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From Oocyte to Neuron: Do Neurotransmitters Function in the Same Way Throughout Development?

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SUMMARY

1. Classical neurotransmitters (such as acetylcholine, biogenic amines, and GABA) are functionally active througout ontogenesis.

2. Based on accumulated evidence, reviewed herein, we present an hypothetical scheme describing developmental changes in this functional activity, from the stage of maturing oocytes through neuronal differentiation. This scheme reflects not only the spatio-temporal sequence of these changes, but also the genesis of neurotransmitter functions, from "protosynapses" in oocytes and cleaving embryos to the development of functional neuronal synapses.

3. Thus, it appears that neurotransmitters participate in various forms of intra- and intercellular signalling throughout all stages of ontogenesis.

1. INTRODUCTION

"Classical neurotransmitters," which participate in synaptic neurotransmission, such as acetylcholine (ACh), dopamine (DA), noradrenaline (NA), adrenaline (A), serotonin (5-HT), and gamma-aminobutyric acid (GABA), have been shown to be multifunctional substances participating in developmental processes

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in all animal species so far investigated. These substances, referred to as "prenervous" neurotransmitters, play regulatory roles throughout ontogenesis, including stages prior to development of the nervous system (Buznikov, 1967; see also Buznikov, 1990a; Lauder, 1993 for reviews). At least some developmental functions of these substances are transient, being expressed only during certain periods of ontogenesis (Buznikov & Shmukler, 1981; Shmukler, 1981, 1993; Lauder *et al.*, 1988; Shuey *et al.*, 1992, 1993; Yavarone *et al.*, 1993a,b).

Such substantial developmental changes in neurotransmitter functions suggest a negative answer to the question posed in the title. At the same time, the organization and genesis of these functions for particular neurotransmitters appear to form continua which can be traced throughout development. These processes have principally the same material basis, since the transmitter signals are precisely directed to their corresponding targets. Thus, prenervous and neuronal transmitters are important components of the biological mechanisms providing the spatio-temporal organization of ontogenesis. The purpose of the present paper is to review the relevant literature and provide concrete examples to substantiate the position that neurotransmitters can have fundamentally different roles throughout development. Particular attention will be paid to the potential roles of neurotransmitters as "morphogens" during development.

"Morphogens" are developmental signals that exert specific effects on receptive cells depending on concentration. In embryos, morphogens are thought to be present in gradients created by the presence of a "source" and a "sink". Developing cells are affected in specific ways along this concentration gradient (reviewed by Lauder, 1988). This concept has traditionally been applied to substances involved in pattern formation and morphogenesis, such as retinoic acid. However, it may also be appropriate to consider neurotransmitters as morphogens when they act as dose-dependent morphogenetic signals in neural and non-neural tissues. Neurotransmitters are known to have these types of actions in primitive organisms and embryos (see above), where they exert their effects using receptors and signal transduction mechanisms similar to those in the adult nervous system. This raises the possibility that the highly specialized roles played by neurotransmitters in synaptic transmission may have evolved from phylogenetically old functions, many of which are recapitulated during development. One example of neurotransmitters acting as morphogenetic signals for vertebrate embryos is that of the monoamines, especially 5-HT, discussed below. For other examples see reviews by Lauder (1988, 1993).

2. DEVELOPMENTAL CHANGES IN NEUROTRANSMITTER FUNCTIONS

2.1. Blastula and Gastrula Stages

Three main events must be noted here: gastrulation itself (with its active cell movements), primary embryonic inductions, and the appearance of the earliest specialized physiological functions. Data have been obtained regarding the participation of ACh and biogenic monamines in these processes in embryos of echinoderms, molluscs and chordates (Brown and Shaver, 1989; Buznikov, 1967; Fluck, 1982; Gustafson and Toneby, 1970; Gustafson, 1989a,b; Laasberg, 1990; Dautov and Nezlin, 1992; Falugi, 1993; Rowe *et al.*, 1993; see also Buznikov, 1990a for a comprehensive review).

It has been established, mainly in sea urchins, that cell movements occurring during gastrulation and post-gastrulation stages are regulated by ACh and biogenic monoamines (Gustafson and Toneby, 1970; Martynova, 1981; Falugi, 1993). Specific antagonists of receptors for these neurotransmitters act as inhibitors or blockers of morphogenetic cell movements during specific phases of gastrulation. For example, 5-HT antagonists are effective throughout this period, whereas ACh antagonists act only during the final phases of gastrulation. LSD and its derivatives among other serotonergic blocking drugs are effective throughout gastrulation (Gustafson and Toneby, 1970), whereas they do not affect cleavage divisons at all (Buznikov, 1967; 1990a). The ability of both 5-HT and ACh to affect gastrulating sea urchin embryos may be indicative of a broadening of the spectrum of neurotransmitters receptors expressed beginning at the time of gastrulation by cells of the primary gut and mesenchyme, both intracellularly and on cell surface membranes (Gustafson and Toneby, 1970; Martynova, 1981).

Histochemical data has confirmed the direct participation of prenervous neurotransmitters in the regulation of morphogenetic cell movements during gastrulation. Acetylcholinesterase (viewed here as a component of the cholinergic system) and 5-HT are localized in echinoderm embryos predominantly in the primary gut where the most active cell movements occur (Markova *et al.*, 1985; Falugi, 1993). Detectable amounts of these substances are absent in other larval cells except the ciliary bands (see below). Biogenic monoamines have been found in *Xenopus* gastrulae in ectoderm and mesoderm, but not in endoderm (Rowe, *et al.*, 1993). This suggests that neurotransmitter specialization of different cell lineages arises already at prenervous stages. A correlation between morphogenetic cell movements and acetylcholinesterase localization has also been reported in the chick embryo (Laasberg, 1990). After embryonic induction, monoaminergic or cholinergic systems in vertebrates appear to be predominantly localized to the complex of axial organs (Buznikov, 1990a; Lauder 1988, 1993), as discussed below.

