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Serotonin regulates mouse cranial neural crest migration

(neurotransmitters/craniofacial development/morphogenesis/receptors)

JULIAN R. D. MOISEIWITSCH*† AND JEAN M. LAUDER*‡

*Department of Cell Biology and Anatomy, School of Medicine, and †Department of Endodontics, School of Dentistry, CB#7090, University of North Carolina, Chapel Hill, NC 27599-7090

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ABSTRACT **Serotonergic agents (uptake inhibitors, receptor ligands) cause significant craniofacial malformations in cultured mouse embryos suggesting that 5-hydroxytryptamine (serotonin) (5-HT) may be an important regulator of craniofacial development. To determine whether serotonergic regulation of cell migration might underly some of these effects, cranial neural crest (NC) explants from embryonic day 9 (E9) (plug day = E1) mouse embryos or dissociated mandibular mesenchyme cells (derived from NC) from E12 embryos were placed in a modified Boyden chamber to measure effects of serotonergic agents on cell migration. A dose-dependent effect of 5-HT on the migration of highly motile cranial NC cells was demonstrated, such that low concentrations of 5-HT stimulated migration, whereas this effect was progressively lost as the dose of 5-HT was increased. In contrast, most concentrations of 5-HT inhibited migration of less motile, mandibular mesenchyme cells. To investigate the possible involvement of specific 5-HT receptors in the stimulation of NC migration, several 5-HT subtype-selective antagonists were used to block the effects of the most stimulatory dose of 5-HT (0.01 μM). Only NAN-190 (a 5-HT_{1A} antagonist) inhibited the effect of 5-HT, suggesting involvement of this receptor. Further evidence was obtained by using immunohistochemistry with 5-HT receptor antibodies, which revealed expression of the 5-HT_{1A} receptor but not other subtypes by migrating NC cells in both embryos and cranial NC explants. These results suggest that by activating appropriate receptors 5-HT may regulate migration of cranial NC cells and their mesenchymal derivatives in the mouse embryo.**

The cranial neural crest (NC) gives rise to most of the mesenchyme (also known as ectomesenchyme) of the craniofacial region (1, 2) and contributes to the endocardial cushions of the heart (3, 4). In the mouse, NC cells start to emerge from the lateral borders of the neuroepithelium or neural folds during early stages of neurulation (5, 6). Although a passive distribution of cranial NC in the mouse has been suggested (7), there is evidence that these cells actively migrate toward the developing pharyngeal arches and frontonasal process (8). Here, they differentiate into mesenchymal derivatives, such as bone, cartilage, dentin, and dental pulp (2, 3, 5).

A number of different signals have been implicated in stimulation and guidance of NC migration, including growth factors, extracellular matrix (ECM) molecules, and their receptors. Using the chick as an experimental model, Bronner-Fraser and others have investigated the involvement of several ECM molecules in NC migration, including tenascin, fibronectin, laminin, and several types of collagen, as well as integrins, which act as receptors for some of these molecules (see ref. 9 for review). From these studies it is apparent that many of these actions are mediated by β_1 integrin subunits that are expressed on the cell surface of migrating NC.

