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Ligand-dependent stimulation of introduced mammalian brain receptors alters spicule symmetry and other morphogenetic events in sea urchin embryos

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Abstract

Zygotes of *S. purpuratus* were injected with synthetic mRNAs encoding rodent brain neurotransmitter receptors, and specific developmental phenotypes were produced on addition to the sea water of the respective ligands. Most of these experiments were carried out with a mouse serotonin receptor (5HT-R) mRNA, though exactly comparable results were obtained with a rat muscarinic acetylcholine receptor (MAChR) mRNA; these receptors are expected to couple to the same endogenous signal transduction system. We show by whole mount in situ hybridization that the injected mRNAs diffuse to all of the early blastomeres, and that they are translated in vivo. Three specific phenotypes were reproducibly observed. The most severe, occurring at highest levels of injected mRNA, was a cleavage arrest phenotype in which no overtly differentiated cells ever appear, though the embryos remain alive for at least 72 h. A gastrular arrest (GA) phenotype is generated in appreciable fractions of embryos developing from eggs injected with lower levels of mRNA. In GA embryos the blastocoel is filled with disorganized mesenchyme cells, including pigment cells and skeletogenic cells; there is no archenteron; and the entire ectoderm expresses an oral ectoderm cell surface marker. The least severe phenotype that we recognized displays an altered arrangement of spiculogenic foci (RSE phenotype), generating a ring of extra spicules that are properly positioned with respect to the animal/vegetal axis, but that lack any reference to the oral/aboral axis. However, use of cytological and molecular markers demonstrates that RSE embryos retain normal spatial patterns of aboral and oral ectoderm. They develop a fully formed archenteron, but fail to form either a stomodaeum or a ciliated band. RSE embryos can be produced in embryos expressing the 5HT-R by exposure to serotonin, beginning as late as 12 h postfertilization (pf). All of the morphogenetic processes affected in RSE embryos depend in normal embryos on intercellular interactions occurring at the blastula-gastrula stages of development.

Key words: Signal transduction; Cellular interaction; Conditional specification; 7TD receptor; Serotonin; Nuclear distribution

1. Introduction

This work was undertaken in an effort to discover a means of perturbing intercellular signaling functions in the early sea urchin embryo. Intercellular signaling clearly underlies the conditional specification processes by which several of the polyclonal territories of the late cleavage embryo are initially established, while the embryonic structures deriving from these territories are generated through morphogenetic interactions that occur later in development (Hörstadius, 1973; Wilt, 1987; Ettensohn, 1992; McClay et al., 1992; Ransick and Davidson, 1993; Davidson, 1986, 1989, 1991, 1993). In this paper we describe an initial attempt to affect morphogenesis experimentally, by ectopically stimulating signal transduction systems, using foreign receptors that might be able to couple to these systems. We chose for this study two rodent brain neurotransmitter receptors that in mammalian cells are known to couple to the phosphatidyl-inositol (PI) signal transduction pathway.

The general involvement of the phosphotidylinositol (PI) signal transduction system in embryonic spatial specification processes is suggested by various kinds of evidence. Lithium ion is teratogenic in many systems and it is believed to inhibit the inositol mono- and biphosphatases that regenerate myo-inositol. Thus, Li^+

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directly affects the operation of the PI cycle (reviewed by Berridge, 1993). A good example has been documented in *Xenopus* embryos where the teratogenic effect of Li^+ can be cancelled by the co-injection of myo-inositol (Busa and Gimlich, 1989). If Li^+ is injected into ventral blastomeres of a 5th-6th cleavage *Xenopus* egg a second dorsal mesodermal axis is generated, while if *myo*-inositol is coinjected, normal development ensues. During this stage, when the initial events of dorsal mesoderm specification are taking place, there occurs a significant increase in the concentration of inositol 1,4,5-trisphosphate, which is Li^+ sensitive (Maslanski et al., 1992). Furthermore, these authors report that the introduction and stimulation of exogenous serotonin receptors in *Xenopus* eggs blocks dorsal axis formation. In sea urchin embryos Li^+ has long been known as a potent teratogen that particularly affects specification processes along the animal/vegetal axis. Li^+ causes respecification of ectodermal lineages as vegetal plate and gut precursors ('vegetalization') in both intact embryos and isolated animal pole ectoderm preparations (early experiments reviewed by Lallier, 1964; Hörstadius, 1973; see Livingston and Wilt, 1989, for experiments utilizing molecular markers). These well known effects also have been interpreted as specific consequences of the inhibition of the PI cycle, which in normal embryos might mediate cellular responses to signaling interactions between blastomeres (Berridge et al., 1989; Davidson, 1989). Recently it was reported that addition of *myo*-inositol to the sea water blocks the teratogenic effects of Li^+ on intact embryos (Giudice et al., 1992). Another current study (Livingston and Wilt, 1992) shows that application of a phorbol ester (TPA) to sea urchin embryo cultures causes excess specification of gut and skeletogenic cells. These effects are much like those of Li^+, and are synergistic with simultaneous Li^+ treatment; TPA acts like diacylglycerol, a major effector generated by stimulation of the PI cycle.

The experiments we report here were carried out with serotonin 1C receptors (5HT-R) from mouse. Both the rat and mouse genes have been cloned from choroid plexus (Lübbert et al., 1987; Julius et al., 1988). We also used an M1 muscarinic acetylcholine receptor (MAChR) cloned from rat cerebral cortex (Bonner et al., 1987). On stimulation these receptors act through trimeric G-proteins to cause the cleavage of membrane bound phosphatidylinositol bisphosphate (PIP_2) by phospholipase C. The products, DAG and IP_3, activate novel cellular functions, including changes in transcription patterns, by two routes (reviewed by Berridge, 1993): the DAG by its stimulatory effect on protein kinase C, and the IP_3 by driving the release of intracellular Ca^{2+} via the PI cycle. Ectopically expressed receptors can couple successfully to the endogenous PI cycle, as shown by a strong increase in the intracellular Ca^{2+} on exposure to serotonin. Julius et al. (1989) showed that transfection of this 5HT-R into fibroblasts that do not normally express it results in a serotonin-dependent transformation of these cells to a tumorigenic phenotype. Similar results have been obtained with the MAChR that we used in some of the following experiments (Lechleiter et al., 1989; Askenazi et al., 1989).

We could not predict what effects, if any, expression and stimulation of these mammalian CNS receptors would produce in developing sea urchin embryos. The results we obtained are striking: we observe three different and unique developmental phenotypes, depending both on the amounts of receptor mRNA introduced, and on stimulation by addition of the appropriate ligand to the embryo culture. In the following we describe parameters for the effective range of receptor mRNA injections, and analyze the least severe of the three phenotypes, both morphologically and with respect to the expression of certain territorial molecular markers.