In all cases investigated, prenervous neurotransmitters have been found to act as local hormones triggering and regulating pre-nervous and non-nervous embryonic motility. Serotonin is synthesized by, or at least accumulated in, the cells of various organs of embryonic motility. It is one of the regulators of ciliary activity in embryos and larvae of molluscs, echinoderms, and hemichordates, sometimes acting together with other pre-nervous neurotransmitters (Buznikov, 1967; Markova *et al.*, 1985; Gustafson, 1989a,b, 1991; Dautov and Nezlin, 1992). These serotonergic functions begin in sea urchins very early, at the midblastula stage, when all the embryonic cells are motile. Specialized organs (e.g., ciliary bands) arise later, during gastrulation, and histochemically detectable 5-HT disappears from ectodermal cells, except those in the ciliary bands (Markova *et al.*, 1985). At this time, 5-HT regulates the *de novo* formation and regeneration of cilia of the ciliary bands and the corresponding rebuilding of the cortical cytoskeleton in sea urchins (Stephens and Prior, 1992; Shmukler, unpublished; see Buznikov, 1990a). It has been shown in nudibranch molluscs that serotonergic receptors present at this stage are localized to cell surface membranes and are similar pharmacologically to classical 5-HT_2 receptors (Buznikov, 1990a). 5-HT also acts as a regulator of one of the earliest physiological functions in sea urchin midblastulae, secretion of the hatching enzyme (Buznikov, 1990a).

Very promising data have been obtained concerning the possible participation of 5-HT in primary embryonic induction in echinoderms (Gustafson and Toneby, 1970). 5-HT, secreted by cells of the primary gut at the final stage of gastrulation, appears to induce the transformation of some cells of the ciliary bands into neuroblasts. It is a pity these interesting investigations were not continued. In particular, it would be very interesting to know the neurotransmitter phenotype(s) of these neuroblasts.

Thus, neurotransmitters appear to be multifunctional regulators at gastrulation stages of development, as well as during earlier cleavage stages of blastulation. The spatio-temporal organization of their functions becomes increasingly complex in connection with neurotransmitter specialization of cells as different receptors are expressed. An interesting question is whether neurotransmitters continue to function in the triggering and regulation of cell divisions at post-blastula stages of development. *A priori,* it would seem reasonable to expect that this function might be present during bursts of cell proliferation in the course of various morphogenetic events. At least for 5-HT, recent evidence in the mouse embryo (discussed below) suggests that this may indeed be the case.

A peculiarity of neurotransmitter systems during the early developmental period (e.g., cleavage to gastrulation) seems to be related to their receptor components. These consist of functional receptors localized both intracellularly and on the cell surface. Neurotransmitters trigger and regulate cleavage divisions via intracellular receptors in sea urchins and amphibians (and possibly in other animal groups also; Buznikov, 1990a). Antagonists for serotonergic, cholinergic and adrenergic receptors have been found to inhibit or block the progression of cleavage divisions, whereas corresponding agonists or transmitters themselves reduce, prevent, or even eliminate this cytostatic action of antagonists (Buznikov, 1967, 1990a; Renaud *et al.*, 1983; Shmukler *et al.*, 1986; Markova *et al.*, 1990; Sadykova *et al.*, 1992). In various kinds of experiments it has been shown that penetration of neurotransmitters and their agonsists or antagonists into cells is required for the protective or cytostatic effects of these substances. New experimental results regarding this requirement (Buznikov, Shmukler, Bezuglov, Whitaker, in preparation) are presented in Fig. 1.

Much data has been obtained indicating that intracellular neurotransmitter

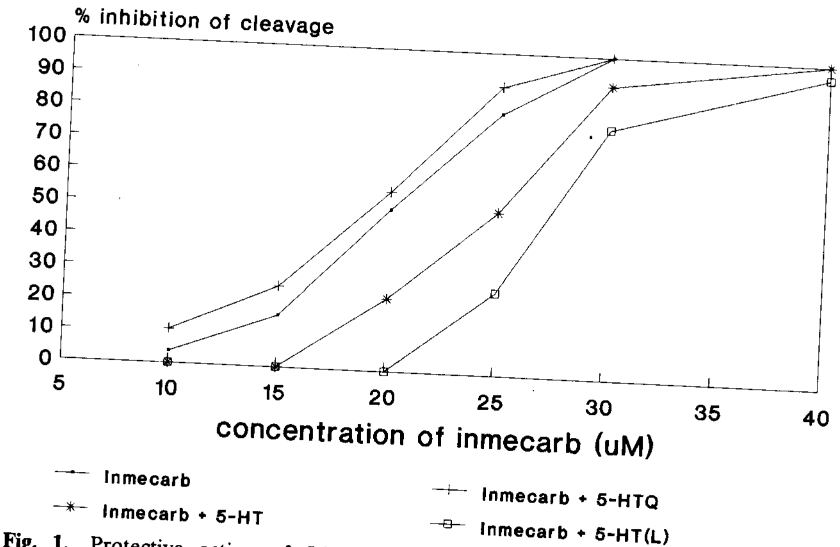

Fig. 1. Protective action of 5-HT and its analogs on the cytostatic action of 5-HT-antagonist inmecarb in sea urchin *Lytechinus pictus* early embryos. division. Abscissa, concentration of inmecarb (μM); ordinate, percentage of inhibition of 1st cleavage. 1—Inmecarb (I); 2—I + 5-HT (50 μM); 3—I + 5-HTQ (50 μM; trimethylserotonin methiodide); 4—I + 5-HT(L) (50 μM; 5-HT analog with high lipophilicity originally synthesized by Dr V. V. Bezuglov).

receptors found in cleaving sea urchin embryos differ in some respects from their typical synaptic counterparts (Buznikov, 1967, 1990a,b). For example, 6-hydroxytryptamine, a synaptically inactive 5-HT-isomer, protects cleaving embryos against cytostatic 5-HT antagonists as effectively as 5-HT itself. Likewise, in experiments with the beta-adrenergic agonist isoproterenol and its cytostatic antagonists (propranolol, alprenolol), a complete lack of stereospecificity was found, which would not be the case for synaptic beta-adrenergic receptors (Buznikov, 1990b).

The investigation of intracellular 5-HT receptors by means of radioligand techniques has revealed two pools of intracellular binding sites in one-cell stage and cleaving sea urchin embryos (Buznikov, 1990a). These appear to represent receptor and non-receptor pools. The latter is of low affinity and extraordinarily high capacity, and may be located in the yolk. This low affinity binding site in yolk may mask the true receptors. For this reason, it was necessary to eliminate most of the yolk in order to detect the receptor pool in the whole sea urchin cells or embryos (Buznikov, 1990a,b). Data on radiolabelling of 5-HT binding sites have been obtained by other investigators in early sea urchin embryos (Brown and Shaver, 1987, 1989). However, in these studies intact embryos were used and, as would be expected, specific binding of [^{3}H]5-HT was only found at postgastrulation stages of development.

As to other neurotransmitter systems, the specific binding of the beta-adrenergic ligands iodocyanopindolol and dihydroalprenolol was shown in early *Xenopus laevis* embryos. Lack of stereospecificity similar to that discussed above, was also demonstrated in these experiments (Shmukler *et al.*, 1988).