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Although less well studied, humoral factors may also regulate or modulate NC migration. Increasing evidence suggests that blood-borne factors, in particular neurotransmitters, may play important roles in the development of craniofacial structures and endocardial cushions of the heart. Specifically, 5-hydroxytryptamine (serotonin) (5-HT), which appears to reach the mouse embryo from maternal sources (10), has been shown to influence development of craniofacial and cardiac mesenchyme in a dose-dependent manner (11). By employing mouse whole-embryo culture (WEC) (12), Lauder and colleagues (13–15) demonstrated the transient expression of low-affinity sites for 5-HT uptake and degradation in developing craniofacial epithelia that exhibited a striking spatiotemporal correlation with key morphogenic events in this region. Recently, the 5-HT uptake transporter has been localized in developing craniofacial mesenchyme of the mouse (16), where it could help to mediate the morphogenetic effects of 5-HT by transporting this neurotransmitter toward epithelial 5-HT uptake sites. Immunohistochemistry with either antibodies to the 5-HT_{1A} receptor (17) or an anti-idiotypic antiserum (which stains most 5-HT receptor subtypes except 5-HT_{1A}; ref. 18) has revealed widespread expression of both 5-HT_{1A} and non-1A subtype receptors in the craniofacial mesenchyme of embryonic day 12 (E12) (plug day = E1) mouse embryos (19). Moreover, WEC studies have demonstrated that craniofacial malformations result from exposure to 5-HT uptake inhibitors (20), as well as agonists or antagonists for 5-HT receptors (19). Likewise, it has recently been reported that craniofacial defects similar to those seen in WEC are caused by targeted disruption of the 5-HT_2 receptor gene through homologous recombination.§ Although 5-HT receptors are widely distributed during craniofacial development, differential effects of subtype-specific receptor ligands have been demonstrated (19). This suggests that, while several cell populations are capable of responding to a serotonergic stimulus, only subpopulations expressing the appropriate cellular mechanisms may be responsible for the malformations seen.

The ability of 5-HT to regulate *in vitro* migration of mesenchymal cells has been studied in the developing palate and heart of the mouse embryo. Zimmerman *et al.* (22, 23) and Wee *et al.* (24) have provided evidence that 5-HT plays an important role in palate development, where it appears to mediate palatal-shelf elevation, mesenchyme contractility, migration, and cellular metabolism. In the developing heart, Yavarone *et al.* (25) demonstrated transient sites of 5-HT uptake and degradation in the myocardium adjacent to developing endocardial cushions and found a dose-dependent inhibition of migration of endocardial mesenchyme cells by 5-HT by using an *in vitro* assay.

Abbreviations: NC, neural crest; 5-HT, 5-hydroxytryptamine (serotonin); ECM, extracellular matrix; WEC, whole-embryo culture, E*n*, embryonic day *n*.

‡To whom reprint requests should be addressed.

§Toth, M., Benjamin, D. & Shenk, T., IUPHAR Satellite Meeting on Serotonin Abstracts, July 30–Aug. 3, 1994, Chicago, Volume 3, p. 37 (abstr.).

Evidence that 5-HT regulates craniofacial and cardiac development is consistent with studies by Buznikov and colleagues (26, 27) using the sea urchin embryo, indicating that either 5-HT or a 5-HT-like substance is involved in early morphogenetic events, such as cleavage and gastrulation, prior to formation of the nervous system. Likewise, 5-HT has been implicated in gastrulation and neurulation during early avian development (28–30). The presence of 5-HT during gastrulation is particularly interesting with respect to NC cell migration, since both processes have been suggested to be pivotal for determination of the body plan (8), and in each case, an ectodermal layer undergoes transformation into a mesenchyme and then proceeds to migrate away from its site of origin. Association of 5-HT uptake capacity with the primitive streak and notochord has led to the suggestion that it plays an important role in gastrulation of the chick embryo (28, 29).

Although much of the evidence implicating 5-HT in regulation of early embryogenesis is circumstantial, the dysmorphogenesis produced by perturbing binding of 5-HT to its receptors or uptake transporter in cultured mouse embryos is compelling. The primary objective of the present study was to elucidate possible mechanisms whereby 5-HT is able to influence a wide range of morphological events during craniofacial development. Dose-dependent effects of 5-HT on migration of cranial NC cells and mandibular arch mesenchyme were investigated by using an *in vitro* migration assay, and immunohistochemistry was performed to localize 5-HT_{1A} and non-1A subtype receptors.

MATERIALS AND METHODS

Timed pregnant ICR mice (Harlan) were mated overnight. Cranial NC cells have been shown to continue to form in the mouse embryo until the 11-somite stage (31). To obtain NC explants, the dams were sacrificed on E9 (5- to 10-somite stage) by cervical dislocation, concepti rapidly removed by cesarean section, and embryos dissected away from extraembryonic tissues. For mandibular arch mesenchyme, dams were sacrificed at E12 (50–55 somites).