2. Results

2.1. Injection of exogenous mRNA into zygotes: global diffusion of injected RNA, and translation in vivo

The egg and early embryo of *S. purpuratus* contains about 30 pg of mRNA (reviewed by Davidson, 1986, p. 71). Perhaps surprisingly, we found that these eggs would tolerate injection of as much as 3 pg of 5HT-R mRNA without affecting embryogenesis, and would still undergo cleavage after injection of 30 pg of this mRNA. In most of the following experiments we operated in the range 0.01 to 1 pg of mRNA injected per egg. For the 2.3 kb 5HT-R message a 1 pg inoculum is about 8×10^5 molecules of receptor mRNA per egg, and for the 2.8 kb MAChR message, about 6.3×10^5 molecules of mRNA per egg.

Two essential conditions for these experiments to be meaningful are that the exogenous mRNA diffuse to all regions of the embryo after injection, and that it be translated in vivo at an acceptable efficiency. In Fig. 1 we display a whole mount in situ hybridization carried out on 16-cell embryos that had been raised from zygotes injected with about 8×10^4 molecules of 5HT-R mRNA. The antisense probe easily detects the exogenous transcript at this level (equivalent to about 50 mRNA molecules/average cell at the end of embryogenesis), and as Fig. 1 clearly indicates, the message is present in every cell. Thus the effects we discuss below cannot be ascribed to adventitious localization of the injected message, and barring any unexpected regional restrictions on translation, the expression of the injected message almost certainly is global as well.

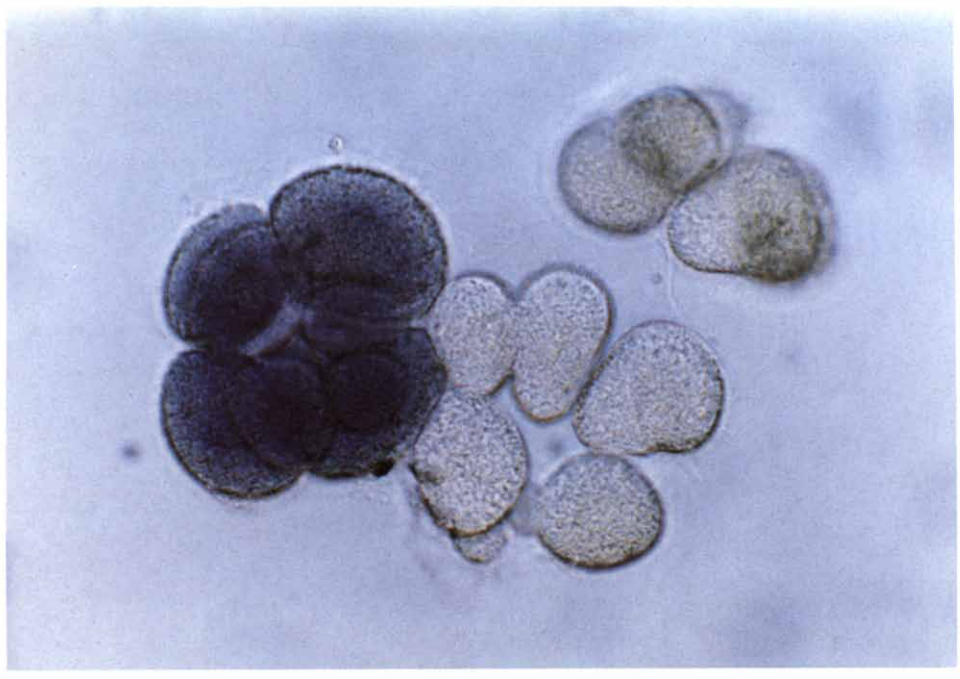

Fig. 1. Whole mount in situ hybridization displaying 5HT-R mRNA in a 16-cell embryo that had developed from an egg injected with about 8×10^4 molecules of the mRNA. The partially disaggregated embryo on the right was not injected. The message appears evenly distributed to all the cells of the injected embryo.

That the message is translated after injection is shown directly in the experiment illustrated in Fig. 2. For this experiment a very large amount (4×10^7 copies) of exogenous message was injected, equivalent to about 100% of the mass of the total endogenous pool of mRNA. This was done so that the translation product would be detectable in a reasonable number of injected eggs. The experiment shown in Fig. 2 obviously cannot be used as a measure of the translation efficiency of exogenous mRNA introduced at the far lower levels utilized in the following experiments. The eggs were grown for only two division cycles, and then harvested. As shown in Fig. 2, a strong band at 51 kDa, the expected size of the 5HT-R polypeptide, appears in the display of proteins from the embryos bearing the 5HT-R mRNA. All other major bands are apparent in both lanes. The 5HT-R band amounted to 5% of the total incorporated label, while the injected message represented 50% of the total estimated mRNA content (i.e., exogenous and endogenous). Given that during cleavage these embryos synthesize about 250 pg of protein $\cdot h^{-1}$ at mid-cleavage stages (Goustin and Wilt, 1981), in the experiment in Fig. 2 about 3×10^8 molecules of 5HT-R protein have been synthesized (assuming 2 h of synthesis). In the experiments that follow, in which we obtained a reproducible phenotype at levels of exogenous mRNA that are 1/3000 those used in this experiment, *at least* 10^5 molecules of

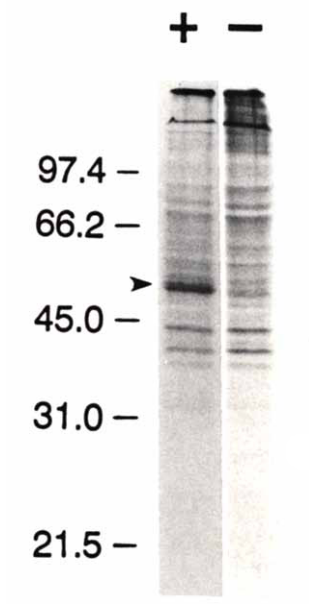

Fig. 2. Autoradiograph of radioactively labeled proteins extracted from embryos that had been injected with either [^{35}S]methionine and 5HT mRNA, or [^{35}S]methionine alone. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The '+' lane contains separated proteins from 522 embryos injected with both 4×10^7 molecules of 5HT mRNA and [^{35}S]methionine, and the '−' lane contains proteins from 658 embryos injected with only [^{35}S]methionine (see Experimental procedures). The prominent 51 kDa band in the + lane (arrow) represents the expected size of the serotonin receptor protein, as determined from the mRNA sequence. All other major bands are identical in both lanes.