Intracellular receptors may be involved in other functions of 5-HT-like compounds during cleavage and blastulation of sea urchins, namely the regulation of cell adhesion. Lipophilic 5-HT antagonists, which readily permeate the cell membrane and reach the cytoplasm, specifically block postcleavage adhesion of blastomeres and evoke cell disaggregation. Conversely, hydrophilic analogs of these antagonists, which poorly permeate into the cytoplasm, are ineffective (Buznikov and Shmukler, 1981).

Pharmacological experiments have also demonstrated the presence of surface membrane neurotransmitter receptors in early sea urchin embryos. In particular, exogenous 5-HT imitates interblastomere signals in isolated blastomeres, whereas its antagonists prevent this effect (Shmukler, 1981, 1993). Because quarternary (i.e., hydrophylic) and tertiary (lipophilic) 5-HT antagonists were equally effective in this regard, it was concluded that these receptors were on the cell surface (Shmukler, 1993). It should be noted that this early function of 5-HT is probably as important as the regulation of cleavage divisions, since blastomere interactions determine the further fate of these cells (Buznikov and Shmukler, 1981; Shmukler *et al.*, 1981, Shmukler, 1993).

The existence of membrane receptors was confirmed by experiments using the 5-HT$_{1A}$ receptor ligand [H^3]8-OH-DPAT which specifically bound to the cell surface of early embryos of the sea urchin *Strongylocentrotus intermedius* (Fig. 2) and had a dissociation constant similar to that of typical 5-HT receptors (Shmukler, 1993). Cell surface binding sites for [^{3}H]5-HT have also been reported in the sea urchin *Arbacia punctulata* at later stages of embryonic development (Brown and Shaver, 1989). Membrane cholinergic receptors, closely related to classical nicotinic receptors, were found in early embryos of the sea urchin *Paracentrotus lividus* (Falugi and Prestipino, 1989; Falugi, 1993). Similar, typical 5-HT$_2$ and D$_2$ receptors were found in trophoblast cells of early human embryos (Vaillancourt *et al.*, 1994b).

It seems highly likely that prenervous neurotransmitter receptors, including intracellular ones, are functionally coupled to standard second messengers. Lypophilic analogues of cAMP exert protective actions against cytostatic transmitter antagonists and are able to imitate interblastomere signalling similar to the effects of 5-HT (Shmukler *et al.*, 1986, Shmukler and Grigoriev, 1984). Adenylyl cyclase, which is active mainly in the cytoplasm (in membranes of the endoplasmic reticulum, filopodia and yolk granules) of cleaving sea urchin embryos (Rostomyan *et al.*, 1985) is activated by dopamine, the major catecholamine present at this stage of development (Capasso *et al.*, 1987, 1988). In addition, adenylyl cyclase is present on surface membranes of the interblastomere cleft immediately after formation of the first cleavage furrow (Rostomyan *et al.*, 1985). This is the very place where neurotransmitter receptors are thought to be localized (Buznikov, 1990a; Shmukler, 1993). Recently, the first evidence for coupling of cell surface serotonergic and cholinergic receptors to Ca^{2+} and diacylglycerol second messengers in sea urchin embryos has been obtained

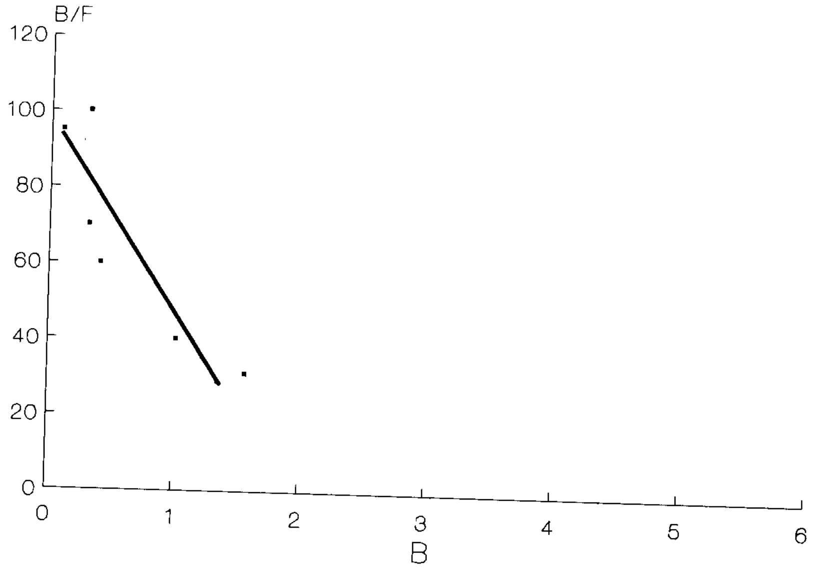

Fig. 2. Scatchard plot of the binding of agonist [H3]8-OH-DPAT by surface membrane 5-HT-receptors of sea urchin *Strongylocentrotus intermedius* early embryos. Abscissa—B, bound labelled ligand concentration; ordinate—B/F, ratio of bound and free labelled ligand concentrations.

(Buznikov *et al.*, 1993a). Thus, independent coupling of intracellular and surface membrane neurotransmitter receptors to second messenger systems appears to be the principal peculiarity of early embryonic development. It is surprising, therefore, that this intersting scenario has not yet attracted wider attention.

Given the above mentioned developmental functions and second messenger coupling, it is natural to progress to the problem of the intracellular targets of prenervous neurotransmitters. Such targets include various components of the sea urchin embryo cytoskeleton, such as the cortical microfilaments forming the contractile ring during cleavage divisions, microfilaments of filopodia involved in blastomere adhesion, and structures involved in the orientation of mitotic spindles of blastomeres (Buznikov and Shmukler, 1981; Buznikov, 1990a,b; Shmukler, 1993). Similar data have been obtained in early embryos of the polychaete *Ophryotrocha labronica* and the neurulating chick embryo (Emanuelsson, 1974, 1992), where it was shown that [H^3]-5-HT binds mainly to elements of the cytoskeleton.

