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5-HT2B receptor-mediated serotonin morphogenetic functions in mouse cranial neural crest and myocardial cells

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Abbreviations: 2,5-Dimethoxy-4-iodophenyl-2-aminopropane: DOI; days post-coitum: d.p.c.; neural crest: NC; N-Acetyl-5-Hydroxytryptamine: NAS; Nile Blue sulphate: NBS; protein kinase C: PKC; phospholipase C: PLC; reverse transcriptase-PCR: RT-PCR; serotonin: 5-HT; serotonin transporter: SERT; transmission electron microscopy : TEM; tryptophan hydroxylase: TPH.

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SUMMARY

During embryogenesis, serotonin has been reported to be involved in craniofacial and cardiovascular morphogenesis. The detailed molecular mechanisms underlying these functions, however remain unknown. From mouse and human species, we have recently reported the cloning of 5-HT2B receptors which share signal transduction pathways with other 5-HT2 receptor subtypes (5-HT2A and 5-HT2C). In addition to phospholipase C stimulation, it appears that these three subtypes of receptor transduce a common serotonin-induced mitogenic activity, which could be important for cell differentiation and proliferation. We have first investigated the expression of 5-HT2 receptor mRNAs in the mouse embryo. Interestingly, a peak of 5-HT2B receptor mRNA expression was detected 8 to 9 days post-coitum, whereas there was only low levels of 5-HT2A and no 5-HT2C receptor mRNA expression at this stage. Expression of this receptor was confirmed by binding assays using a 5-HT2-specific ligand which revealed a peak of binding on membrane preparation from 9 days post-coitum embryos. In addition, whole mount in situ hybridisation and immunohistochemistry on similar staged embryos detected 5-HT2B expression in neural crest cells, heart myocardium, and somites. The requirement for functional 5-HT2B receptors between 8 and 9 days post-coitum is supported by culture of embryos exposed to 5-HT2-specific ligands; 5-HT2B-high affinity antagonist such as ritanserin, induced morphological defects in the cephalic

region, heart and neural tube. These antagonistic treatments interfere with cranial neural crest cell migration, induce their apoptosis, and are responsible for abnormal sarcomeric organisation of the subepicardial layer and for the absence of the trabecular cell layer in the ventricular myocardium. This report indicates for the first time that 5-HT_{2B} receptors are actively mediating the action of serotonin on embryonic morphogenesis, probably by preventing the differentiation of cranial neural crest cells and myocardial precursor cells.

INTRODUCTION

Serotonin (5-Hydroxytryptamine, 5-HT) is one of the well known monoamine neurotransmitters, mitogens, and hormones, which mediate a wide variety of physiological effect, including peripheral and central actions. The large variety of 5-HT functions is paralleled by the pharmacological complexity of 5-HT receptors which can be classified into different families depending on their signalling pathways. The family including 5-HT₁ and 5-HT₅ receptors interacts negatively with adenylyl cyclase; the 5-HT₂ receptor family is coupled to the activation of phospholipase C (PLC); the family, including 5-HT₄, 5-HT₆ and 5-HT₇ receptors, activates adenylyl cyclase, whereas the 5-HT₃ receptor is a ligand gated ion channel.

Recently, we have cloned the 5-HT_{2B} receptor cDNA from the mouse (Loric et al., 1992) and human (Choi et al., 1994). These receptors are functionally coupled to G_q and to the ras signalling pathway, and can be considered as a ligand dependent oncogene acting on protein kinase C (PKC) and MAPKinase activation (Launay et al., 1996). The development of a subtype-specific antiserum allowed a precise mapping of the distribution of the mouse 5-HT_{2B} receptors. In adult mice, the major sites of expression of 5-HT_{2B} receptors are the gut and heart, and there is also detectable levels of expression in the brain and kidney (Choi and Maroteaux, 1996). Interestingly, the expression of 5-HT_{2B} receptor mRNA is observed as early as 8 days post-coitum (d.p.c.) of mouse embryonic development (Loric et al., 1992). Additionally, the 5-HT₂ receptor homologue in *Drosophila* is expressed during gastrulation (Colas et al., 1995), and the 5-HT_{2B} receptors are also expressed during the serotonergic differentiation of the mouse teratocarcinoma derived 1C11* cell lines (Loric et al., 1995; Kellermann et al., 1996).

Several line of evidence indicate that 5-HT possess developmental functions (Lauder, 1988; Lauder, 1993). For instance, it has been shown that 5-HT is present early in mammalian embryonic development and is probably maternally derived (Yavarone et al., 1993a). Moreover in the mouse, the ability to take up 5-HT is detected at early embryonic stages (8.5 d.p.c., 10 somite-pairs) in the heart myocardium (Shuey et al., 1993) and in the rhombencephalic neuroepithelium where it is restricted to rhombomeres 3 and 5 (Shuey et al., 1993). The physiological relevance of these findings is stressed by the data showing that embryos grown in the presence of high levels of 5-HT or 5-HT uptake blockers develop deficient head mesenchyme, hypoplastic mandibular arches and forebrain, open cranial neural folds and abnormal eyes. These malformations overlap those observed in whole embryo culture following exposure of mouse embryos to 13-cis-retinoic acid (Lauder, 1988; Lauder et al., 1988). 5-HT has also been shown to participate in rat cranio-facial development (Van Cauteren et al., 1986) and to be involved in the formation and migration of the cranial neural crest (NC) cells (Moiseiwitsch and Lauder, 1995) as well as in chicken and mouse cardio-vascular morphogenesis (Huether et al., 1992; Yavarone et al., 1993b).

Here we used molecular biology, pharmacology and whole embryo culture techniques to present evidence for involvement of 5-HT_{2B} receptors in mouse embryonic development at the neurulation stage.

MATERIALS AND METHODS

RT-PCR and Binding Assay

Total RNA from 8 to 13 d.p.c. mouse embryos were isolated using guanidinium-thiocyanate method. Reverse transcriptase-PCR (RT-PCR) experiments were performed using specific oligonucleotides for mouse 5HT2A, 2B, 2C, receptors and for the 5-HT transporter (SERT) as follows:

5-HT2A-AAGCCTCGAACTGGACAATTGATG/AATGATTTTCAGGAAGGCTTTGGTT
5-HT2B-CAGAAGACATGTGATCACCTGATC/TGTAATCTTGATGAATGCAGTAGCC
5-HT2C-GTTTGGCAGTTTCGATATTCCATA/GGCAATCTTCATGATGGCCTTAGTC
SERT-ACATCTGGCGTTTTCCCTACAT/TTTTGACTCCTTTCCAGATGCT

To avoid the possible amplification of genomic DNA, oligonucleotides were designed in different exons. RT-PCR reaction was tested with different amounts of RNA (3, 4, 5 mg/50 ml reaction) to keep the reaction in the exponential phase for quantitative analysis of mRNA, and its specificity was verified by Southern blotting and hybridisation to a third primer.

Binding assays were performed with embryo membranes using [¹²⁵I]2,5-Dimethoxy-4-iodophenyl-2-aminopropane ([¹²⁵I]DOI), alone or in presence of ketanserin, mesulergine, ritanserin (10⁻⁸ M) as described (see Loric et al., 1992). Membrane extracts were also used for SERT quantification by binding experiments using [³H]paroxetine and for tryptophan hydroxylase (TPH) activity dosage by enzymatic assay according to published procedures (Buc-Caron et al., 1990).