5HT-R protein would be expected to have been synthesized (12 molecules of protein per mRNA). However this is indeed a minimal estimate, and the true value, which we could not directly measure in zygotes injected with only 0.01–1 pg of mRNA, could easily be more than an order of magnitude higher: (i), because at much lower levels of exogenous mRNA the efficiency of translation should be closer to its molar proportion of the total message (i.e., up to 10 × higher than in the experiment of Fig. 2); and (ii), because the

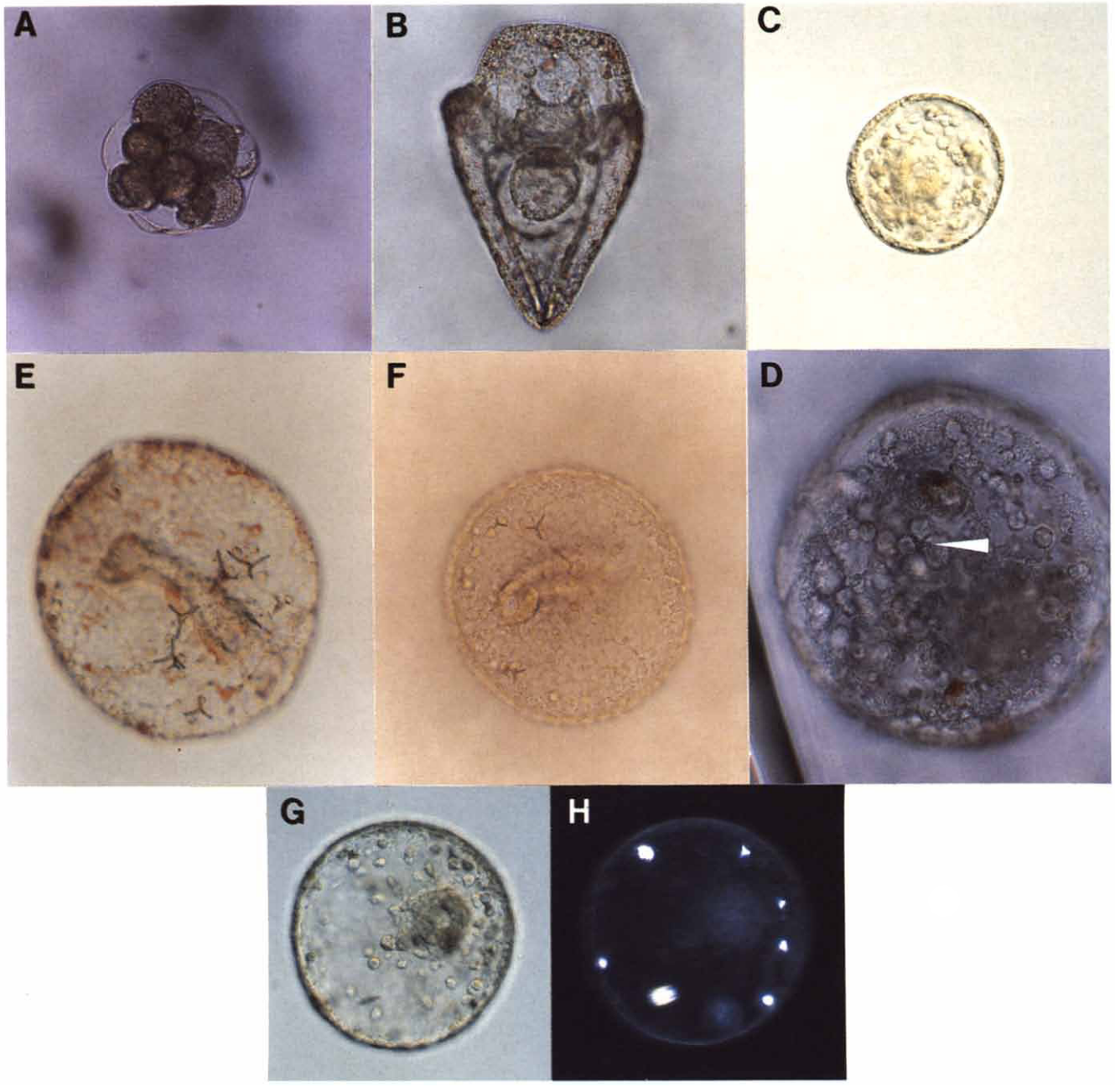

Fig. 3. Abnormal 5HT-R phenotypes observed at 72 h pf. Except where noted, DIC images are shown. (A) CA phenotype generated by exposure of an embryo bearing 8×10^5 molecules of 5HT-R mRNA to 10 μM 5HT 0–12 h pf. (B) Normal 72 h pluteus. This embryo contained no 5HT-R mRNA, but was grown continuously in 100 μM 5HT, 0–72 h pf. (C,D) Two GA phenotype embryos. That depicted in C developed from an egg injected with 8×10^4 molecules of 5HT-R mRNA, exposed to 30 μM 5HT 0–72 h pf; that in D from an egg that had been given 8×10^5 molecules of 5HT-R, and exposed to 1 μM 5HT 24–72 h pf. The arrowhead indicates a small spicule primordium. (E-H) RSE phenotype embryos. (E) and (F) are two RSE embryos, in which the fully extended archenteron, and the multiple spicules can easily be observed. These embryos developed from eggs injected with 8×10^4 molecules of 5HT-R mRNA; the embryo in E was exposed to 5HT 0–72 h pf (30 μM added at T_o) and F was exposed to 1 μM 5HT 0–72 h pf (added at T_o). (G) and (H) are the same embryo viewed from the vegetal pole (H) in polarized light to display the radial arrangement of the 7 triradiate spicule elements. This embryo developed from an egg injected with 8×10^3 molecules of 5HT-R mRNA, that was exposed to 30 μM 5HT 0–72 h pf (30 μM added at T_o).

5HT-R mRNA may continue to be actively translated for many hours into development rather than for just the 2 h of cleavage rate synthesis in the experiment of Fig. 2.

2.2. Embryonic phenotypes induced by stimulation of the exogenous 5HT-R

Three different morphological phenotypes are produced in embryos bearing exogenous 5HT-R mRNA on treatment with serotonin (5HT). In order of decreasing severity these phenotypes are *cleavage arrest* (CA); *gastrula arrest* (GA); and *radialized spicule elements* (RSE). In this section we provide illustrations and brief morphological descriptions of these phenotypes.

CA embryos consist of from 2 to approximately 50 undifferentiated blastomeres usually tightly compacted within the fertilization envelope. These embryos exhibit no overt signs of differentiation, such as pigment cells, spicules or cilia. CA embryos remain alive in this state beyond 72 h pf. An example of a cleavage arrest embryo visualized at 72 h is shown in Fig. 3A, compared to the normal 72 h pluteus shown in Fig. 3B. As in the example illustrated, CA embryos often fail to hatch. This phenotype is generated on exposure to high