It is well known that the state of cortical microfilaments involved in cleavage divisions can be evaluated by measuring the rigidity of the cell surface (Yoneda and Schroeder, 1984). Immediately before the first cleavage division in sea urchin embryos, 5-HT antagonists have been shown to decrease this rigidity, whereas beta-adrenergic blockers increase it (Fig. 3) (Buznikov, 1989; Buznikov and Grigoriev, 1990). Serotonin strengthens the contraction of cortical microfilaments

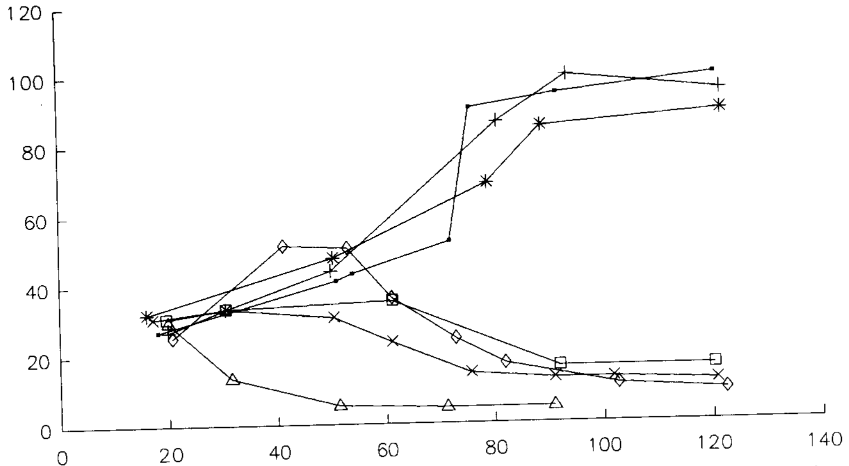

Fig. 3. Effect of beta-adrenergic (1–3) and serotonergic (4–6) antagonists on the rigidity of sea urchin *Paracentrotus lividus* early embryos (from Buznikov, 1989). 1—Alprenolol (400 μM); 2—propranolol (200 μM); 3—dichloroisoproterenol (500 μM); 4—cyproheptadine (35 μM); 5—DPTC (75 μM); 6—cytochalasin B (10 μM; for comparison). Abscissa: time from fertilization (min); Ordinate: rigidity (din × cm^2/um). —■— 1 + 2 —✳— 3 —⊟— 4 —×— 5 —◇— 6 —△— 7.

evoked by exogenous ATP administration in permeabilized one-cell embryos, whereas adrenaline (A) inhibts this contraction, and antagonists of the two transmitters have the expected opposite effects (Buznikov, 1990b). Thus, intracellular 5-HT and A may play opposite roles (i.e., act as functional antagonists) during the first cell cycles of sea urchin blastulae.

These data are of additional interest in relation to the spatio-temporal organization of prenervous neurotransmitter processes. The reaction of cortical microfilaments of intact and permeabilized sea urchin embryos to serotonertic and adrenergic neurotransmitters and their antagonists was spatially generalized. However, later (during cleavage furrow formation) cortices began to contract locally under the action of 5-HT and beta-adrenergic blockers, probably, in the area of the contractile ring (Buznikov, 1990b). In other words, this reaction of the embryonic cells became directed. The microinjection of A into *Xenopus laevis* blastomeres also evoked a direct reaction, namely some acceleration of normal cleavage furrow formation. This was in contrast to cAMP and Ca^{2+} that evoked diffuse changes in the cell surface (Shmukler *et al.*, 1987). The most probable reason for such differences is the spatial distribution of neurotransmitter receptors involved in cleavage divison regulation and their coupling to appropriate second messenger systems.

Neurotransmitter receptors taking part in blastomere adhesion may also be spatially organized, as indicated by the asymmetry of the adhesion process. It had also been found that corresponding receptors appear later than those regulating cleavage divisions (Buznikov and Shmukler, 1978).

As to direct blastomere interactions, the presence of an embryonic "protosynapse" has been hypothesized [Shmukler, 1993; see Fig. 7(3]. In this case, neurotransmitter receptors on membranes forming the interblastomere cleft (Shmukler, 1993) and the increased concentration of 5-HT there (see Markova *et al.*, 1985) could form an internal asymmetry of blastomere reactivity to serotonergic compounds. Interestingly, sister blastomeres may be the actual source of neurotransmitter as well as the target of it. Cell-cell interactions between blastomeres may also form a barrier that prevents leakage of neurotransmitter from the interblastomeric cleft. Based on the above evidence, it would appear that all prenervous neurotransmitter functions may be both spatially and temporally organized.

In the invertebrates and vertebrates alike, prenervous neurotransmitters are synthesized by the usual pathways, but at unusual sites. For example, biogenic monoamines, especially 5-HT or 5-HT-like substances, are synthesized in the yolk of early polychaete, sea urchin, amphibian and chick embryos. There is also some evidence related to the existence of cholinergic substances in the quail embryo yolk (Emanuelsson *et al.*, 1988; Buznikov, 1990a; Emanuelsson, 1992; Kaltner *et al.*, 1993).

3. NEUROTRANSMITTERS AS MORPHOGENS IN VERTEBRATE EMBRYOS

3.1. Monoamines and Early Embryogenesis

In the early rodent embryo, monoamines (5-HT, catecholamines) are present in the fertilized egg, and appear to regulate early cleavage divisions (Burden and Lawrence, 1973; Pienkowski, 1977; Sadykova *et al.*, 1992) as they do in sea urchins. These neurotransmitters are synthesized by yolk granules and notochord of the neurulating chick and frog embryo, and are also actively taken up by the neural tube during neurulation (Wallace, 1982; Strudel *et al.*, 1977; Kirby and Gilmore, 1972; Newgreen *et al.*, 1981; Lawrence and Burden, 1973; Godin and Gipouloux, 1986). Analogous to the case of sea urchins, these prenervous neurotransmitters may regulate morphogenetic cell movements and cell shape changes necessary for neural tube closure, since exposure of chick embryos to monoamine uptake inhibitors (MAO inhibitors) or receptor ligands produce a variety of malformations, including neural tube defects (Palen *et al.*, 1979). One mechanism whereby these effects might occur is by binding of monoamines to cytoskeletal elements within neuroepithelial cells, as reported for 5-HT (Emanuelsson *et al.*, 1988). These cytoskeletal elements might constitute one of the pools of intracellular 5-HT binding sites described in sea urchin embryos (Brown and Shaver, 1987; Buznikov and Shmukler, 1981). Another possiblity is that notochord induction of brainstem 5-HT neurons (see Ruiz i Altaba, 1994) may involve uptake of 5-HT into the floorplate.

Recent studies in the neurulating mouse embryo have provided evidence that 5-HT acts as a morphogen during craniofacial (Lauder *et al.*, 1988; Shuey *et al.*, 1992, 1993) and cardiac development (Yavarone *et al.*, 1993a). In these studies, whole embryo culture was used to present embryos with 5-HT (added to the medium), followed by anti-5-HT immunocytochemistry to detect sites of 5-HT uptake. This approach revealed transient expression of 5-HT uptake sites between days 9–12 of gestation (E9–12; day of insemination = E1) in craniofacial epithelia, hindbrain, and myocardium (heart). Serotonin appeared to be rapidly degraded following uptake, since it was necessary to add an MAO inhibitor together with 5-HT to visualize these sites. Therefore, these uptake sites could be viewed as constituting a "sink" for 5-HT.

