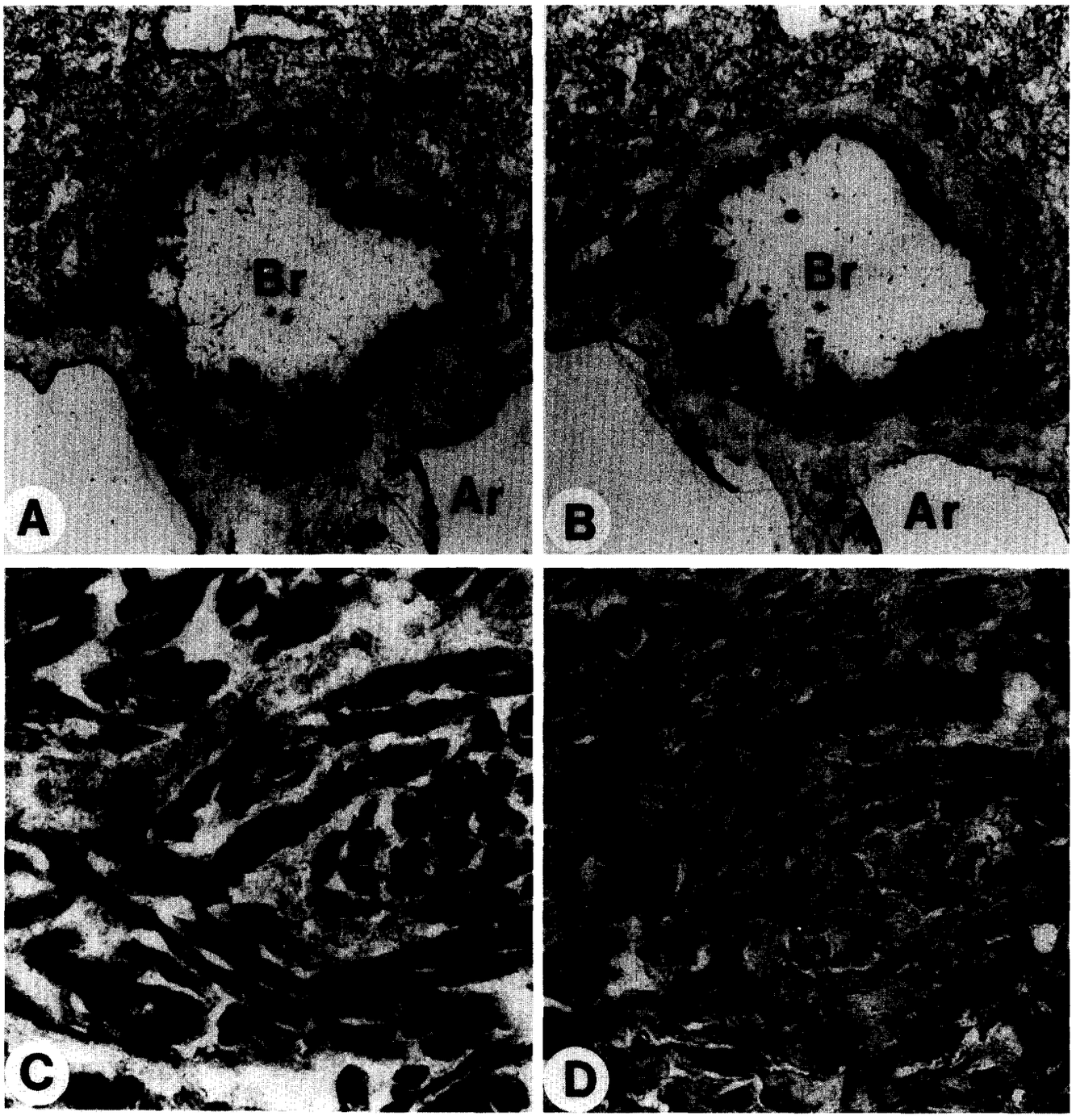

Fig. 3. Expression of the 5-HT2B receptor in the lung and heart. Sections of lung (A, B) and of heart (C, D) were incubated with 5-HT2B antibodies alone (A, C) or in the presence of an excess of immunising peptide (B, D) and revealed by VIP staining, and counterstained with haematoxylin. The smooth muscle cell layer surrounding the bronchial epithelium is indicated (SM) as well as the bronchial lumen (Br) and the artery lumen (Ar). The staining observed over the secreting epithelium in the control is not specific (B). Finally, muscle fibres of the myocardium are positively stained whereas the connective tissue is not (C, D). The original magnification is 200× (A, B) and 400× (C, D).