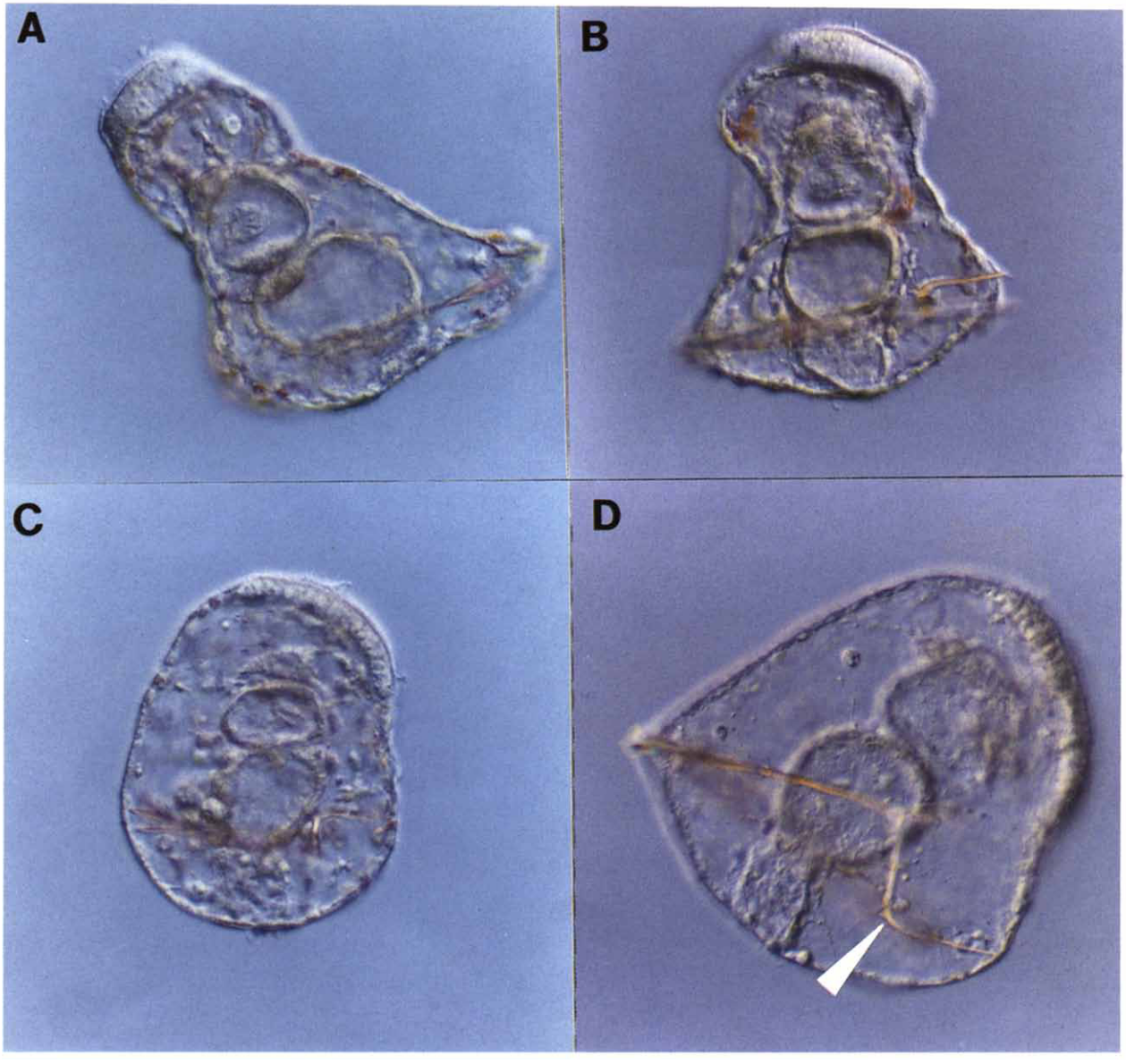

Fig. 4. RSE embryos at 7 days of development. DIC images are shown. These are all side views, with blastopore towards bottom. These examples display the final RSE phenotype: viz, tripartate gut; thickened oral ectodermal region; no ciliated band and no mouth; abnormally disposed, extra spicule rods. The embryo in (D) has one spicule with the anterolateral arm extending in the opposite direction from normal (arrowhead). These embryos developed from eggs injected with 8×10^4 molecules of 5HT-R mRNA, that had been exposed to 20 μM 5HT 0–72 h pf.

levels of 5HT from fertilization onward in embryos bearing relatively high levels of 5HT-R mRNA.

GA embryos consist of an apico-basally polarized, blastula-like spherical epithelium, more or less filled

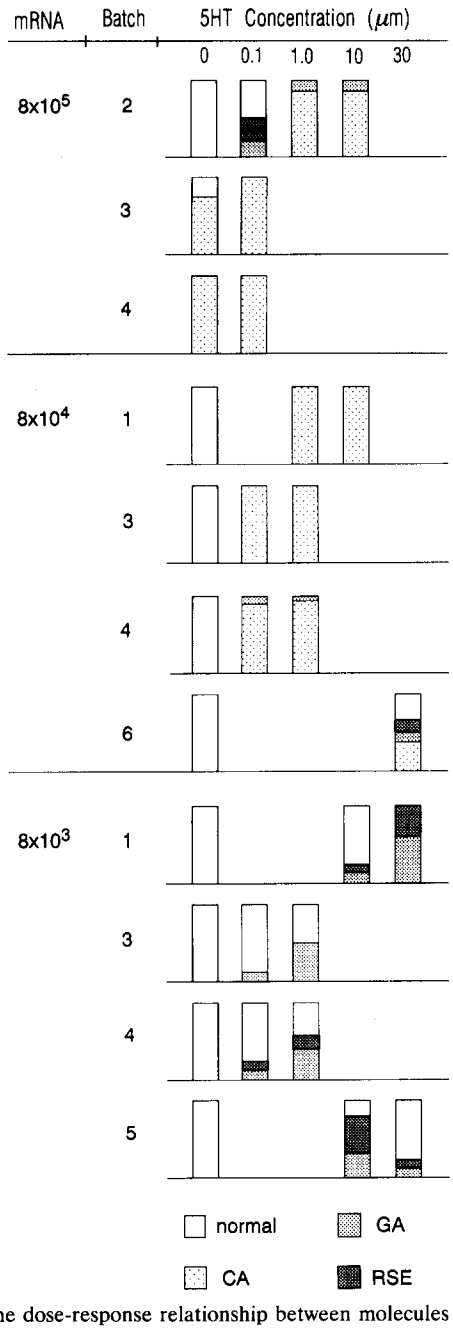

Fig. 5. The dose-response relationship between molecules of 5HT-R mRNA injected into zygotes, the concentration of 5HT to which the embryos were exposed, and the frequency of abnormal phenotypes observed. The observations are presented as 100% bars subdivided according to the proportion of each phenotype recorded. For each experiment or batch of zygotes used, the bars are arranged horizontally by concentration of 5HT used and the batches are grouped by amount of mRNA injected. For example, embryos from two batches (3, 4) were injected with all three amounts of mRNA and exposed to several concentrations of 5HT at each mRNA amount. Clear bars = normal, light stipple = cleavage arrest; medium stipple = gastrula arrest; dark stipple = radialized spicule foci.

with loose, disorganized mesenchymal cells. Sometimes differentiated cells can be observed among these, e.g., echinochrome-containing pigment cells, and skeletogenic mesenchyme cells that secrete spicule rudiments. Two examples are shown in Fig. 3C,D. Even at 72 h pf GA embryos have failed to expand fully their blastocoels (expansion normally occurs at 24 h). The archenteron may be partially invaginated but its morphogenesis does not proceed past the initial stage at which the thickened vegetal plate buckles inward. Cellular rearrangement and elongation of the archenteron do not occur in GA embryos. However, unlike CA embryos, these embryos always hatch out of the fertilization envelope. Development of GA embryos is apparently normal through the mesenchyme blastula stage, and they enter a permanent state of arrest early in gastrulation, though accumulation of mesenchymal cells within the blastocoel may continue. This phenotype occurs at intermediate and lower levels of 5HT-R mRNA and 5HT stimulation.