Whole mount in situ hybridisation, immunohistochemistry

For whole mount in situ hybridisation, embryos were fixed with 4% paraformaldehyde followed by dehydration in serial dilutions of methanol and stored at -20°C in methanol until use. Hybridisation experiments were performed as described (see Wilkinson and Dourish, 1991), using whole 5-HT2B cDNA sense or antisense probe. An affinity-purified antiserum specific for 5-HT2B receptors was used for whole mount immunohistochemistry after fixation of the embryos with 3% paraformaldehyde, and then as described (see Mark et al., 1993; Choi and Maroteaux, 1996)

Whole embryo culture

CD1 strain mouse embryos were cultured as described (see Morriss-Kay, 1993). Briefly embryos were dissected in cold Tyrode's solution, and then placed in 30 ml sterile glass bottle with 2.5 ml rat serum, 2.5 ml Tyrode's buffer, 0.1 ml penicillin/streptomycin. The bottles were filled with a mixture of 90% nitrogen, 5% oxygen, 5% carbon dioxide. Embryos of 8 d.p.c. (around 5 somite-pairs) were incubated for 24 hours on a rotator wheel (30 revs min⁻¹) at 37.5°C (Bavik et al., 1996). Drugs were dissolved in water or ethanol and then diluted at least 1000X (5ml/5ml) in culture medium. The morphological analysis is based on that described in (New, 1990). Experiments have been performed at least 4 times with 4 to 5 treated embryos each.

Histological and electron microscopic analysis

Embryos were fixed by immersion in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) overnight at room temperature and washed in cacodylate buffer for further 30 min followed by post fixation with 1% osmium tetroxyde in 0.1 M cacodylate buffer for 1 hour at 4°C. Embryos were dehydrated through graded alcohol (50, 70, 90, 100%) for 30 min each and embedded in Epon 812. Semithin sections were cut at 1.5 μm and stained with toluidine blue, and histologically analysed by light microscopy. Ultrathin sections were cut at 70 nm and contrasted with uranyl acetate and

lead citrate, and examined with a Philips 208 electron microscope. For scanning electron microscopy, embryos were fixed, dehydrated as above, dried with critical point-drying apparatus, and the mounted on aluminium stubs coated with palladium-gold using a cold sputter-coater and observed with a Philips XL-20 microscope.

Nile Blue sulphate staining

Nile Blue sulphate (NBS) dye staining of apoptotic cells was performed as described in (Jeffs et al., 1992) except that phosphate buffer saline was used as incubation medium.

Embryo staging

The staging of the mouse embryos follows that described in (Hogan et al., 1994) with the following criteria: 8 d.p.c. corresponds to 1-7 somite-pairs, 8.5 d.p.c. to 8-12 somite-pairs (embryo turning), 9 d.p.c. to 13-20 somite-pairs, 9.5 d.p.c. to 20-30 somite-pairs and 10 d.p.c. to 30-35 somite-pairs.

RESULTS

5-HT2B receptors are highly expressed at 8 to 9 d.p.c.

Previous studies have indicated that different 5-HT2 receptors are expressed early during rodent embryonic development (Loric et al., 1992; Hellendall et al., 1993). We have refined these data by performing semi-quantitative RT-PCR on RNA extracts from staged embryos using a set of amplimers specific for the 5-HT2A, 2B and 2C receptors. A representative results of such experiments are shown in (Fig. 1 A, B). We observed a strong expression of 5-HT2B mRNA at 8 d.p.c. followed by a sharp decreased by 10 d.p.c.. In contrast, low levels of 5-HT2A mRNA were detected at 8 d.p.c. and raised slowly. The expression of 5-HT2C mRNA appeared only at 13 d.p.c. in parallel with a second wave of 5-HT2B mRNA expression. Interestingly, elevated levels of SERT mRNA and protein (as [3H]paroxetine binding sites) were detected from 8 to 10 d.p.c. (Table I-Fig. 1A). Binding experiments performed on protein extracts from similarly staged embryos, using [125I]DOI, a specific 5-HT2 radioligand, revealed a peak of specific binding at 9 d.p.c. (Fig. 1 C). The specific binding of [125I]DOI was completely blocked by ritanserin, partially reduced by mesulergine and totally insensitive to ketanserin. Given the respective affinity of these drugs for the different 5-HT2 receptors (Table II), these results indicate that the peak of [125I]DOI binding detected at 9 d.p.c. is mainly due to the expression of 5-HT2B receptors.

The 5-HT2B receptor protein is strongly expressed in brain and heart at the neurulation stage

Further refinement of the 5-HT2B receptor expression was obtained by studying its mRNA distribution by whole mount in situ hybridisation: Strong expression of the 5-HT2B receptor mRNA was detected at 8 d.p.c. in the neural fold, neural tube, and heart primordia (Fig. 2 A). This expression persisted in the cephalic region, neural tube and heart at 9 d.p.c. (Fig. 2 B). This mRNA expression was confirmed by immunostaining whole mount embryos using a 5-HT2B C-terminal-specific antiserum (Choi and Maroteaux, 1996): 5-HT2B receptor protein appears to be expressed in the somites, neural tube and heart region at 8.5 d.p.c. (Fig. 3 B, D), and persisted in the same regions with addition of the otic and optic vesicles, and pharyngeal arches at 9.5 d.p.c. (Fig. 3. F, H).

5-HT_{2B} receptor blocking between 8 and 9 d.p.c. induces embryonic defects

Early embryonic expression of 5-HT_{2B} receptors raised the possibility that these receptors are involved in 5-HT-dependent morphogenetic processes. We initiated, therefore, a series of experiments to test this hypothesis by performing whole embryo culture. Embryos at about the 5 somite-pairs stage were incubated for 24 hours in medium containing 1:1 rat serum/Tyrodes salt solution and various serotonergic drugs. Normal rat serum contains nearly micromolar amount of 5-HT (0.5-1 mM) and dialysed serum cannot support embryonic development in our experimental conditions, even supplemented by 1 mM 5-HT. Therefore, in order to block the action of 5-HT_{2B} receptors, we selected specific antagonists of the 5-HT₂ subtypes of receptors, ritanserin, methysergide, mesulergine, ketanserin and mianserin, for their differential affinity for these receptors (Table II). No defects were detected in embryos incubated in normal serum, whereas treatment with antagonist induced several reproducible embryonic defects which were characterised by optical microscopy (Fig. 4 A-C), and by scanning electron microscopy (Fig. 4 D-F). As shown in (Fig. 4 B, E) ritanserin treated embryos show a strong growth retardation as compared to controls (Fig. 4. A, D). The yolk sac circulation was also impaired as indicated by the formation of few blood islands. Moreover, the cephalic region showed apparent defects in flexure, and the forebrain, hindbrain, and the first pharyngeal arch presented abnormal development. In addition, the epicardial layer of the ventricular wall was swollen. Defects in embryonic turning, in somite number and shape, and in neural tube shape and closure were also frequently observed after ritanserin treatment (Fig. 4. B, E-Table III). Ritanserin induced these defects in all treated embryos at 1 mM concentration (Table III), whereas at 100 nM of ritanserin nearly 20% of the embryos were already affected (not shown). Defects could be visually detected at 6 hours after the beginning of treatment. Addition of 5-HT (Fig. 5-Table III) or of the highly-specific 5-HT_{2B} agonist N-Acetyl-5-HT (NAS) (Colas et al., 1996), prevented the onset of these defects (Table III). Interestingly, among the other 5-HT antagonists tested (Table III) only those having a high affinity for 5-HT_{2B} receptors (Table II), gave a similar phenotype to that of ritanserin. In contrast, the antagonists mianserin (not shown) and ketanserin which have a lower affinity for 5-HT_{2B} than for 5-HT_{2A} or 5-HT_{2C} receptors (Table II), gave a milder phenotype even at a concentration of 10 mM (Table III-Fig. 4 C, F), pointing out a 5-HT_{2B}-mediated action.