In the heart, 5-HT uptake was initially expressed throughout the myocardium, but became progressively restricted to regions immediately adjacent to developing endocardial cushions in the outflow tract and atrioventricular canal (which later give rise to valves; Yavarone *et al.*, 1993a). The endocardial cushions contain an extracellular matrix known as the cardiac jelly or myocardial basement membrane, produced by the myocardium, which becomes populated by cardiac mesenchyme (CM) cells as they proliferate and migrate toward the myocardium (Markwald *et al.*, 1990). Exposure of cultured mouse embryos to 5-HT uptake inhibotors (fluoxetine, sertraline) during heart development severely inhibited proliferation of these cells. In the context of the above model for a "morphogen" (see Fig. 4), these effects were interpreted as resulting from blocking the "sink" (i.e., myocardial 5-HT uptake and degradation), while not affecting the "source" (the blood; see below), thereby building up excess levels of 5-HT in developing endocardial cushions (Yavarone, 1991; Yavarone *et al.*, 1993a). Using an *in vitro* cell migration assay, high doses of 5-HT (10–100 μM) significantly inhibited and low doses (0.01–0.1 μM) had a tendency to stimulate, migration of CM cells isolated from outflow tracts of E12 embryos (Yavarone *et al.*, 1993a). Therefore, myocardial uptake may provide a means for

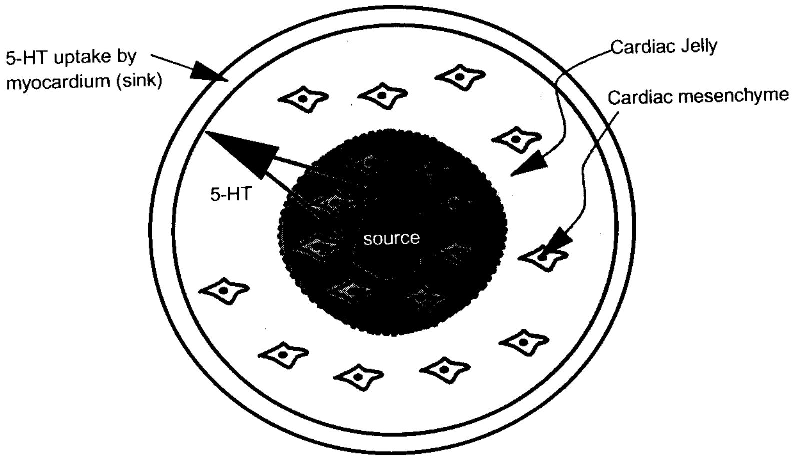

Fig. 4. Serotonin as a morphogen for cardiac mesenchyme cells in developing endocardial cushions of the embryonic mouse heart. (Adapted by J. R. D. Moiseiwitsch from Fig. 6.1 in Yavarone, 1991; based on data presented in Yavarone *et al.*, 1993a.)

maintaining appropriate levels of 5-HT important for regulating proliferation and migration of CM cells during development of endocardial cushions. In addition, uptake of 5-HT could regulate myocardial production of endocardial jelly. These findings may be relevant to cardiac malformations present in Down syndrome, and in a murine model for Down's, the Trisomy 16 mouse (Epstein, 1991; Webb *et al.*, 1994).

After an extensive search for sites of 5-HT synthesis in E9–12 mouse embryos, and finding that the ectoplacental cone and placenta can transport 5-HT toward the embryo, it was concluded that the primary source of 5-HT for the neurulating mouse embryo *in vivo* is the maternal-embryonic circulation (Yavarone *et al.*, 1993b). In whole embryo culture, rat serum in the medium provides μM amounts of 5-HT (Shuey, Lauder and Tamir, unpublished).

In the craniofacial region, 5-HT uptake/degradation sites were found to be transiently expressed in epithelia and other ectodermal derivatives during the period of most active morphogenesis and cell proliferation (E10–12). In addition, a serotonin binding protein (SBP; Tamir and Gershon, 1990) was expressed by underlying mesenchyme. This expression became progressively more restricted with age such that by E11 SBP was only located in mesenchyme immediately subadjacent to and in register with epithelial uptake sites. Structures exhibiting 5-HT uptake in E9 embryos included rhombomeres 3 and 5 of the hindbrain, the rhombic lips of the E10 hindbrain, and, in E11–12 embryos, the invaginating lens vesicle, fusing nasal prominences, otocyst, thyroid (Shuey *et al.*, 1993), ectomesenchyme surrounding the tooth germ and the palatal shelves (Lauder and Zimmerman, 1988). Where examined, uptake was found to be negatively correlated with cell proliferation (^{3}H-thymidine labelling), suggesting that 5-HT may normally inhibit cell proliferation (and possibly promote differentiation) in these craniofacial structures (Shuey *et al.*, 1993).

When cultured mouse embryos were exposed to 5-HT uptake inhibitors from E9–11 or E10–11, malformations of the craniofacial region were observed that involved structures expressing 5-HT uptake sites. Moreover, cell proliferation was severely curtailed and cell death increased in mesenchyme of these regions, especially in cells not expressing SBP (which may have provided protection from deleterious effects of excess 5-HT; Shuey *et al.*, 1992). As in the heart, these results were interpreted in the context of the "morphogen" model, whereby excess levels of 5-HT in mesenchyme resulted from inhibition of 5-HT uptake and degradation in adjacent epithelia (see Fig. 5).

These findings may be relevant to craniofacial abnormalities found in Down syndrome and Trisomy 16 mice (Epstein, 1991; Grausz *et al.*, 1991). They should also be considered in light of previous reports indicating that 5-HT, L-tryptophan or tricyclic antidepressants can cause malformations of the skull, brain, spinal cord or vertebral column in rodents and humans (Guram *et al.*, 1982; Idänpään-Heikkila and Saxen, 1973; Jurand, 1980; Van Cauteren *et al.*, 1986). However, recent evidence suggests that highly specific 5-HT uptake inhibitors such as Prozac taken in pregnancy are not teratogenic in humans (Vorhees *et al.*, 1994).