RSE embryos generate a fully expanded blastocoel that contains a normal complement of skeletogenic mesenchyme cells, and they carry out an apparently normal process of archenteron invagination. By 72 h pf the archenteron reaches all the way across the blastocoel. Furthermore, the distal end of the archenteron often exhibits the normal lateral coelomic pouch evaginations. However, RSE embryos are abnormal in several identifiable respects. Neither a mouth nor a ciliated band is ever formed in these embryos, and the ectodermal wall preserves a spherical form rather than the trapezoidal form characteristic of normal prism and pluteus stage embryos (cf. Fig. 3B). The most striking feature of this phenotype is the appearance of supernumerary (3–9) regular tri-radiate spicules distributed radially around the base of the ectoderm. These spicules often appear arranged in a ring if they are present in sufficient number (i.e., > 5). If fewer are present they may lie haphazardly in the ring of mesenchyme cells, and pairs of spicules may occur at any one location. Examples of the RSE phenotype, visualized at 72 h, are shown in Fig. 3E-H.

If RSE embryos are allowed to develop to 7 days pf they usually produce a tripartite gut possessing the constrictions normally evident at 72 h, and a thickening opposite the blastopore that resembles the normal oral hood, which, however, fails to develop further. Typical examples are shown in Fig. 4A–D. Neither the mouth nor the ciliated band ever form. Often the small supernumerary spicules have by this time coalesced into 3–4 large spicules, two of which lie in approximately normal positions, while the others are disposed haphazardly in the blastocoel. Those spicules not in ectopic positions may be branched as in normal embryos, but sometimes they develop incorrectly. For example, as shown in Fig. 4D the spicule branch which should have

formed the anterolateral or oral arm rod is extended abnormally in an anal direction, rather than in the normal oral direction.

The non-coding 5HT-R transcript has no effect whatsoever, even in high concentration. Thus, e.g., in one experiment eggs bearing 8×10^4 molecules of 5HT-R antisense RNA that were exposed to 10 μM 5HT 0–72 h pf, developed completely normally, while 100% of eggs of the same batch receiving the same amount of 5HT-R mRNA, that were exposed similarly to 5HT, all displayed the drastic CA phenotype. Furthermore, as illustrated in Fig. 3B, continuous exposure of normal eggs that had not been injected with 5HT-R mRNA to even 100 μM 5HT (0–72 h) does not visibly perturb normal development.

2.3. 5HT-R mRNA levels and the concentrations of 5HT to which they are exposed determine the embryonic phenotype

We next tested the responses of a number of different batches of eggs, over a 100 × range of injected 5HT-R mRNA concentrations. Zygotes were exposed to 5HT concentrations varying from 0–30 μM, in experiments in which the ligand was present over the whole period 0–72 h pf. Different batches of eggs responded to the treatments slightly differently. At the highest inocula of 5HT-R mRNA tested in this series of experiments, 8×10^5 molecules of mRNA per egg, only the CA phenotype is usually observed, as e.g., with batches 3 and 4 of Fig. 5. Occasional batches, such as batch 2 of Fig. 5, appear less sensitive and behave more like the majority of egg batches injected with lower levels of message. The CA phenotype was generated in batches 3 and 4 in 80% and 100% of eggs, respectively, even without any addition of the 5HT ligand, suggesting that at this level of expression there is sufficient background stimulation of the foreign receptor to trigger the internal events that lead to cleavage-stage arrest. However, an important result shown in Fig. 5 is that at inocula of 8×10^4 and 8×10^3 molecules of 5HT-R mRNA per egg, none of the abnormal embryonic phenotypes is ever observed unless 5HT is added to the culture medium.

The most interesting results in Fig. 5 are those obtained at 8×10^3 and 8×10^4 molecules of injected 5HT-R mRNA per egg. At these levels various frequencies of GA and RSE phenotypes are generated, though there is considerable batch-to-batch variability. Within each batch, however, the results tend to be consistent. For example, batch 3 eggs produced only the CA phenotype when injected with 8×10^4 molecules of 5HT-R mRNA per egg, and exposed to either 0.1 M or 1.0 M 5HT, but these same eggs produced significant fractions of GA phenotype embryos when injected instead with 8×10^3 molecules of 5HT-R mRNA and then exposed to 0.1 μM or 1 μM 5HT. Batches 1, 4 and 5 generated significant fractions of GA and RSE embryos after injection of only 8×10^3 molecules of 5HT-R mRNA, while in other batches these phenotypes were produced in large fractions of embryos developing from eggs that received inocula of 8×10^4 mRNA molecules. In the course of the experiments described below we frequently encountered batches bearing this amount of 5HT-R mRNA in which > 70% of the embryos produced the RSE phenotype on stimulation with 10–30 μM 5HT.

2.4. Eggs bearing muscarinic acetylcholine receptors also generate CA and RSE phenotypes on stimulation with carbachol

Despite the batch-to-batch variability displayed in Fig. 5 it is clear that the abnormal phenotypes occur within certain ranges of 5HT-R mRNA inocula, and at certain concentrations of 5HT ligand, and that within these ranges neither the mRNA nor the ligand alone produces these phenotypes. The implication is that these phenotypes result from ectopic stimulation of the endogenous embryonic signal transduction systems to which the 5HT-R couples. To test this implication further we turned to an entirely different receptor, the MAChR, which is also coupled by way of a G protein to the PI system. Like the 5HT-R, the MAChR has also been shown to activate this system when introduced into cells that normally do not express it (see Introduction).