5-HT_{2B} receptor antagonists block cranial NC cell migration and/or differentiation

The defects induced by 5-HT antagonists were further characterised by light and transmission electron microscopy (TEM) on thin sections of these embryos. Toluidine blue stained sections showed that ritanserin-treated embryos have hypoplastic pharyngeal arches and an irregular neural tube with dilated blood vessels (Fig. 6B-H). It is important to point out that the reduced size of pharyngeal arches is indicative of impairment of NC cell migration and/or proliferation. The NC cells in the ritanserin-treated embryos, seemed not to migrate properly and remained in a more dorsal aspect of the cephalic region (Fig. 6 F) than in the control embryos (Fig. 6 E). Figure 7 presents higher magnification of the neural crest region of these sections: Although dividing cells can be seen in the epithelium, densely stained pyknotic nuclei, which are indicative of dying cells, were specifically observed in NC cells of the first pharyngeal arch and in the neuroepithelium near the optic stalk. Several characteristics of apoptosis were identified, cells presenting electron dense nuclei with condensed chromatin, dark cytoplasm and fragmented cells, whereas no necrotic cells could be evidenced after ritanserin treatment (Fig. 7 B-D). Counting the number of apoptotic NC cells on these sections gave an average of 52% of apoptotic cells in ritanserin-treated embryos whereas only 6% were observed in vehicle-treated embryos. In order to confirm the apoptotic nature of these cells we stained embryos with the NBS dye which has been frequently used to

visualise patterns of cell death during embryogenesis (Lumsden et al., 1991; Jeffs et al., 1992). The cephalic region of the ritanserin-treated embryos present extensive NBS staining over the pharyngeal arch 1, forebrain region and optic vesicle (Fig. 8B, D) in addition to the staining observed in control embryos over the rhombomeres 3 and 5 (Fig. 8A, C) already described (Graham et al., 1993; Graham et al., 1994; Lumsden and Graham, 1996). Furthermore, in treated embryos, the kinked neural tube and somites present more labelled cells than control embryos (Fig. 8C, D)

5-HT_{2B} receptor antagonist induces heart defects

Similar studies of the cardiac region were performed (Fig. 9). In ritanserin-treated embryos, the heart structure was disorganised (Fig. 9 B, D). In particular, the bulbo-ventricular groove was not well defined and the swollen atrio-ventricular canal showed reticulocytes accumulation indicating an inefficient circulation (Fig. 9 B). The subepicardial layer was thin and the cardiac trabecular cells were absent in the ventricle. Elongated cells, however, were present in this layer which normally contains myocardial trabecular stem cells (Fig. 9 D). TEM analysis of thin sections of ritanserin-treated embryos (Fig. 9E, F) indicated that this compound induces abnormal differentiation of myofilament sarcomeres in the subepicardial layer. These anomalies are not detected in vehicle-treated embryos at the same stage. This results suggest either a modification of the differentiation program of the myocardial stem cells and/or a deficient migration of the precursors of the trabecular cells.

DISCUSSION

The presence of 5-HT has been detected during early mammalian embryonic development (Lauder et al., 1988). We observed that the limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase (TPH) cannot be detected on embryos between 8 to 10 d.p.c. (Table I), substantiating its suspected maternal origin (Yavarone et al., 1993a). Similarly, we detected the presence of binding sites at 8 to 10 d.p.c. for the SERT-specific ligand [3H]paroxetine (Table I), corroborating the 5-HT uptake activity previously described at the 10 somite-pairs stage (8.5 d.p.c.) (Lauder et al., 1988) in the neuroepithelium and myocardium (Shuey et al., 1993). Furthermore, 5-HT has been shown to affect rat and mouse cranio-facial (Van Cauteren et al., 1986; Shuey et al., 1993) and chicken and mouse cardio-vascular morphogenesis (Huether et al., 1992; Yavarone et al., 1993b). Some of these effects have been reported to be mediated by 5-HT₂-like receptors (Van Cauteren et al., 1986; Huether et al., 1992).

We describe here, an extensive identification of 5-HT receptors present at the neurulation stage of early mouse embryogenesis. We have focused on the 5-HT₂ receptor family since the mitogenic activity of 5-HT has been linked to 5-HT₂ receptor-dependent stimulation of PLC/PKC: (i) 5-HT has mitogenic effects on NIH3T3 fibroblast expressing high density of 5-HT_{2A} or 5-HT_{2C} receptors (Julius et al., 1989), as well as on LMTK- cells expressing 5-HT_{2B} receptors which in response, form foci and induce tumours in nude mice (Launay et al., 1996). (ii) We have shown that, in these later cells, 5-HT_{2B} receptors are functionally coupled to IP₃ stimulation via the heterotrimeric alpha subunit of G_q and to the ras signalling pathway via the beta-gamma subunit of the same protein. These trigger the effects of 5-HT_{2B} selective agonists on PKC and MAPKinase activity in these cells (Launay et al., 1996). (iii) 5-HT acts on the serotonergic differentiation of the teratocarcinoma-derived cell line 1C11* which express 5-HT_{2B} receptors before 5-HT_{2A} receptors and regulates the final phenotype of these cells (unpublished; Loric et al., 1995; Kellermann et al., 1996). These overall properties strongly suggest that 5-HT_{2B} receptors mediate some trophic functions of 5-HT.

In the present study, we have observed that, by both mRNA analysis (Fig. 1 A, B) and receptor protein binding (Fig. 1C), the 5-HT_{2B} subtype is the major 5-HT₂ receptor expressed between 8 and 9 d.p.c.. Whole-mount *in situ* hybridisation (Fig. 2) and immunohistochemistry experiments (Fig. 3) reveal expression of this receptor on the neural tube, cranial neural crest cells, on somites, and in the heart tube at 8.5 d.p.c. (Fig. 3 B, D). At 9.5 d.p.c., the forebrain region, the pharyngeal arch 1 and the otic and optic vesicles are also positively labelled (Fig. 3 F, H). At a later stage of development, 11.5 d.p.c., the cardiac staining is restricted to the trabecular cells of the myocardium (not shown). Interestingly, the 5-HT_{2B} staining at 8.5 d.p.c. is observed in the region of migratory NC cells (Fig 3D), and the staining observed at 9.5 d.p.c. over the pharyngeal arch is suggestive of that of the cranial NC cells after migration. The 5-HT_{2B} receptor staining over the somites, neural tube and cranial neural crest is overlapping with that of the paired box gene Pax-3 (Goulding et al., 1993; Pourqui_ et al., 1995). This transcription factor is responsible for splotch phenotype in mice and the Waardenburg syndrome in human (Wehr and Gruss, 1996) which affect primarily neural crest cells derivatives and its gene has a similar localisation as the 5-HT_{2B} receptor on chromosome 2q36 (LeConiat et al., 1996).