Exposure of cultured mouse embryos to selective 5-HT receptor ligands (agonists or antagonists) caused craniofacial malformations similar to those seen

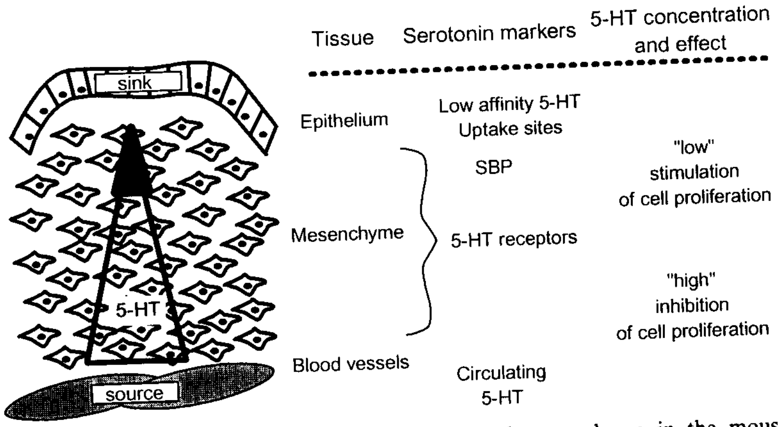

Fig. 5. Serotonin as a morphogen for craniofacial mesenchyme in the mouse embryo. (Adapted by J. R. D. Moiseiwitsch from Fig. 8.1 in Shuey, 1991; based on data presented in Shuey *et al.*, 1992.)

with uptake inhibitors, implicating receptors in the morphogenetic actions of 5-HT (see Lauder *et al.*, 1994). This is supported by a recent study reporting craniofacial malformations in transgenic mice where expression of 5-HT_2 receptors was "knocked out" (Toth *et al.*, 1994). Two classes of 5-HT receptors have been localized in mouse craniofacial mesenchyme using immunocytochemistry with antibodies recognizing 5-HT_{1A} and non-5-HT_{1A} receptor subtypes. These receptors appear to be coordinately expressed with other "morphoregulatory molecules" thought to be involved in craniofacial development, such as tenascin, S-100ß, and insulin-like growth factors (see Fig. 1 in Lauder *et al.*, 1994). Interestingly, different receptor ligands were not equipotent in their teratogenic capacities, suggesting that these effects resulted from activation of particular 5-HT receptor subtypes.

Taken together, these studies provide evidence that 5-HT acts as a dose-dependent regulatory signal or "morphogen" during both craniofacial and cardiac development in the mouse embryo. Possible mechanisms underlying this activity are currently under investigation. To date, these *in vitro* studies suggest that activation of 5-HT_{1A} receptors regulates migration of cranial neural crest cells (Moiseiwitsch and Lauder, 1995), and that antagonists for 5-HT_{1A}, 5-$HT_{1C/2}$ (5-$HT_{2C/2A}$), and 5-HT_3 receptors differentially regulate expression of S-100ß, tenascin, and cartilage proteoglycan core protein (Moiseiwitsch and Lauder, 1993; Moiseiwitsch and Lauder, 1996).

3.2. Serotonin and Neurogenesis

Cells of the serotonergic raphe nuclei are generated early in the embryonic rat brain, prior to most of their target cells. As soon as they are formed, these neurons begin to send axons rostrally, where they soon encounter their earliest targets (e.g., dopamine neurons of the substantia nigra). Depletion of transmitter in developing 5-HT neurons by treatment of pregnant rats with pCPA, an irreversible inhibitor of tryptophan hydroxylase, has been found to delay the onset of differentiation (time of last cell divison) of neurons developing along the serotonergic pathway, including mesencephalic dopamine neurons and 5-HT neurons of the dorsal raphe nucleus. On the contrary, the stress of the daily maternal vehicle injection caused early onset of neuronal differentiaton in the same regions. These results imply that 5-HT (presumably released from growth cones; Ivgy-May *et al.*, 1994) acts as a "differentiation signal" for appropriately placed embryonic target cells (reviewed by Lauder, 1990). This function appears to also apply in some invertebrate embryos, such as the snail *Helisoma,* where depletion of 5-HT in developing serotonergic neurons by treatment with the neurotoxin 5,7-dihydroxytryptamine disrupts dendritic outgrowth by targets of serotonergic axons (Goldberg and Kater, 1989).

The ability of 5-HT to regulate development of its target cells may be mediated by specific 5-HT receptor subtypes. In the rat embryo, it has been demonstrated that prenatal exposure to pCPA, or the general 5-HT_1 agonist 5-methoxytryptamine (5-MT), alters postnatal expression of 5-HT receptors in brain (Whitaker-Azmitia *et al.*, 1987). A recent *in situ* hybridization study has shown that embryonic monoamine neurons and other neuronal populations affected by *in utero* exposure to pCPA express mRNA transcripts encoding 5-HT_{1C} (5-HT_{2C}) and 5-HT_2 (5-HT_{2A}) receptors (Hellendall *et al.*, 1993). Moreover, the 5-$HT_{1C/2}$ (5-$HT_{2C/2A}$) agonist DOI promotes growth of cultured E14 embryonic brainstem 5-HT neurons and mesencephalic dopamine neurons (Liu and Lauder, unpublished). These results must be considered in light of a recent immunocytochemical study suggesting that 5-HT_2 (5-HT_{2A}) receptors do not appear until just before birth (Morilak and Ciaranello, 1994). However, since this study did not examine embryos younger than E19, an earlier peak of 5-HT_2 receptor expression would not have been detected.

Serotonin appears to autoregulate development of cultured E14 5-HT neurons (see Lauder, 1990; and Whitaker-Azmitia *et al.*, 1990, for reviews), and can initiate and autoamplify its own synthesis in hypothalamic cultures (De Vitry *et al.*, 1986). Further evidence for an autoregulatory role of 5-HT comes from *in vivo* studies demonstrating that *Drosphila* mutants incapable of 5-HT synthesis, and adult snails depleted of 5-HT, exhibit aberrant growth of serotonergic and other axons (Baker *et al.*, 1993; Budnik *et al.*, 1989). Similar effects are seen in rats treated prenatally with the 5-HT receptor agonist 5-MT (Whitaker-Azmitia *et al.*, 1990). Taken together, these studies indicate that altered levels of this neurotransmitter may affect development of the serotonergic system in vertebrate and invertebrate embryos.