Results from these experiments, which were carried out with the same protocol as those just reviewed, are shown in Fig. 6 (batches 1 and 2). Carbachol, a potent

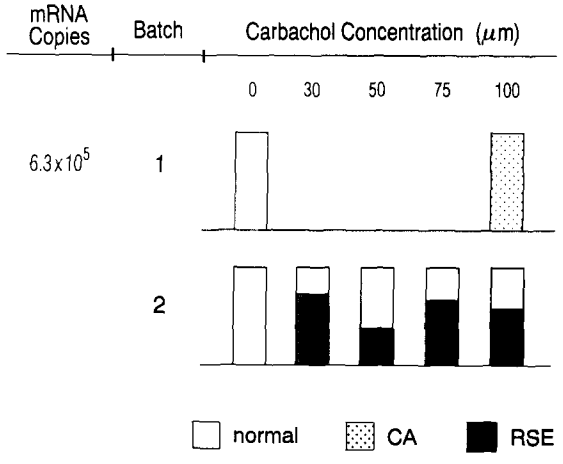

Fig. 6. The frequency of abnormal phenotypes resulting from injection of MAChR mRNA and exposure to carbachol. The bars each represent 100% of the observed embryos at each concentration of carbachol. Two separate batches of zygotes were injected at 6.3×10^5 copies of mRNA. Open bars = normal; lightly stippled bars = cleavage arrest; darkly stippled bars = RSE.

agonist for this receptor, has no effect on control embryos not expressing the MAChR. As in the case of the 5HT-R, the alterations in phenotype resulting from MAChR and carbachol are variable between batches. Exposure of embryos developing from zygotes injected with 6×10^5 molecules of MAChR mRNA to 100 μM carbachol produced 100% CA phenotype embryos in batch 1, but in batch 2, exposure to carbachol concentrations between 30 and 100 μM produced the RSE phenotype in almost 3/4 of the embryos. Other batches (data not shown) produced both GA and RSE embryos. This observation strongly supports the argument that in sea urchin embryos, as in mammalian cells, both receptors couple to the same intracellular pathway(s), and that it is ectopic stimulation of these pathways that lead frequently to the observed embryological phenotypes.

2.5. *Onset of sensitivity: how early in development must the stimulus be applied to produce the RSE phenotype?*

In normal embryos the bilateral pair of skeletogenic foci appears only at the early gastrula stage (> 30 h pf), when they can first be discerned as two clusters of skeletogenic mesenchyme cells at the base of the archenteron on the future oral side. The radially arranged, ectopic skeletogenic foci could be formed in RSE embryos by interference with the initial mechanism by which the oral/aboral axis is established. This occurs early in cleavage, and the specification of the particular lineages that generate the oral and aboral ectodermal territories is completed by mid-late cleavage (Cameron et al., 1987, 1989; Davidson, 1989). In this case production of the RSE phenotype in embryos bearing the 5HT-R mRNA might require exposure to 5HT during cleavage. To determine the latest time when embryos bearing the 5HT-R could be subjected to 5HT stimulation and yet produce the RSE phenotype, we carried out two experiments, results of which are summarized in Fig. 7. In one experiment 30 μM 5HT was added at 0, 12 and 24 h after injection of 8×10^3 molecules of mRNA; in the other, 10 μM 5HT was added at 0, 6, 12, 18 and 24 h after injection of 1.6×10^6 molecules of mRNA. Both experiments show clearly that the RSE phenotype, and the GA phenotype as well, can be produced at maximum frequencies in eggs even when exposed to no ligand whatsoever until 12 h pf. By this time cleavage is complete, and all the initial territorial specifications of the embryo have occurred (Davidson, 1989; Cameron and Davidson, 1991). It follows that the RSE phenotype is not the consequence of interference with the initial embryonic processes by which the spatial domains of the oral and aboral ectoderm are established.

In Fig. 7 it can be seen that the efficiency of the 5HT-R stimulation producing the RSE and GA phenotypes declines sharply if the ligand is not added until 18 or 24 h pf. This is consistent with the possibility that the sensitive period is confined to the early-mid blastula stage, but such a conclusion cannot be drawn in the absence of additional data demonstrating the continuing presence and functionality of the receptor this many hours after injection of the mRNA. There is no direct evidence on this point, and we can adduce only a qualitative observation that may be relevant. Fig. 8 shows a typical embryo bearing 8×10^4 molecules of injected 5HT-R mRNA, which was exposed to serotonin (30 μM) only at 24 h pf. On addition of the ligand such embryos immediately exhibit disruption of the normal blastula wall and an easily visible, change in epithelial cell shape from an apically polarized epithelium cell type to a rounded cell type. The 5HT-R would therefore appear to have been present and functional, though to what relative extent we cannot say. In this same experiment, sibling zygotes injected with the same amounts of 5HT-R and initially exposed to 30 μM 5HT were arrested in cleavage and while those not exposed to 5HT developed normally.

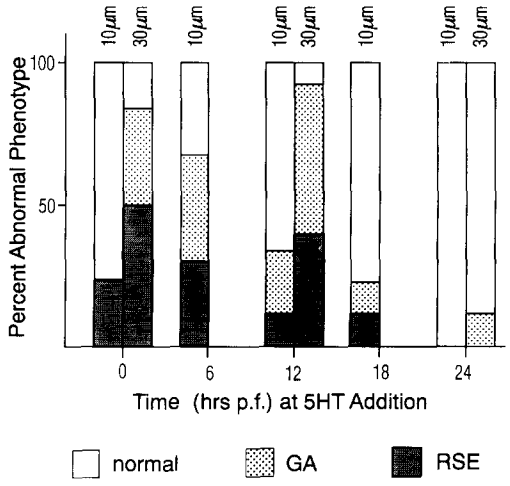

Fig. 7. Onset of sensitivity to 5HT-R stimulation for the generation of GA and RSE phenotypes. Two experiments are shown. The bars marked 30 μM represent phenotype frequency in embryos developing from a batch of eggs injected with 8×10^3 copies of 5HT-R mRNA, and exposed to 30 μM 5HT beginning at 0, 12 and 24 h pf. The bars marked 10 μM represent a separate experiment in which the eggs were injected with 1.6×10^6 molecules of 5HT-R mRNA, and the embryos were exposed to 10 μM 5HT beginning at 0, 6, 12, 18 and 24 h pf. Observations were made at 72 h, as in Fig. 3. Legend: clear bars are normal, stippled bars represent GA and dark bars are RSE.

2.6. *The aboral ectoderm is normally positioned and expresses normal regulatory functions in RSE embryos*

The RSE embryos shown in Figs. 3E,F,G,H and 4 could be interpreted as generally radialized, since their skeletogenic foci are arranged in a circle, and they

retain an overall spherical shape. This was our initial impression on encountering this phenotype. Truly radialized embryos would lack an oral/aboral axis, which in normal embryos is manifested by the bilateral disposition of the oral and aboral ectodermal territories that define this axis ab initio. At first these territories can be visualized in normal embryos only by their expression of distinct molecular markers, but as gastrulation begins, they become morphologically differentiated. The normal pair of skeletogenic foci is among the earliest indications of the future oral side, followed by the thickening of the ectodermal wall on that side, and ultimately the fusion of secondary mesenchyme cells emanating from the extending archenteron with the stomodeal region of the oral ectoderm (reviewed by McClay et al., 1992). The mouth forms either in the portion of the oral ectoderm contributed by the *No* eight-cell blastomere or at the boundary between the progeny of the *VO* and *No* eight-cell blastomeres (Cameron et al., 1987); the exact position has not been established by lineage tracing. The ciliated band arises at the boundary of the oral and aboral ectoderm territories (Cameron et al., 1993). RSE embryos could be regarded as radialized, or axis deficient, since they lack