5-HT_{2B}-specific antagonist treatments induce developmental defects

The treatment of embryos with high affinity 5-HT_{2B} receptor antagonist induce a highly reproducible phenotype which includes strong growth retardation (Fig. 4 B, E), abnormal flexure of the cephalic region, underdeveloped forebrain and hindbrain, small pharyngeal arches and distended epicardial layer (Fig 6, 8). Defects in embryo turning, in somite number and shape, and in neural tube shape and closure are also frequently observed after these treatments (Table III). This phenotype, including small brain and pericardial oedema, is very similar to that observed in the mouse mutated for the fused gene (Perry III et al., 1995), and in the knock-out mice phenotype of Ras GTPase-activating protein (Henkemeyer et al., 1995) and of the neurofibromatosis type-1 gene which is a regulator of the ras signal transduction pathway (Brannan et al., 1994). Finally, it has to be noticed that a human pathology, showing similar embryonic defects to those we observed, has been described in the embryos of phenylketonuria mothers (Lenke and Levy, 1980), and seems to be associated with low levels of blood 5-HT (Roux et al., 1995).

All the affected areas of the embryos express high levels of 5-HT_{2B} receptors (Fig. 2-3). In addition, the effects induced by ritanserin or mesulergine seem to be specific: First, morphological modifications induced by antagonist treatment are dose- (Table III) and time-dependent, and can be prevented by the treatment with NAS a highly selective 5-HT_{2B} receptor agonist or with 5-HT (Fig. 5-Table III); Second, the yolk sac circulation impairment is not directly involved since nearly normal embryos were associated with blood island in ritanserin plus 5-HT treatment (Table III); Third, a general toxic effects of these compounds can be excluded since the defects are tissue-specific and, despite the presence on sections of apoptotic cells, dividing cells can still be observed (see Fig. 7 B); Finally, the non-migrating NC cells are undergoing cell death with typical apoptotic but not necrotic morphology (Fig. 7 D). The possibility that ritanserin act at other 5-HT receptor subtypes cannot be completely excluded but is very unlikely since these compounds are typical 5-HT₂-specific ligands (Hoyer et al., 1994) ; The only known serotonin receptors having fairly high affinity for ritanserin are the rat 5-HT₆ and 5-HT₇ subtypes but their affinity for ritanserin is at least 10 times lower than that of mouse 5-HT_{2B} receptors (Boess and Martin, 1994).

5-HT_{2B} specific antagonists trigger differentiation of the heart myocardium

The 5-HT action on early embryonic development includes head mesenchyme and pharyngeal arch

formation, neural tube closure, eye and heart development, and is partially overlapping with retinoic acid teratology (Lauder, 1988). The ritanserin treatment of embryos induced a modification of the differentiation program of the myocardial stem cells and/or a deficient migration of the precursors of the trabecular cells (Fig. 9). The absence of trabeculation in the myocardium is similar to that reported in neuregulins knock-out mice (for review see Lemke, 1996), and in RXRa mutated animals (Kastner et al., 1994). In both cases, the thickness of the ventricular wall is markedly decreased. The retinoic acid-induced transcription factor AP-2, when knocked-out, also gives a similar cardiac phenotype (Shorle et al., 1996; Zhang et al., 1996). Since the promoter region of the 5-HT_{2B} receptor contains consensus binding-sequence for retinoic acid receptor and AP-2 (unpublished), we propose, therefore, that the 5-HT_{2B} receptors are interacting with these transcription factors.

5-HT_{2B} specific antagonists activate apoptosis in cranial NC cells

In mouse, the NC cells emerge by epitheliomesenchymal transformation (Newgreen and Minichiello, 1995), from epithelial cells on both sides of the neural tube at the neurulation stage (Le Douarin, 1982; Hall and H_{rstadius}, 1988). There is strong evidence that the cranial NC cells are actively migrating toward the developing pharyngeal arches and the fronto-nasal process (Morriss-Kay and Tan, 1987; Hall and H_{rstadius}, 1988; Selleck and Bronner-Fraser, 1996). Here, they differentiate into mesenchymal derivatives such as bone, cartilage and muscles (Le Douarin, 1982; Hall and H_{rstadius}, 1988). Several factors have been implicated in stimulation and guidance of NC migration including cell adhesion molecules (tenascin, fibronectin, laminin) and their receptors (integrins) (Bronner-Fraser, 1993). Humoral factors such as growth factors (Le Douarin et al., 1993; Selleck and Bronner-Fraser, 1996) or neurotransmitters are also implicated in NC migration. Evidences that 5-HT and some of its receptors participate in these processes have also been reported (Moiseiwitsch and Lauder, 1995).

In our experiments of embryos treatment with 5-HT_{2B} antagonist, widespread cell death was detected in the hindbrain, in cranial NC and in disorganised somites (Fig. 7, 8). The site of expression of the 5-HT_{2B} receptor in cranial NC cells and the phenotype observed after antagonist treatment, where NC cells present induced-cell death with typical apoptotic nuclei (Fig. 7 D, F), suggest a role for 5-HT_{2B} receptors in NC migration, cellular proliferation and/or survival. One possibility is the involvement of 5-HT in the formation of cranial NC cells by epitheliomesenchymal transformation which has been shown to be dependent on PKC activity (Newgreen and Minichiello, 1995), and is under the control of some members of the transforming growth factor β superfamily (Selleck and Bronner-Fraser, 1996). Conversely, 5-HT is involved in cranial NC cell migration (Moiseiwitsch and Lauder, 1995), and previous experiments have suggested the involvement of a 5-HT₂-like receptor since the migration of mesenchymal cells can be blocked by methysergide (Lauder and Zimmerman, 1988). The contribution of 5-HT_{2C} receptors in these effects can nevertheless be ruled-out, since 5-HT_{2C} knock-out mice do not show any developmental deficits (Tecott et al., 1995). Interestingly, we have detected SERT mRNA (Fig. 1) and binding (Table I) in extracts from embryos at 8-10 d.p.c. and SERT uptake activity has been localised in the neuroepithelium at the 12 somite-pair stage in rhombomeres r3 and r5 (Shuey et al., 1993). These data along with the observation that apoptotic NC cells are present in the same rhombomeres of chicken embryos at the same stage (Graham et al., 1993; Lumsden and Graham, 1996) indicate that rhombomeres r3 and r5 are associated with apoptotic NC cells and that local uptake activity reduces the levels of 5-HT. It is therefore, tempting to speculate that treatment by 5-HT_{2B}-antagonist mimics the absence of 5-HT stimulation resulting normally from the local uptake of 5-HT and therefore generalises the apoptosis of NC cells (Fig. 8). This would predict that

5-HT acting on the 5-HT_{2B} receptors exert a trophic action on cranial NC cells to prevent the induction of programmed cell death.