Although some developmental actions of 5-HT may involve direct activation of neuronal receptors, others may involve activation of glial receptors. In the rat embryo, ependymal glial adjacent to brainstem 5-HT neurons, and radial-like glial cells located along the rostrally projecting serotonergic pathway, both express immunoreactivity for 5-HT_{1A} receptors and S-100ß (see Fig. 1 in Lauder and Liu, 1994). S-100ß acts as a growth factor for 5-HT neurons (Whitaker–Azmitia, 1991; Liu and Lauder, 1992; Ueda *et al.*, 1994), and is released from postnatal astrocytes in response to 5-HT_{1A} agonists (reviewed by Whitaker–Azmitia and Azmitia, 1994). Therefore, it is possible that developing 5-HT neurons could stimulate adjacent glial cells to provide trophic support for themselves by activating 5-HT_{1A} receptors. Evidence in favor of this hypothesis has recently been obtained in studies demonstrating regulation of S-100ß by activation of 5-HT_{1A} receptors in glial cells from embryonic rat brainstem. These cells respond to the 5-HT_{1A} agonist 8-OH-DPAT by increasing intracellular levels of S-100ß protein and cAMP (Lauder and Liu, 1994), and by releasing factor(s) into culture medium that stimulate growth of 5-HT neurons (Lauder, 1993). Although the S-100ß gene has a cAMP regulatory element, second messengers linked to other 5-HT receptors may also regulate its expression, since the 5-$HT_{2A/2C}$ agonist DOI (which does not stimulate cAMP), also upregulates S-100ß in these cells (Liu and Lauder, unpublished).

Developing 5-HT neurons may also regulate production of trophic factors for dopamine neurons by activation of appropriate receptors on mesencephalic glial cells. Growth of cultured embryonic dopamine neurons is stimulated by insulin-like growth factor-II (IGF-II), which does not affect 5-HT neurons, whereas S-100ß promotes growth of 5-HT neurons, but not dopamine neurons (Liu and Lauder, 1992). Preliminary evidence indicates that treatment of embryonic mesencephalic glia with 5-HT, DPAT or the 5-$HT_{2A/2C}$ agonist DOI increases intracellular levels of IGF-II (Liu and Lauder, unpublished).

These studies support the hypothesis that serotonergic activation of appropriate receptors promotes the production of glial-derived factors important for the growth of developing 5-HT neurons and their presumptive target cells. If this is the case, it could represent a general mechanism whereby developing neurons regulate the availabiltiy of neurotrophic factors required for construction of their own neural circuitry.

4. OOCYTES

Evidence for the complex progression from prenervous to neuronal (definitive) neurotransmitter systems was considered above (Sections 2 and 3). Inasmuch as it probably concerns a cyclical process, the return from definitive transmitter systems to prenervous functions must also exist. It was suggested earlier (Buznikov, 1967) that the onset of prenervous functions coincides with the starting point of embryonic development, i.e., fertilization. This point of view seems to be valid, since there is ample evidence, albeit rather indirect, suggesting the possible participation of prenervous neurotransmitters in fertilization events, in particular the demonstrated presence of 5-HT, ACh, and catecholamines in spermatozoids (see Buznikov, 1990a for review). It was also found recently that at least cholinergic and serotonergic systems are necessary for gamete interaction during fertilization (Falugi and Prestipino, 1989; Jaffe, 1990; Falugi, 1993).

Based on further evidence, discussed below, it has become evident that neurotransmitter systems actually exhibit prenervous functions during oocyte maturation (i.e., prior to fertilization). For example, preliminary data suggest that ACh, 5-HT and catecholamines are synthesized by mature, intact (i.e., follicular-envelope enclosed) unfertilized oocytes of starfish and amphibians. In fact, these substances or their close relatives are reportedly present and functional in one-cell and cleaving embryos of various invertebrate and vertebrate species (Buznikov, 1967, 1990a; Emanuelsson, 1992; Falugi, 1993; Rowe *et al.,* 1993; Burden and Lawrence, 1973; Pienkowski, 1977; Sadykova *et al.,* 1992). Further evidence obtained using pharmacological methods indicates that in starfish, oocytes as well as early embryos express both receptor and non-receptor intracellular binding sites for neurotransmitters and their antagonists (Buznikov, 1990a,b, 1993b; Nikitina *et al.,* 1993). Moreover, full-grown oocytes of various animal groups, including starfish and amphibians express classical neurotransmitter receptors for 5-HT, ACh and catechloamines on their surface and on the surface of the follicular envelope. These receptors have mainly been studied with electrophysiological methods (e.g., Kusano *et al.*, 1982; Eusebi *et al.*, 1984; Dascal *et al.,* 1984; Miledi and Woodward, 1989; Greenfield *et al.,* 1990; Yoshida and Plant, 1991; Krantic *et al.,* 1993; Fujita *et al.,* 1993; Arellano and Miledi, 1993; Ji *et al.,* 1993; Durieux, 1993; Sakuta, 1994). Thus, the neurotransmitter systems of the full-grown oocyte have some characteristics typical of the prenervous period of ontogenesis, in particular, the presence of intracellular receptor and non-receptor binding sites, and both intracellular and classical extracellular membrane-bound transmitter receptors. This provides evidence for the reutrn to prenervous neurotransmitter systems in unfertilized, full-grown oocytes.

Concerning the possible functional activity of neurotransmitters during oocyte maturation, these cells are normally blocked in prophase of their first meiotic division, and need to receive a signal for the reinitiation of meiosis. As discussed below, evidence suggests that neurotransmitters can serve as reinitiation signals in those species that do not have special maturation hormones to play this role (e.g. marine bivalve molluscs). Alternatively, in animals such as starfish or vertebrates that employ maturation hormones secreted by follicular cells to accomplish this task, neurotransmitters may act as modulators of these signals.

The first scenario has been established only for 5-HT, which removes the block of meiosis in oocytes of *Spisula* and other marine bivalve molluscs, whereas certain 5-HT antagonists prevent this action (Abdelmajid *et al.,* 1994; Deridovich and Reunova, 1993; Guerrier *et al.,* 1993; Krantic *et al.,* 1993; Togo *et al.,* 1993; Juneja *et al.,* 1994). The 5-HT receptors located on the oocyte surface have been described as a new receptor subtype (5-HT_5), but they actually appear to be similar to known 5-HT receptors (5-HT_{1A} and 5-HT_2; Krantic *et al.,* 1993). Although it is unknown whether 5-HT that acts as a trigger of oocyte maturation is synthesized in the oocytes themselves, there is evidence for a maternal source of 5-HT deriving from serotonergic terminals surrounding ovarian follicles in bivalves (Paulet *et al.,* 1993; Ram *et al.,* 1992). In any case, the cyclical return from definitive to prenervous transmitter functions can be demonstrated by this example, at least from a functional point of view.