function of the 5-HT2B receptor in serotonin-mediated gut contractions. In addition, we detected mouse 5-HT2B receptor expression in bronchial SMC (Fig. 3). Bronchial expression of the 5-HT2B receptor may correspond to the 5-HT receptor implicated in bronchoconstriction, recently described as antagonistic of histamine action [28] and bronchial asthma [29]. The peripheral expression of 5-HT2 receptor subtypes is still a matter of controversy. If the 5-HT2C receptor is considered to be mainly restricted to the CNS, 5-HT2A receptor expression in the periphery has been reported in platelets, SMC of blood vessels [17,30] and myometrium [31], but not gut or lung [32]. Therefore the 5-HT2B receptor may represent a major 5-HT2-like receptor in SMC. In addition, we detected its expression in the cell line DDT-MFII, hamster

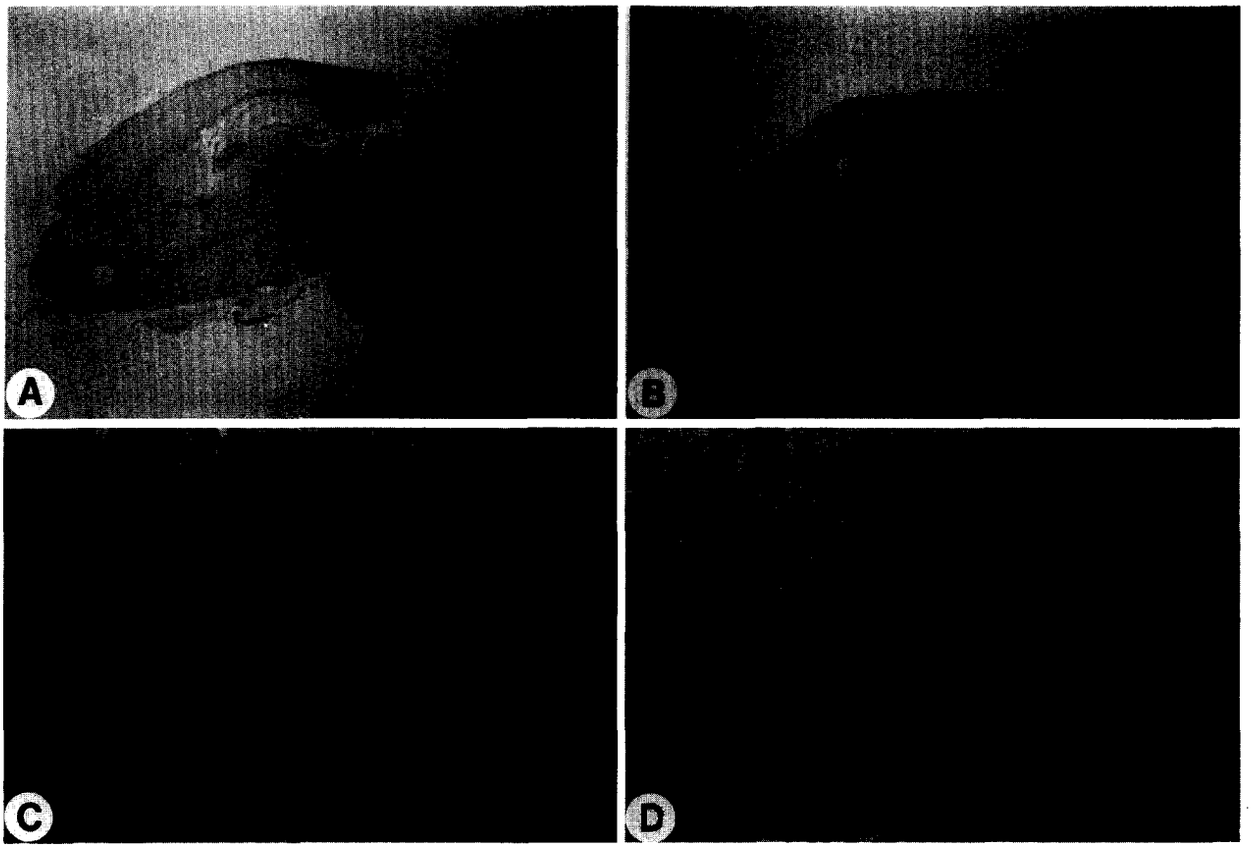

Fig. 4. Brain expression of the 5-HT2B receptor. Sections of mouse brain were incubated with 5-HT2B antibodies alone (A, C), or in the presence of an excess of immunising peptide (B, D) and revealed by DAB staining and counterstained with haematoxylin. The major staining is over the cerebellum region where cerebellar nuclei are positively revealed, more precisely the interpositus nucleus, shown by a bracket in A and B and at higher magnification in C and D. The Purkinje cells are stained (Pu), as well as their axonal projecting area in the interpositus nucleus (Ip) where cell bodies are also revealed. This seems to be correlated by the positive staining observed in the projecting area of the interpositus nucleus such as the red nucleus (not shown). The granular cells layer (Gc) is also stained and may correspond to projection from the lateral reticular nuclei or from dorsospino cerebellar tract innervation. The original magnification is 2× (A, B) and 400× (C, D).

vas deferens leiomyosarcoma, and in both human and Mastomys carcinoid tumours [24] which are derived from enterochromaffin cells of the gut.

The cardiovascular expression of the 5-HT2B receptor is detected in both the myocardium and the vascular endothelium (Fig. 3, Table 1). In the heart, function and distribution of 5-HT receptors have been shown to be complex and species dependent [33]. The presence of 5-HT2B receptors may explain partially this species dependence since the pharmacology of this receptor subtype shows a very strong species specificity [34]. In the myocardium, the 5-HT2B receptor may be similar to the one mediating the positive inotropic response to 5-HT in the atria [35]. In blood vessels, this expression may correspond to the 5-HT endothelial receptor (Fig. 3A) [17,36]. Serotonergic receptors are known to act in synergy with growth factors to mediate SMC contraction and proliferation. SMCs regulate gut contraction, blood pressure and participate in angiogenesis. The SMC proliferation is involved in coronary arterial diseases, hypertension and