In conclusion, our data indicate that 5-HT_{2B} receptors participate in the regulation of cranial NC cells, by affecting their migration, cellular proliferation and/or survival, as well as migration and/or inhibition of cellular differentiation of the heart myocardium during early mouse embryogenesis. Investigations with gene targeted (knockout) mice should further validate these findings about the functions of 5-HT_{2B} receptors during early embryonic development.

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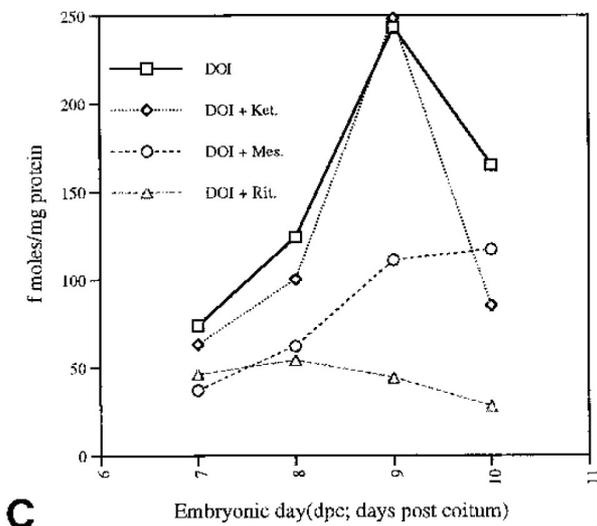
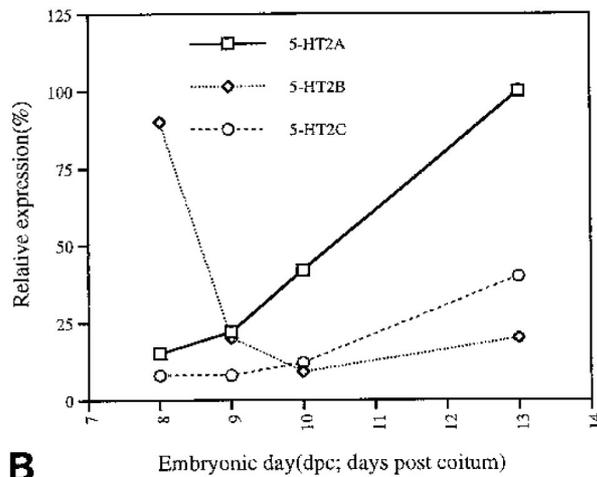
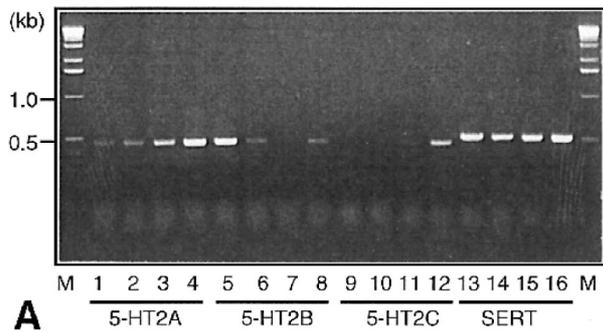


Fig. 1. 5-HT2B receptors are expressed at 8 and 9 days post coitum. (A) mRNA expression levels of 5-HT2 receptors and SERT. The expression pattern of 3 different subtypes of 5-HT2 receptors and SERT during mouse embryogenesis (8, 9, 10, 13 d.p.c.) have been analysed by quantitative RT-PCR: 8 d.p.c.-lane 1, 5, 9, 13; 9 d.p.c.-lane 2, 6, 10, 14; 10 d.p.c.-lane 3, 7, 11, 15; 13 d.p.c.-lane 4, 8, 12, 16. (B) Densitogram analysis of quantitative RT-PCR, 100% represent the highest level of expression (5-HT2A at 13 d.p.c.). 5-HT2A mRNA: squares, 5-HT2B mRNA: diamonds, 5-HT2C mRNA: circles. (C) Specific binding of [125I]DOI to 5-HT2 receptors alone (squares) or in the presence of 10⁻⁸ M of Ketanserin (Ket-diamonds), Mesulergine (Mes-circles) or Ritanserin (Rit-triangles).

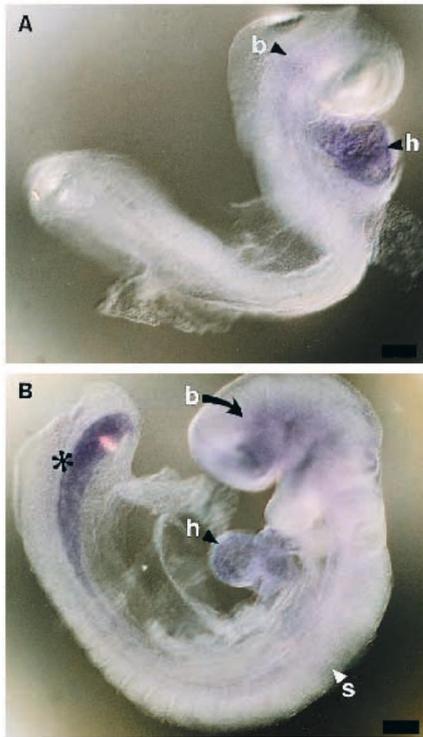


Fig. 2. 5-HT2B receptor mRNA is expressed in brain and heart region. Whole mount in situ hybridisations were performed using digoxigenin-labelled 5-HT2B antisense RNA and show that 5-HT2B receptors expression is mainly localised in brain and heart regions. (A) 8 d.p.c. embryo. Scales bar = 50 μm (B) 9 d.p.c. embryo. Scale bar =100 μm . Abbreviations; b- cephalic neural plate region, h- heart region, s- somites. *: labelling of the caudal region including hind gut which was sometime observed with the sense probe and therefore may be non-specific.