As to the possibility that neurotransmitters modulate hormonally evoked oocyte maturation, data have been obtained suggesting that 5-HT acts as a positive modulator of 1-methyladenine, the maturation hormone of starfish oocytes, whereas 5-HT antagonists inhibit 1-methyladenine action (Buznikov *et al.,* 1993b; Shilling *et al.,* 1994). In starfish oocytes, 5-HT could be synthesized by follicular cells or some maternal tissues. These actions of both 5-HT and its antagonists appear to involve second messengers (such as cAMP and Ca^{2+}) and are mediated through membrane 5-HT receptors expressed by the oocyte rather than by any receptors on follicular cells (which produce 1-methlyadenine; Buznikov *et al.*, 1990a,b, 1993b). It is quite possible (but not yet proven) that the same receptors take part in the above-mentioned blastomere interactions during the first cleavage division when the blastomeres themselves are the source of 5-HT (or 5-HT-like substances).

In amphibians, 5-HT acts as negative modulator of the oocyte maturation hormone, progesterone. 5-HT inhibits the action of progesterone in both intact and denuded oocytes. Likewise, 5-HT antagonists potentiate the action of progesterone and actually induce maturation of denuded amphibian oocytes (Nikitina *et al.*, 1988, 1993; Buznikov *et al.*, 1993b). It is thought that 5-HT is the main endogenous inhibitor of meiosis in full-grown oocytes, and continues to play that role until the start of the breeding season. Moreover, it is possible that progesterone, as it evokes reinitiation of meiosis, actually acts as a 5-HT antagonist. This functional antagonism could be produced at the level of second messenger systems (i.e., intracellularly). This possibility is supported by the finding that 5-HT inhibits oocyte maturation evoked both by progesterone and an activator of protein kinase C, phorbol myristate acetate (Nikitina and Buznikov, in preparation).

Multiple 5-HT receptor subtypes (as yet unidentified) appear to exist in amphibian oocytes. These receptors are expressed in at least three locations: the surface of follicular cells, the surface of oocytes, and in the ooplasm (Buznikov *et al.*, 1993b). Hypothetically, follicular cell surface receptors should only be accessible to 5-HT from maternal sources, whereas intracellular receptors are probably accessible only to 5-HT synthesized by the oocyte itself, as for prenervous 5-HT in blastomeres, discussed above. On the other hand, oocyte cell surface receptors are probably targets of 5-HT from both sources. This multiplicity of potential ligand-receptor interactions may have functional significance. For example, experiments on oocytes exposed to the 5-HT-antagonist inmecarb methiodide have shown that sensitivity to its progesterone-like effects undergo seasonal changes that are not the same in intact and denuded oocytes (Fig. 6), suggesting that the presence of follicular cells in intact oocytes may contribute to these differences. Therefore, the action of 5-HT as an endogenous inhibitor of oocyte meiosis could change between the beginning and end of the breeding season by the successive switching over of extracellular and perhaps intracellular receptor pools. These studies also indicate that intact and denuded amphibian oocytes can be very useful for investigating processes regulated by neurotransmitters during the reversion from definitive to prenervous signalling mechanisms.

Synaptic neurotransmitters must be present in sufficient quantities to interact with the appropriate postsynaptic receptors. Similarly, sufficient quantities of these substances must be present in the space between the follicular envelope and the surface of the full-grown oocyte, (i.e., in the follicular fluid) to regulate oocyte maturation. Although this has yet to be directly demonstrated, it has recently been found that physiological concentrations of biogenic amines,

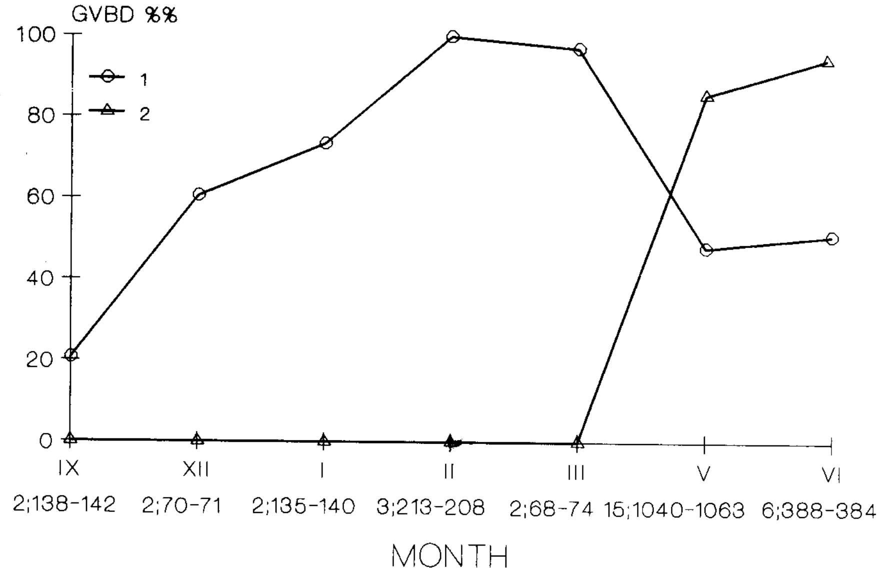

Fig. 6. The seasonal changes of the sensitivity of intact (1) and denuded (2) *Bufo viridis* oocytes to the 5-HT antagonist inmecarb methiodide (200 μM). The number of females, intact oocytes and denuded oocytes are given for each month (first, second and third number, respectively). Abscissa, months; ordinate, percentage of oocytes where germinal vesicle breakdown occured (GVBD).

including 5-HT, do occur in human follicular fluid (Bodis *et al.*, 1993a). Moreover, 5-HT, A and DA control the secretion of progesterone by human follicular cells (Bodis *et al.*, 1992, 1993b). Serotonin appears to participate in such regulation in telosts as well (Iwamatsu *et al.*, 1993). These findings suggest the presence of corresponding receptors at the surface of these cells and the possible participation of biogenic amines in the regulation of oocyte maturation. In addition, ACh is known to be a positive modulator of progesterone action on mature amphibian oocytes. This action is mediated by second messengers, in particular cGMP, linked to activation of muscarinic receptors located on the folliclar and oocyte cell surfaces (Dascal *et al.*, 1984; Miledi and Woodward, 1989; Matus-Leibovich *et al.*, 1993). There are also data suggesting the possible participation of the cholinergic system in the maturation of starfish (Falugi, 1993) and human oocytes (Malinger *et al.*, 1989). Unfortunately, the existence of intracellular receptive sites for ACh similar to those for 5-HT has not yet been investigated.

Neurotransmitters participate not only in the control of oocyte maturation, but also appear to be involved in the preparation for events following fertilization, i.e., for embryonic development. Asymmetry in the distribution of cholinergic receptors on the *Xenopus* oocyte membrane probably reflects the participation of these receptors in ooplasm reorganization following fertilization that is important for predetermining the animal-vegetative axis of the embryo (Oron *et al.*, 1988; Matus-Leibovitch *et al.*, 1993).