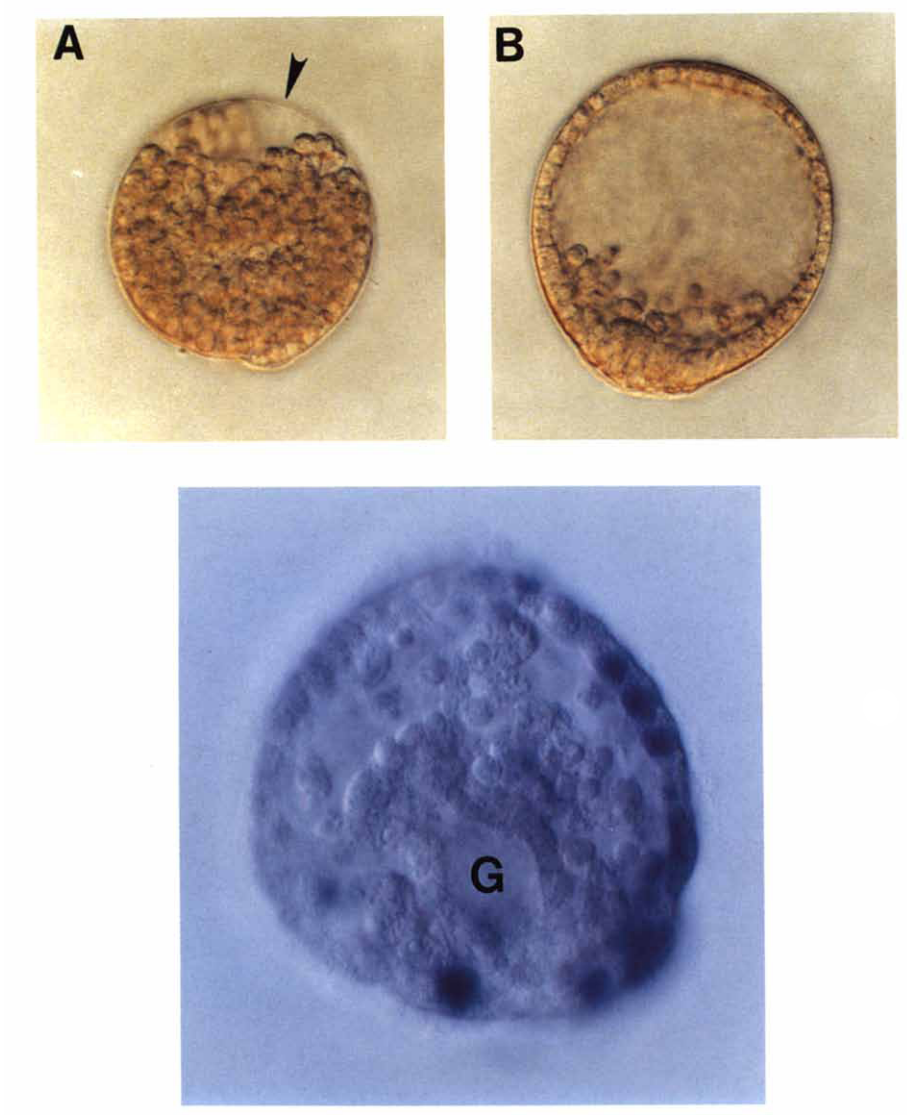

Fig. 8. Effect on blastula epithelial wall of late exposure to serotonin. Eggs were injected with 8×10^4 molecules of 5HT-R mRNA. (A) 30 μM 5HT added at 24 h, photographed shortly afterwards. (B) Normal appearing 24 h late blastula, not exposed to 5HT; the arrowhead indicates the hyaline layer where cells have completely lost contact.

Fig. 9. Whole mount in situ hybridization displaying aboral ectoderm expression of *CyIIIa · CAT* genes in RSE embryos. Eggs were injected with 8×10^3 molecules of 5HT-R mRNA + *CyIIIa · CAT* (see Experimental procedures), exposed to 30 μM 5HT, and at 72 h pf fixed for whole mount in situ hybridization with an antisense CAT RNA probe. The embryo is viewed from the front, with the oral ectoderm out of focus toward the viewer. Only aboral ectoderm is in focus. The blastopore is downward.

all of the following definitive oral features: these embryos remain spherical rather than assuming a tetrahedral form; their archenterons never fuse with the oral ectodermal wall, and neither the stomodaeum nor the ciliated bands ever form. Since the radial arrangement of spicules could result from a complete loss of early oral-aboral ectodermal specification, it is important to examine the spatial patterns of expression for markers of artifacts in RSE embryos.

To investigate aboral ectoderm functions in these embryos we used the *CyIIIa · CAT* reporter gene, the expression of which has been shown to be confined to aboral ectoderm clones (Hough-Evans et al., 1988, 1990; Zeller et al., 1992; and unpublished whole mount in situ hybridization data). We injected this reporter construct together with 8×10^4 molecules of 5HT-R mRNA, and exposed the embryos to 30 μM 5HT. Embryos displaying the RSE phenotype were selected, and prepared for whole mount in situ hybridization with a CAT antisense RNA probe. Approximately 100 embryos that displayed CAT transcript were obtained in two different experiments. Normal spatial patterns of expression of CAT mRNA were revealed in these embryos by whole mount in situ hybridization. That is, except for five embryos in which single cells were labeled in the oral ectoderm, all displayed patches (i.e., clones) of stained cells that were confined entirely to the aboral ectoderm, here defined as those regions of the embryonic ectoderm *other* than the surface toward which the archenteron inclines (RSE embryos retain a very clear association of the extended archenteron with one ectodermal wall, which we show explicitly in the following section is the oral ectodermal wall). An example is illustrated in Fig. 9. These experiments thus reveal that, in direct contrast to embryos radialized by treatment with $NiCl_2$, the RSE embryos retain a more or less normally positioned and normally extensive aboral ectoderm. Furthermore, the cells of this ectodermal territory are capable of presenting the *trans*-regulatory factors required to differentially activate an exogenous aboral ectoderm-specific regulatory system, just as in normal embryos.

2.7. RSE embryos retain a normally positioned oral ectoderm

We utilized two different features to identify the oral ectoderm in RSE embryos, viz, a previously unnoticed but clearly distinct oral ectoderm cell shape character; and the spatial disposition of the Ecto V antigen discovered by Coffman and McClay (1990).