NC Explants. Explants were prepared from the portion of the developing neural tube that gives rise to the rhombencephalon, as described by Lumsden (5). Briefly, that portion of the embryo rostral to the first somite was divided from the remainder of the embryo and split sagittally. The forebrain expansion was removed, and the free edge of the neuroepithelium dissected away from the anterior neural plate and the developing cardiac tube (dissected tissue indicated by box in Fig. 1*B*). This tissue was used to assay motility of cranial NC and for immunocytochemical staining. To test the possibility that trypsinization might in some way modify the NC response, preliminary studies were carried out with and without trypsin. No difference in cell migration was seen between the groups; however, fewer cells were viable if trypsinization was used. Therefore, NC explants were prepared without trypsinization for the experiments reported here.

Mandibular Arch Mesenchyme. Mandibular arches were dissected away from other developing craniofacial structures by one cut at the commissure of the oral cavity and another rostral to the hyoid (second) arch. The arches from an entire litter (10–12 embryos) were pooled, and the cells were dissociated by light trypsinization (0.2% trypsin; 10 min.; 37°C) in the presence of 0.1% EDTA with gentle agitation. The epithelium remained intact after this treatment and was removed by filtration through a Nitex mesh. The remaining mesenchymal cells were centrifuged, pelleted, and resuspended at 10^4 cells per ml. Trypan blue exclusion was used to establish viability and to produce the correct density of cells plated directly into the migration chamber.

Immunohistochemistry. For *in vivo* analysis of 5-HT-receptor expression, 10-μm sections were obtained from neurulating mouse embryos (E9; 9 somites) that had been fixed by immersion in 0.67 M phosphate-buffered 4% paraformalde-

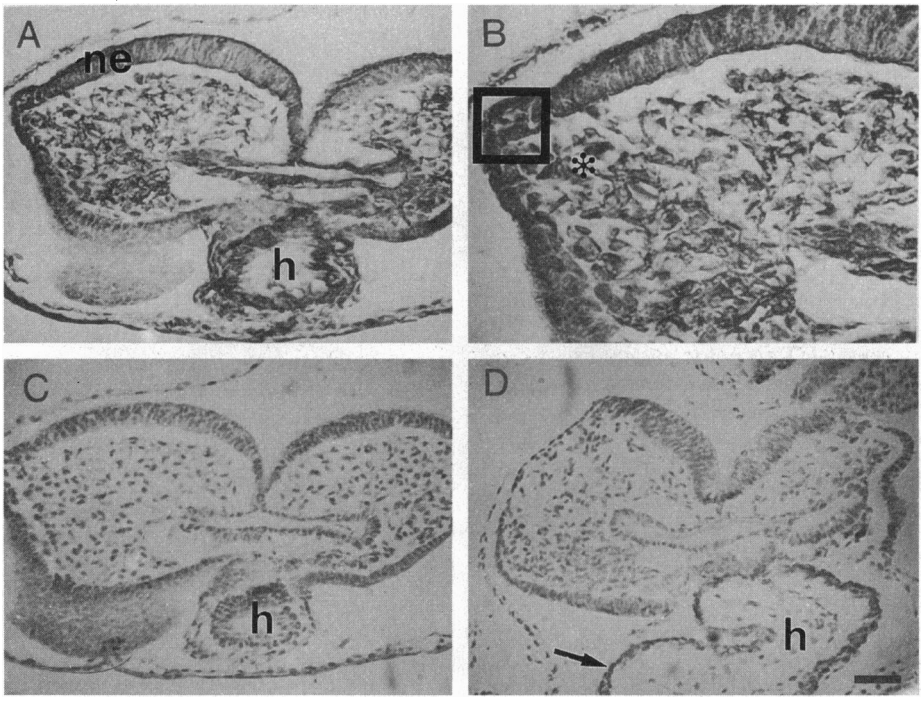

FIG. 1. Immunohistochemical expression of 5-HT receptors in an E9 (9 somites) mouse embryo. (*A*) Immunoreactivity against 5-HT_{1A} is widely expressed within mesenchyme of the developing head folds. (*B*) Higher magnification shows staining of what appear to be cranial NC cells emerging from the lateral edge of the neural fold (*). Box represents tissue included in NC explants. (*C*) Anti-5-HT_{1A} staining is completely blocked by preadsorption with 5-HT_{1A} peptide. (*D*) No staining is seen in migrating NC cells or head-fold mesenchyme with an anti-idiotypic antibody that recognizes 5-HT receptors other than the 5-HT_{1A} subtype; however, specific staining is present within the conotruncal region of the heart (arrow). ne, Neuroepithelium; h, heart. Bar = 60 μm.