atherosclerosis, all of which are, amongst others, under serotonergic control. The contractile response of SMC involves intracellular Ca^{2+} release under the control of the second messengers IP3 and DAG released by phospholipase C and is controlled by phosphorylation of the myosin light chain (MLC) by the MLC kinase; its relaxation is induced by phosphatase activation regulated by intracellular cGMP and cAMP levels [37]. We have recently shown that, in addition to IP3 production, stimulation of the 5-HT2B receptor is mitogenic via Ras and MAP kinase stimulation and transforms the mouse fibroblast $LMTK^-$ cells [24]. Therefore, this receptor may be involved in MAP kinase stimulation in SMC where it may also be mitogenic. Furthermore, nitrogen oxide (NO), a positive effector of guanylyl cyclase, is a major vasodilator [38] and seems to be released from vascular endothelium [37]. Experiments indicating that the 5-HT2B receptor also regulates NO synthase activity by 5-HT in carcinoid tumour cells may also be related to NO functions in blood vessels (unpublished results). Although these results have to be refined, the localisation of the 5-HT2B receptor in blood vessel endothelium confirms the previous pharmacological and mRNA analysis [17,39]. We also detected the human 5-HT2B cDNAs in aortic and internal mammary artery libraries and the pharmacology of the 5-HT2B receptor correlates with that of the human saphenous vein [10]. The 5-HT2B receptor may, therefore, participate in the 5-HT endothelial-dependent vasorelaxation which is lost when endothelium is damaged [40]. In addition, the 5-HT2B receptor may participate in the serotonin-induced contraction of the cerebral arteries [41]. 5-HT and 5-HT2B/2C receptor

are suspected to play a role in migraine attacks [3,42], a pathology which results from abnormal 5-HT-induced vasoconstriction of the cerebral arteries.

Finally, the expression of the 5-HT2B receptor in the brain is mainly localised in cerebellar nuclei (Fig. 4). This expression may relate the 5-HT2B receptor to motor controls known to utilise 5-HT, such as the inhibition of spontaneous locomotor behaviour by the 5-HT2B/2C receptor or head twitches, wet-dog shakes, and back-muscle contractions by the 5-HT2A/2B receptor [43]. These observations may explain the hypolocomotion induced by the 5-HT2B-specific antagonist [6].

To conclude, we report for the first time the detailed sites of 5-HT2B receptor expression, detected by specific antiserum, in the cerebellum where it could be involved in motor control, in the gut or lung SMC where it probably regulates 5-HT-dependent contraction, and in heart myocardium where it could participate at the inotropic response to 5-HT. This work opens new possibilities for investigating the different physiological functions of 5-HT and to develop specific drugs affecting these functions.

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