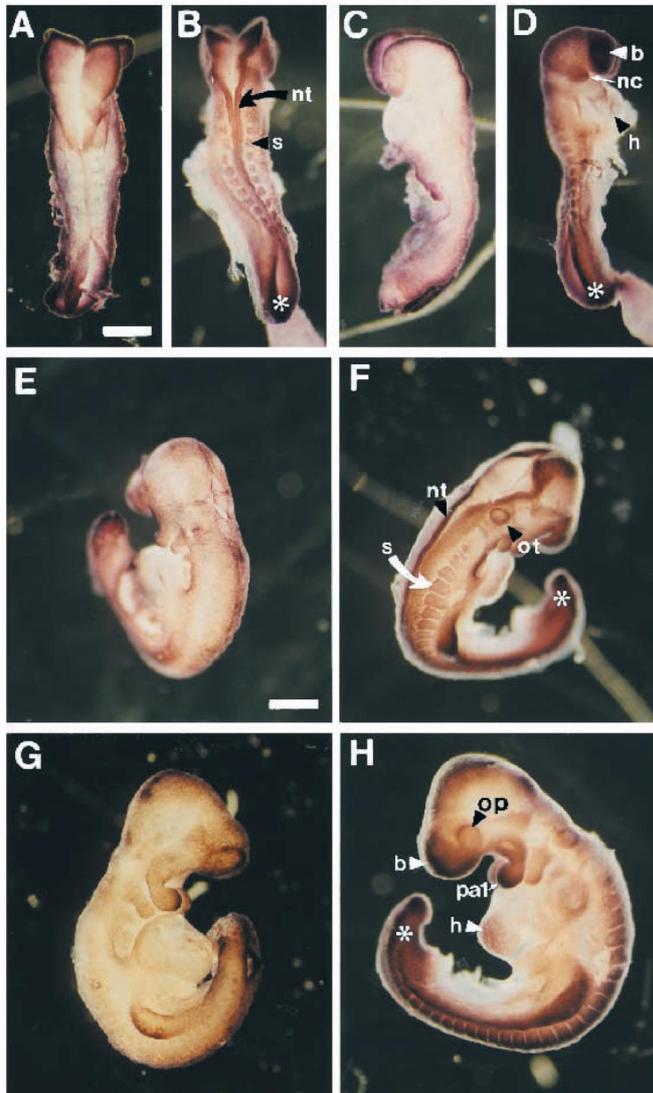


Fig. 3. 5-HT2B receptor protein is expressed in forebrain, heart, neural tube, and somites, at 8.5 and 9.5 d.p.c..

Whole mount immunohistochemical localisation using an affinity purified antibody directed to the C-terminal peptide sequence of the 5-HT2B receptor (B, D, F, H) reveals expression of the receptor over the neural tube, pharyngeal arch 1 and heart. (A, B, C, D) 8.5 d.p.c. embryos, (A, C) Control embryos performed by incubation in presence of the 5-HT2B antiserum with an excess of the immunising peptide. (E, F, G, H) 9.5 d.p.c. embryos, (E, G) Control embryos. Scale bar=200 μ m. Abbreviation: nt- neural tube; s- somites; b- forebrain region, h- heart; nc- neural crest cells ot- otic vesicle; op- optic vesicle; pa1- first pharyngeal arch; *-staining of caudal region sometime revealed in the control embryos and therefore may be non-specific .

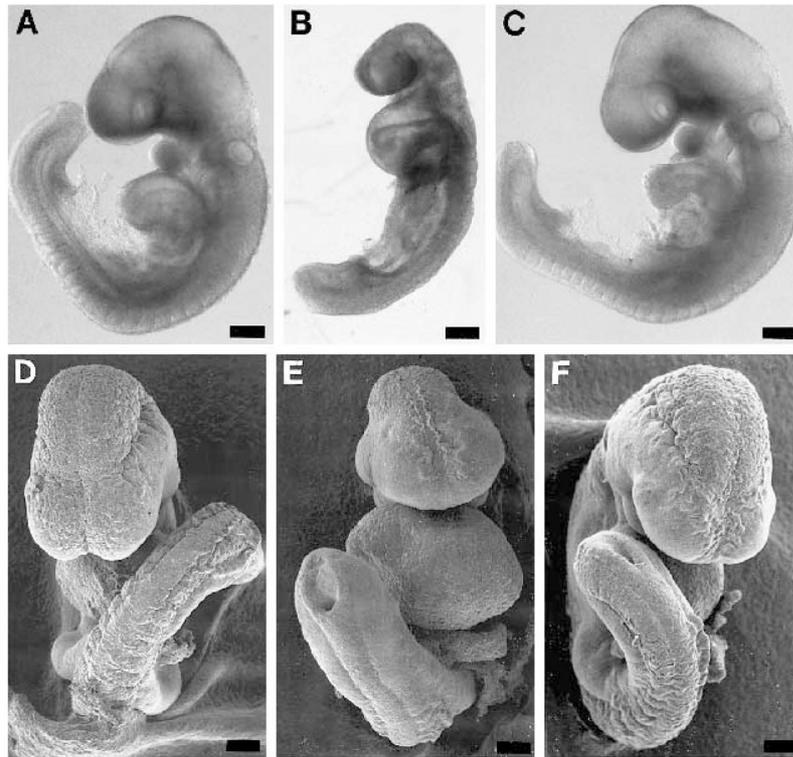


Fig. 4. Morphological analysis of whole embryo culture using light and scanning electron microscopy. CD1 strain mouse embryos were cultured with different 5-HT₂-specific antagonists from 8 to 9 d.p.c. (5 to 20 somite-pairs) for 24 hours. (A, D) Control embryos. (B, E) Ritanserin (1 mM) treated embryos showing morphological defects: The brain, the pharyngeal arches, and the somites are not properly developed, the heart pericardium is swollen and the neural tube is not closed. (C, F) Ketanserin (10 mM) treated embryos show a nearly normal development except over the hindbrain region. Scales bar; (A, B, C)- 145 μ m, (D, F)- 130

μ m, (E)- 100 μ m

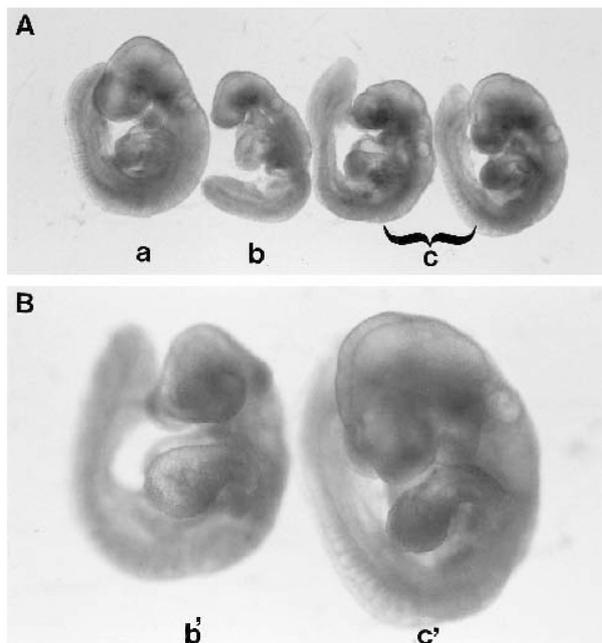


Fig. 5 . Ritanserin-induced morphological defects can be prevented by addition of 5-HT. (A) Comparison of the different phenotypes: a- control embryo; b- ritanserin (0.5 μ M) treated embryo showing morphological defects in cephalic and heart region; c-addition of 5-HT (10 mM) to ritanserin (0.5 μ M) treated embryo showing partial morphological improvement. (B) Comparison between ritanserin and 5-HT plus ritanserin treated embryos: b', c' are the enlargement of b, c and the 5-HT plus ritanserin-treated embryos present a phenotype close to the untreated embryo.