hyde (pH 6.8) and embedded in paraffin. For immunohistochemistry of cultured NC cells, explants were cultured for 18 h on fibronectin-coated plastic coverslips in CMRL culture medium supplemented with 5% (vol/vol) NuSerum (GIBCO) in a humidified CO_2 incubator prior to fixation in phosphate-buffered 4% paraformaldehyde. Permeabilization of sections was achieved by trypsinization (0.6 mg/ml) for 30 s. Explants were permeabilized by graded dehydration in ethanol and rehydration. Both sections and explants were stained with polyclonal antibodies specific for the 5-HT_{1A} receptor subtype (1:500; gift from John Raymond; ref. 17) or an anti-idiotypic antibody recognizing 5-HT receptors of multiple subtypes, excluding 5-HT_{1A} (designated non-1A receptors; 1:100; gift from Hadassah Tamir; ref. 18). Immunohistochemistry was performed using the avidin–biotin complex horseradish peroxidase/diaminobenzidine method. Antibody specificity was demonstrated by preadsorption with antigen—e.g., 5-HT_{1A} peptide; Fig 1*C* and ref. 32—or by fixing embryos with 4% paraformaldehyde to which several non-1A antagonists were added (anti-idiotypic 5-HT receptor antiserum; J.M.L., unpublished data).

Migration Assay. Assays for migration of NC cells and mandibular mesenchyme were carried out in a modified Boyden chamber. The apparatus consisted of two acrylic sheets with matching holes. In one sheet the holes extended to the complete depth of the sheet, whereas in the other, they extended only two-thirds of the depth to create a bottom well. The upper and lower wells were separated by placing an 8-μm pore size polycarbonate filter between them.

Prior to performing the assay, the polycarbonate filter was precoated with rat tail type I collagen (33) and 5% bovine serum fibronectin (Sigma) and stored in a humidified incubator until required. The bottom wells were filled with a chemoattractant [10% (vol/vol) fetal calf serum in CMRL medium] and covered by the precoated filter, and the upper segment was clamped into place, thereby forming the upper well. The apparatus was sealed with a silicone gasket and clamped together with a thumb screw at each corner.

Freshly dissected NC explants or suspensions of dissociated mandibular mesenchyme cells were plated onto the surface of the filter in the upper well in 10 μl of CMRL medium supplemented with 5% NuSerum and allowed to attach to the filter in a humidified CO_2 incubator. After 30 min, the upper well was carefully flooded with 90 μl of this medium. Serotonergic antagonists, as well as 5-HT, were added to the medium in the upper well, either singly or in combination at the time of flooding. For the initial set of experiments, 5-HT was added in concentrations ranging from 0.01 μM to 100 μM. To inhibit the effect of the most stimulatory dose (0.01 μM) on NC migration, different 5-HT receptor antagonists were added to the culture medium at the same concentration in conjunction with 5-HT. Antagonists used in this study were as follows: NAN-190 (5-HT_{1A}; 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl]piperazine hydrobromide; Research Biochemicals, Natick, MA), mianserin (5-$HT_{1C/2}$; 1,2,3,4,10,14b-hexahydro-2-methyldibenzo[c,f]pyryzino[1,2-a]azepine hydrochloride; Research Biochemicals), and Zofran (5-HT_3; ondansetron hydrochloride; Glaxo).

For all assays, the migration chamber was incubated overnight in a humidified atmosphere containing 5% CO_2, followed by removal of the filter, which was then fixed in methanol, stained with hematoxylin, dehydrated, and mounted on slides with Permount and a coverslip. Migration was assessed by counting on migrated cells (on the undersurface of the filter) and unmigrated cells (those remaining on the top surface) and expressing the number of migrated cells as a percentage of total number of cells (migration index). All counts were made blind as to the treatment group.

RESULTS

Expression of 5-HT Receptors by NC Cells. Expression of 5-HT receptors in the 5- to 10-somite (E9) embryo was investigated by using antibodies that specifically bind to 5-HT_{1A} or non-1A 5-HT receptors. The 5-HT_{1A} receptor was found to be widely distributed throughout the mesenchyme and in NC cells emerging from the neural folds of the elevating neural plate of E9 mouse embryos (Fig. 1 *A* and *B*), whereas immunoreactivity for non-1A receptors was only demonstrated in the developing heart at this age (Fig. 1*D*). This is in contrast to the expression of multiple 5-HT receptor subtypes by mandibular mesenchyme of the E12 mouse embryo (see figure 1 of ref. 19). Staining with the 5-HT_{1A} receptor antiserum was specifically blocked by preincubation of the antibody with the 1A peptide (Fig. 1*C*), indicating that immunoreactivity was specific. NC cells appear to undergo transformation from neuroectoderm into ectomesenchyme while expressing the 5-HT_{1A} receptor (Fig. 1*B*). Immunoreactivity for the 5-HT_{1A} receptor was also demonstrated in cultured NC explants (data not shown).

Serotonergic Regulation of Cell Migration. Effects of 5-HT on migration of craniofacial NC and its mesenchymal derivatives were investigated at E9 and E12, respectively (Fig. 2 *A* and *B*). In each case, the effects of five concentrations of 5-HT, ranging from 0.01 μM to 100 μM, were compared with controls (no additives). This dose range included low concentrations of 5-HT—e.g., 0.01 μM—that should activate receptors (34), as well as higher concentrations—e.g, 10 μM—that would be expected to elicit intracellular uptake by binding to the 5-HT transporter (14).

E9 (5–10 Somites). When NC explants were treated with control culture medium, a 5% migration index was exhibited, whereas culture medium supplemented with the lowest concentration of 5-HT (0.01 μM) produced a 2-fold increase in

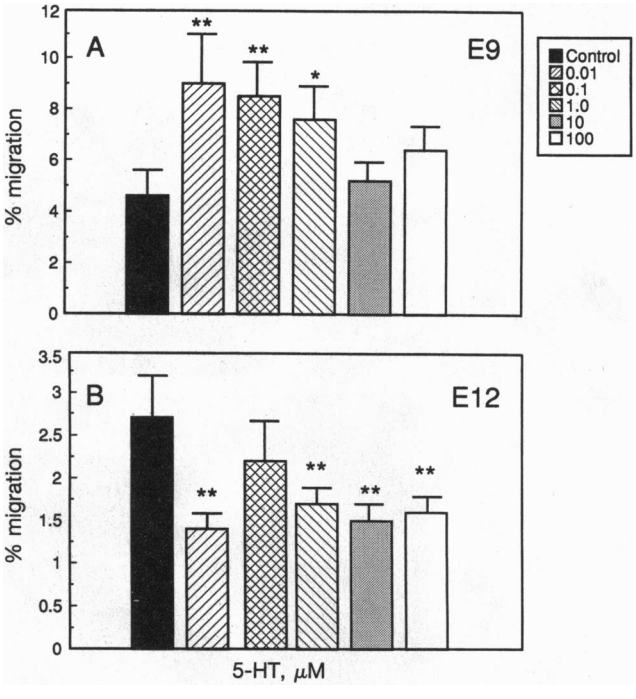

FIG. 2. Dose-dependent effects of 5-HT on cell migration. (*A*) Migration of NC cells from explants from E9 mouse (5–10 somites) embryos is stimulated by 5-HT in an inverse dose-dependent manner, where the greatest effect is produced by the lowest dose tested (0.01 μM 5-HT; $n = 25$). There is no significant effect of the two highest doses of 5-HT (10 and 100 μM). (*B*) Migration of mandibular mesenchyme cells from E12 embryos (50–55 somites; $n = 25$). Most concentrations of 5-HT inhibit migration. *, $P < 0.05$, **, $P < 0.01$ (vs. control).