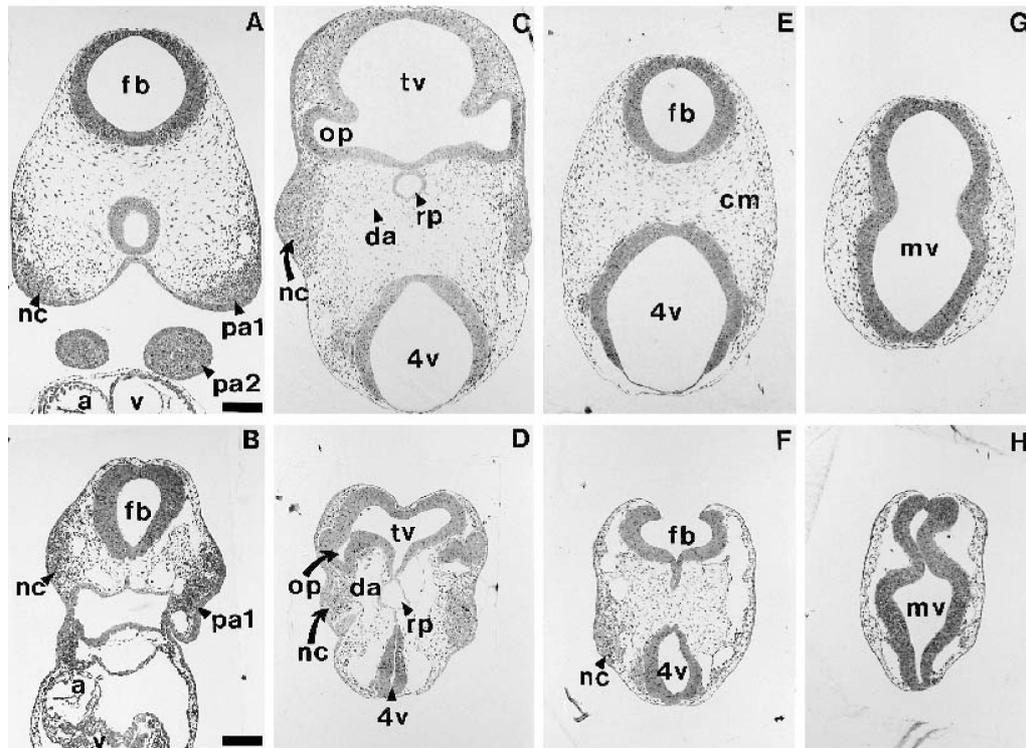


Fig. 6.

Histological analysis of the cephalic region of cultured embryos. Transverse thin sections of control embryos (A, C, E, G) and of ritanserin treated embryos (B, D, F, H) have been performed after fixation and embedding in Epon. The treated embryos shows irregular organisation of the neural tube which is not completely closed (F, H); The fourth ventricle is reduced and blood vessels are dilated; NC cells are observed at higher position than in the control (F) and the pharyngeal arches are not well formed (B). The magnification is the same for all the sections, scale bars; (A, B)- 150 μ m. Abbreviations; a- atrium, cm- cephalic mesenchyme, da- dorsal aorta, fb- forebrain, mv- mesencephalic vesicle, nc- neural crest, op- optic vesicle, pa- pharyngeal arch, rp- Ratke pouch, tv- telencephalic vesicle, v- ventricular chamber, 4v- fourth ventricle.

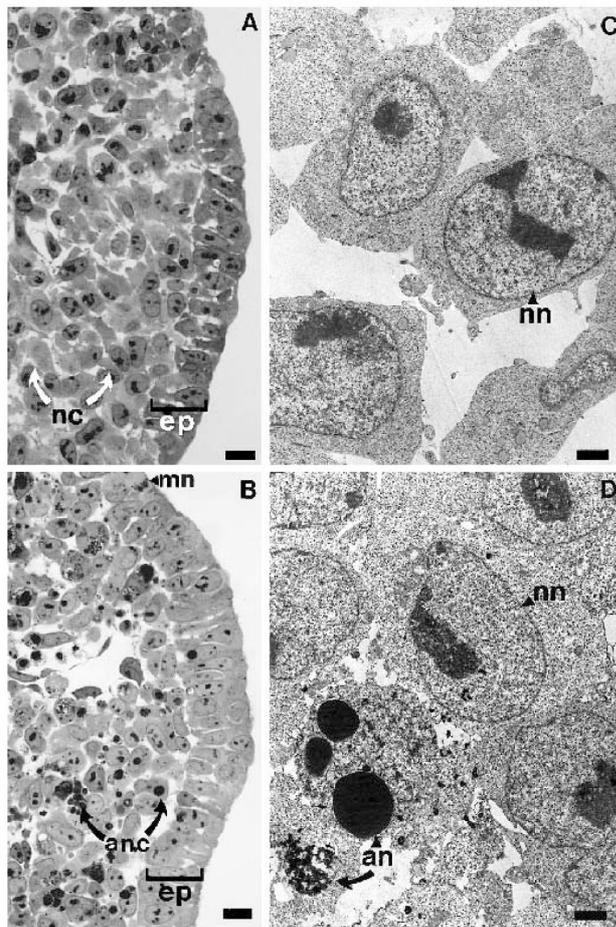


Fig. 7. Histological and electron microscopical analysis of the NC region of cultured embryos. In control embryos (A, C) the NC cells present a normal morphology. In the ritanserin (1mM) treated embryos (B, D), the morphology of NC cells is modified, with several fragmented nuclei and nuclei with condensed chromatin whereas mitotic nuclei are visible in the epithelium. At the EM level, several characteristics of apoptosis can be identified, including the appearance of electron dense chromatin masses, convolution of nuclear membrane, cell shrinkage and darkening of the cytoplasm. Apoptotic bodies containing intact organelles and fragmented chromatin can be observed. Some cell fragments are phagocytosed by macrophages or neighbouring cells. Scale bars; (A, B)- 10 μ m, (C, D)- 1 μ m. Abbreviations: an- apoptotic nucleus, anc- apoptotic NC cells, ep- epithelium, mn- mitotic nuclei, nc- neural crest, nn- normal nucleus.

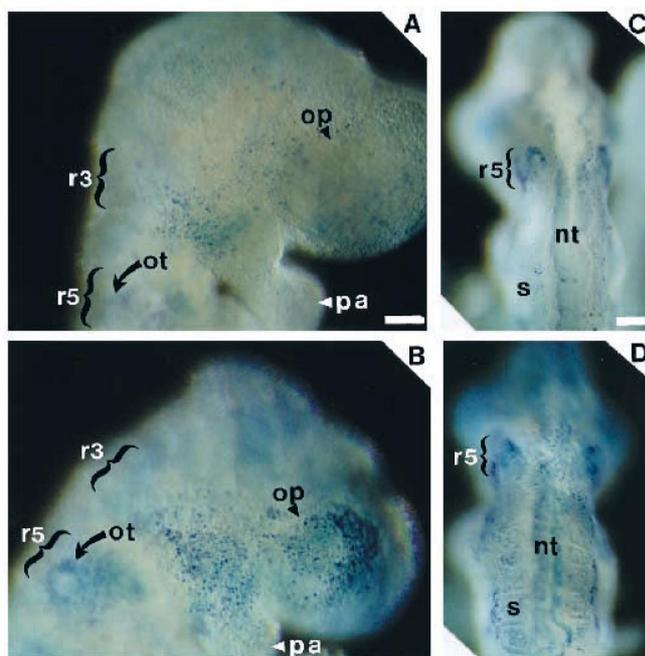


Fig. 8. Cephalic region of embryos labelled with NBS dye. Control embryos (A, C) and ritanserin (1mM) treated embryos (B, D) have been stained using NBS dye, a procedure that has been used to visualise apoptotic cells during embryogenesis (Lumsden et al., 1991; Jeffs et al., 1992). The known regions of apoptotic NC cells are visualised at the level of rhombomeres 3 and 5 in both treated and control embryos whereas in the ritanserin-treated embryos, extensive labelling appears over the pharyngeal arch, in the forebrain region (B), and over the neural tube and somites (D). The magnification is the same in A and B, and in C and D, respectively, and the scale bars represent 160 μ m. Abbreviations: nt- neural tube, op- optic vesicle, ot- otic vesicle, pa- pharyngeal arch 1, r3- r5- rhombomeres 3 and 5, s- somites.