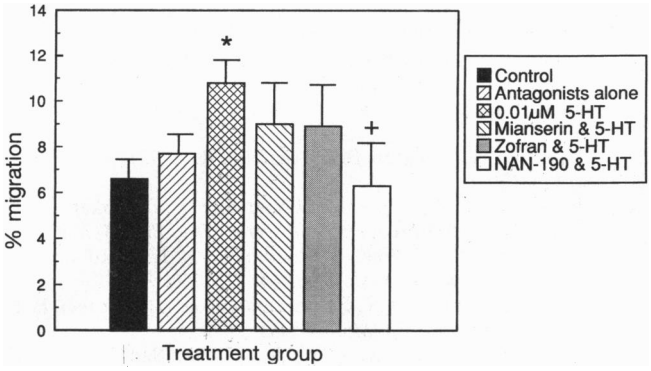

FIG. 3. NC explants cultured in the presence of the most stimulatory dose of 5-HT (0.01 μM) with or without antagonists selective for different 5-HT receptor subtypes. Only the 5-HT_{1A} antagonist (NAN-190) significantly decreases the stimulatory effect of 5-HT on NC migration, which is reduced to control levels. Two other antagonists, mianserin (5-$HT_{1C/2}$) and Zofran (5-HT_3), have no significant effects (n = 10–17). *, $P < 0.05$ (vs. control); +, $P < 0.05$ (vs. 5-HT).

migration compared to controls—i.e., 10% migration. This effect was significant at $P < 0.01$. As the concentration of 5-HT was increased to 1 μM, stimulation of migration was still observed but to a lesser degree: at 0.1 μM and 1 μM there was a 1.8-fold (8.5% migration; $P < 0.01$) and 1.5-fold (7.5% migration; $P < 0.05$) increase in migration, respectively. However, the stimulatory effect disappeared at 5-HT concentrations of 10 and 100 μM. Thus, an inverse, dose-dependent stimulation of migration from NC explants was produced by 5-HT (Fig. 2*A*). To assess underlying mechanisms, the ability of several 5-HT receptor antagonists to block the stimulatory effects of 0.01 μM 5-HT was investigated. When 0.01 μM NAN-190, a 5-HT_{1A} receptor antagonist, was added, the increase in migration produced by 0.01 μM 5-HT was negated. However, antagonists for the 5-$HT_{1C/2}$ (mianserin), and 5-HT_3 (Zofran) receptors, did not significantly inhibit the effect of 0.01 μM 5-HT (Fig. 3).

E12 (50–55 Somites). By this stage, mandibular mesenchyme cells (mainly derived from the NC) have migrated into the developing visceral arches and are nearing the end of or have completed their migratory phase. For this reason, control levels of migration were significantly lower than in NC explants, such that untreated cells exhibited a migration index of 2.7%. Despite the low level of migration, most concentrations of 5-HT reduced this migration index further to between 1.4% and 1.7% (Fig. 2*B*). Unfortunately, given the small amount migration, it was not technically feasible to determine the mechanism of these effects by using receptor antagonists, as was done with NC explants.

DISCUSSION

Using an *in vitro* assay system, we have demonstrated that 5-HT produced a dose-dependent stimulation of cranial NC migration and a general inhibition of migration by mandibular arch mesenchyme. The stimulatory effect of low doses of 5-HT on NC migration was reversed by the addition of the 5-HT_{1A} antagonist NAN-190 but not by other 5-HT antagonists tested, implying that the 5-HT_{1A} receptor may solely mediate this effect. The fact that a dose of 0.01 μM 5-HT was most stimulatory is consistent with the EC_{50} for the 5-HT_{1A} receptor (34). By using immunohistochemistry, this receptor was shown to be expressed by craniofacial NC, both *in vivo* and *in vitro*, while non-1A receptors were shown to be absent from the developing craniofacial region at this stage. Although the 5-HT_{1A} receptor was widely distributed throughout the cranial mesenchyme of the E9 embryo, only the motile neural crest cells appeared capable of responding to activation of this receptor in the migration assay.