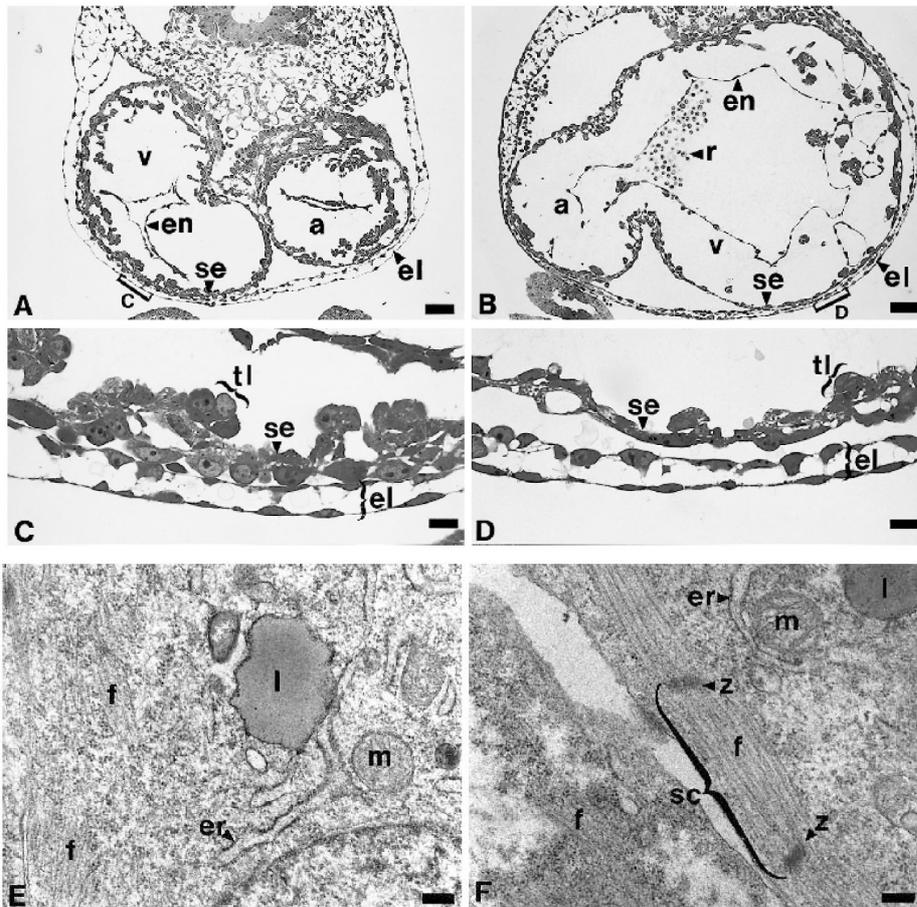


Fig. 9. Histological and electron microscopical analysis of the heart region of cultured embryos. Control embryos show normal subepicardial layer with round cells (A, C, E), whereas ritanserin (1mM) treated embryos (B, D, F) have inflated heart with defects of trabecular layer formation and distended endocardium with accumulation of reticulocytes. The subepicardial layer contains elongated cells presenting myofilaments with sarcomeric organisation (F) which not yet organised in the control (E). Scales bar; (A, B)- 50 μ m, (C, D)- 10 μ m, (E, F)- 0.14 μ m. Abbreviations: a- atrial chamber, el- epicardial layer, en- endothelium, er- rough endoplasmic reticulum, f- myofilaments, l- lipid drops, m- mitochondria, r- reticulocytes, sc- sarcomeric organisation, se- subepicardial layer, tl- trabecular layer, v- ventricular chamber, z- z band.

Table I.
Serotonergic properties of embryonic extracts

Embryonic stage	Paroxetine binding sites (fmoles/mg prot.)	Tryptophan hydroxylase (fmoles/hours/mg prot.)
8 d.p.c.	89.6	<0.3
9 d.p.c.	88.7	<0.3
10 d.p.c.	86.8	<0.3
13 d.p.c.	61.7	1.4

Extracts of embryos at various stages have been analysed for the presence of SERT sites using [³H]paroxetine as labelled compound for binding experiments on membrane fractions, and for TPH enzymatic activity dosage, according to published procedures (see Buc-Caron et al., 1990).

Table II.
Binding affinity of several antagonists to 5-HT₂ receptors

Receptors	Antagonist				
	Ritanserin	Methysergide	Mesulergine	Ketanserin	Mianserin
5-HT _{2A}	9.28±0.12	8.58±0.13	8.21±0.07	8.74±0.21	8.09±0.18
5-HT _{2B}	8.39±0.17	7.86±0.13	7.67±0.22	6.74±0.15	6.42±0.10
5-HT _{2C}	8.68±0.08	8.25±0.10	8.77±0.18	6.90±0.15	7.91±0.24

Data correspond to pK_i values (-logK_i) obtained by competition of [¹²⁵I]DOI binding with various compounds on membranes from mouse L cells transfected with mouse 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C} receptor cDNA. Values represent K_i±s.e.m. of experiments performed three times in triplicate.

Table III.
Summary of Whole Embryo Culture

Treatment	Concentration of drug(μ M)	Number of tested embryos	Yolk Sac circulation	Morphological Defects (%)			
				Brain	Heart	Neural Tube	Growth
Vehicle	none	20	-	0(0%)	0(0%)	0(0%)	0(0%)
Ritanserin	0.5	20	++	16(80%)	18(90%)	15(75%)	11(55%)
	1.0	20	++	20(100%)	20(100%)	18(90%)	18(90%)
Methysergide	10	16	+	9(56%) ¹	9(56%)	6(37%)	5(31%)
Ketanserin	10	20	-	18(90%) ²	2(10%)	2(10%)	2(10%)
Ritanserin +5-HT	0.5 10	18	++	8(44%)	5(27%)	4(22%)	3(16%)
Ritanserin +NAS	0.5 10	16	+	5(31%)	7(43%)	5(31%)	4(25%)

Cultures of CD1 strain mice embryos were started at 8 d.p.c. (4-9 somite-pairs) and incubated for 24 hours in 5 ml culture medium (50% rat serum, 50% Tyrode's saline) containing about 0.5-1 mM endogenous 5-HT. The number of treated embryos represents at least four independent experiments where 4-5 embryos have been treated at a time. The morphological analysis is based on that described in (New, 1990). The yolk sac observation corresponds to blood island formation after treatment, - not detected, + occasional blood island, ++ dispersed but reproducible blood island. The brain defects are mainly microcephaly, 1: Defects of forebrain -3 (19%) and hindbrain-6 (37%), 2: Defects in hindbrain only. The heart defects are essentially distended pericardium with defect in chambers formations. The neural tube defects correspond to irregular and often kinked neural tube. Finally, the growth defect is reduced size of the embryos.