These results indicate that one of the mechanisms underlying serotonergic influences on craniofacial development may be regulation of cell migration in a temporally specific, dose-dependent, and receptor-mediated manner. Thus, as NC cells emerge from the neural folds (at E9), their migration may be stimulated by low to medium concentrations of 5-HT, whereas higher concentrations may be inhibitory or have no effect. In E12 embryos, where these cells have already migrated into the visceral arches and become craniofacial mesenchyme, most concentrations of 5-HT inhibit migration. Since cranial NC cells express 5-HT_{1A} receptors, it is likely that they mediate the stimulatory effects of low doses of 5-HT on these cells, whereas at higher doses intracellular uptake of 5-HT may counteract the effects of receptor activation. Such modulatory effects of 5-HT on migration have previously been observed in other cell types, but the precise action seems to depend on the inherent motility of the cell type tested. For example, 5-HT stimulates migration of motile vascular smooth muscle cells (35, 36), whereas migration of bovine endothelial cells (35, 37) is inhibited, an effect that appears to be associated with stimulation of actin cable formation. The *in vitro* motility of dissociated embryonic palatal mesenchyme (22) is also increased by 5-HT, suggesting that this substance may be involved in palatal shelf elevation. Together with the present results, these findings suggest that 5-HT may promote the inherent motility or nonmotility of a variety of cell types, including those of the developing craniofacial region.

Many parallels have been drawn between NC cells of the craniofacial region and somitic mesenchyme of the trunk. Quite apart from subserving similar structural functions by giving rise to skeletal components of their respective regions, both cell types are produced by a similar process of epithelial–mesenchymal transformation and migrate widely prior to their final differentiation (8). Besides stimulating motility, 5-HT might promote the tendency of NC cells to transform from neuroepithelium into ectomesenchyme. In this way, appropriate levels of 5-HT would increase the population of NC cells being formed from the neuroepithelium. In the migration assay used in the present study, this would be manifest as an increased number of cells available to migrate toward the chemoattractant located in the bottom wells of the chemotaxis chamber. Selleck and Bronner-Fraser (38) have reported that various neuroepithelial cells may be stimulated to form NC when grafted adjacent to nonneuronal ectoderm. This supports previous suggestions (8) that NC formation is determined by a balance between neuronal and ectodermal inductive influences. In this schema, low doses of 5-HT could promote this process by activating 5-HT_{1A} receptors, such that neuroepithelial cells would be stimulated to transform into neural crest cells. This hypothesis is supported by previous work on sea urchins (21, 39), where 5-HT was found to be an important stimulus for epithelial–mesenchymal transformation during gastrulation. Similarly, Wallace (29) demonstrated the transient ability of the floor plate of the neural tube to take up 5-HT synthesized by the notochord in stage 10–12 chick embryos. This ability is expressed not only at the rostral end of the neural tube but also at the caudal end, where gastrulation is still ongoing in association with the persistent primitive streak. It is possible, therefore, that 5-HT may augment or facilitate epithelial–mesenchymal transformation, perhaps by increasing susceptibility to inductive factors.

The results of this study suggest that migration of highly motile cells, such as the cranial NC, may be stimulated by activation of appropriate 5-HT receptors, whereas a wide range of concentrations of 5-HT may promote the cessation of migration in less motile mandibular arch mesenchyme. We propose that both of these mechanisms are present in the developing mouse embryo during neurulation and organogen-

esis. During NC formation, activation of 5-HT_{1A} receptors may mediate serotonergic stimulation of motility, epithelial–mesenchymal transformation, or both. Once NC cells have been formed, 5-HT uptake and/or receptor activation may be involved in down-regulating the inherent migratory activity of NC cells as they differentiate into craniofacial mesenchyme. Thus, 5-HT may play a dual role in regulating migration and differentiation of craniofacial cells of NC origin.

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