During normal development the cells of the aboral ectoderm flatten from their originally cuboidal shape to form a squamous epithelium. This phenomenon does not occur in the oral ectoderm, where the cells remain thickened. Hence there is a higher nuclear density in the oral ectoderm, i.e., a shorter average distance between nuclei, which can easily be detected if the embryos are stained with the nuclear fluorescent dye Hoechst 33342. The distinction between oral and aboral ectoderm in nuclear packing density is illustrated in normal embryos in Fig. 10A,B and in Fig. 10C–E an RSE embryo is shown. In Fig. 10A,B, the embryos are oriented so that the one on the left displays aboral ectoderm as seen from the posterior side and the one on the right shows oral ectoderm as seen from the frontal view. The frontal view also displays the archenteron just below the ectoderm. The nuclei as revealed by staining and epifluorescent illumination are clearly distributed more sparsely in the aboral ectoderm than in the oral. The DIC image in Fig. 10C shows an RSE embryo viewed from the side, with the blastopore toward the bottom of the picture. The skeletogenic mesenchyme cells lie in a circle around the blastopore and are associated in seven skeletogenic foci, each with a nascent spicule, as can be seen in polarized light, in Fig. 10D. Fig. 10E,F shows epifluorescent images of the same embryo at two different focal planes after staining with Hoechst 33342. Fig. 10E is focused at the near surface in the region where the archenteron approaches the ectoderm. Here there is a discrete patch of cells with closely distributed nuclei. On the far surface of the embryo seen in Fig. 10D, the nuclei are more widely distributed, just as seen in the aboral ectoderm of normal embryos (cf.

Fig. 10. Nuclear density in oral and aboral ectoderms of normal and RSE embryos. (A,B) Normal embryos, stained with Hoechst 33342, and viewed in DIC or epifluorescent light. Two normal embryos seen in DIC (A) or epifluorescent illumination (B). The embryo on the left is a posterior view showing aboral ectoderm and the embryo on the right is an anterior view showing oral ectoderm and the archenteron just below. In epifluorescent light nuclei, which appear light against the blue background, are much more closely packed in the oral ectoderm. (C–F) RSE embryo, also stained with Hoechst 33342, that developed from an egg injected with 9×10^4 molecules of 5HT-R mRNA, and exposed to 30 μM 5HT 0–72 h pf. (C) DIC image of the embryo with the vegetal pole upward. The skeletogenic mesenchyme cells form a prominent ring around the blastopore. (D) Polarized light image of the same embryo. Two large and 5 small spicules lie in the ring of skeletogenic cells. (E) Epifluorescent image of the same embryo with the focus on the region toward which the archenteron lies, shown towards the lower right. The relatively dense distribution of nuclei in this area is evident and a dashed line shows the boundary between the two regions. (F) An epifluorescent image of the embryo with the opposite side of the ectoderm in focus. Here the nuclear distribution is much less dense than in D.

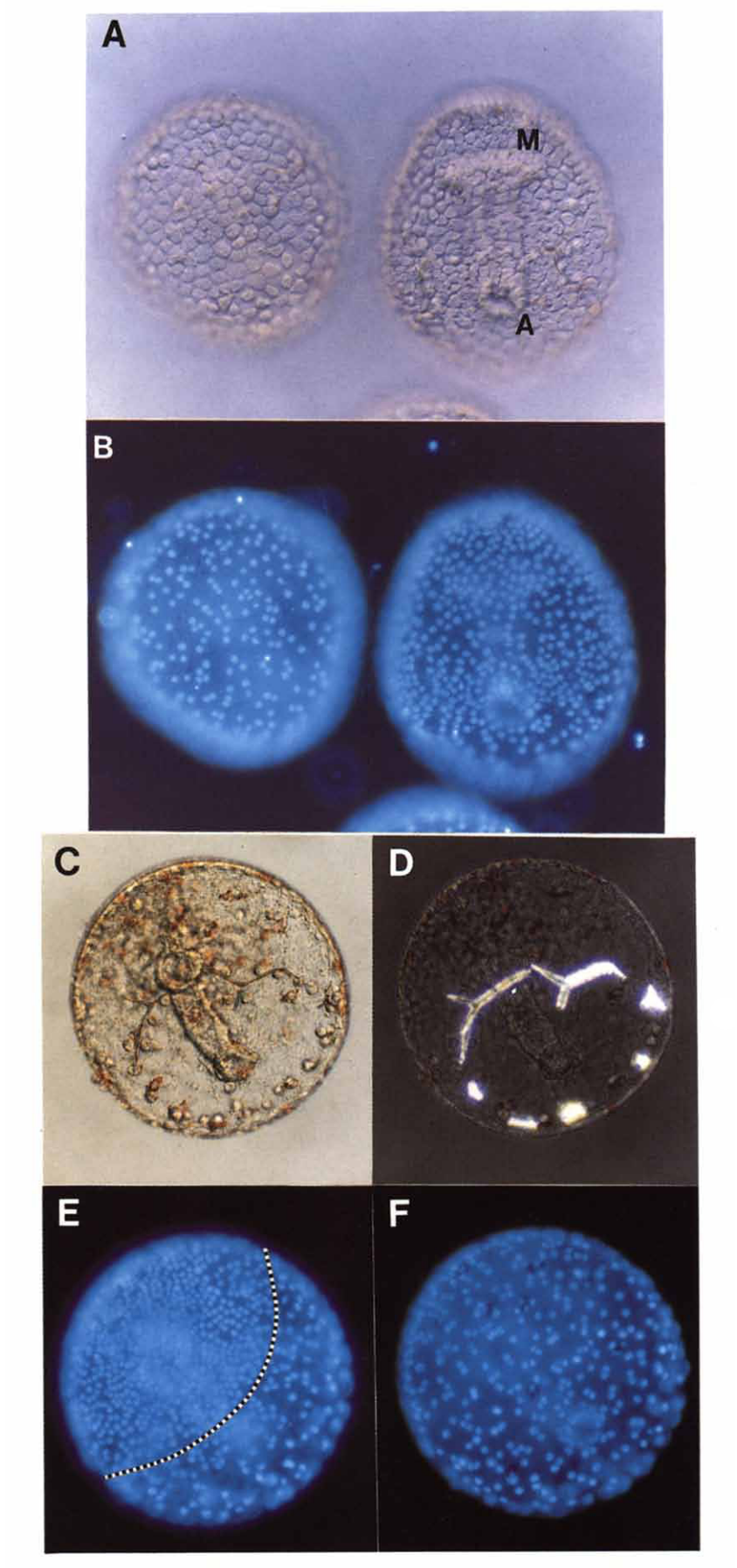
A
M
A
B
C
D
E
F

Fig. 10B). Observations such as these show clearly that in RSE embryos the region identified by proximity to the archenteron displays the same dense nuclear packing as does the oral ectoderm of normal embryos. Concomitantly, the other ectodermal surfaces of the RSE embryo display the more dispersed nuclear distribution typical of the aboral ectoderm of a normal embryo. We note that since the aboral ectoderm is not elongated by the extending skeletal rods in the spherical RSE embryos, the flattening of the squamous epithelium must be driven internally, that is, by cell shape changes, rather than passively, by stretching.

Fig. 11 confirms unequivocally that RSE embryos retain an essentially normal oral ectoderm territory.

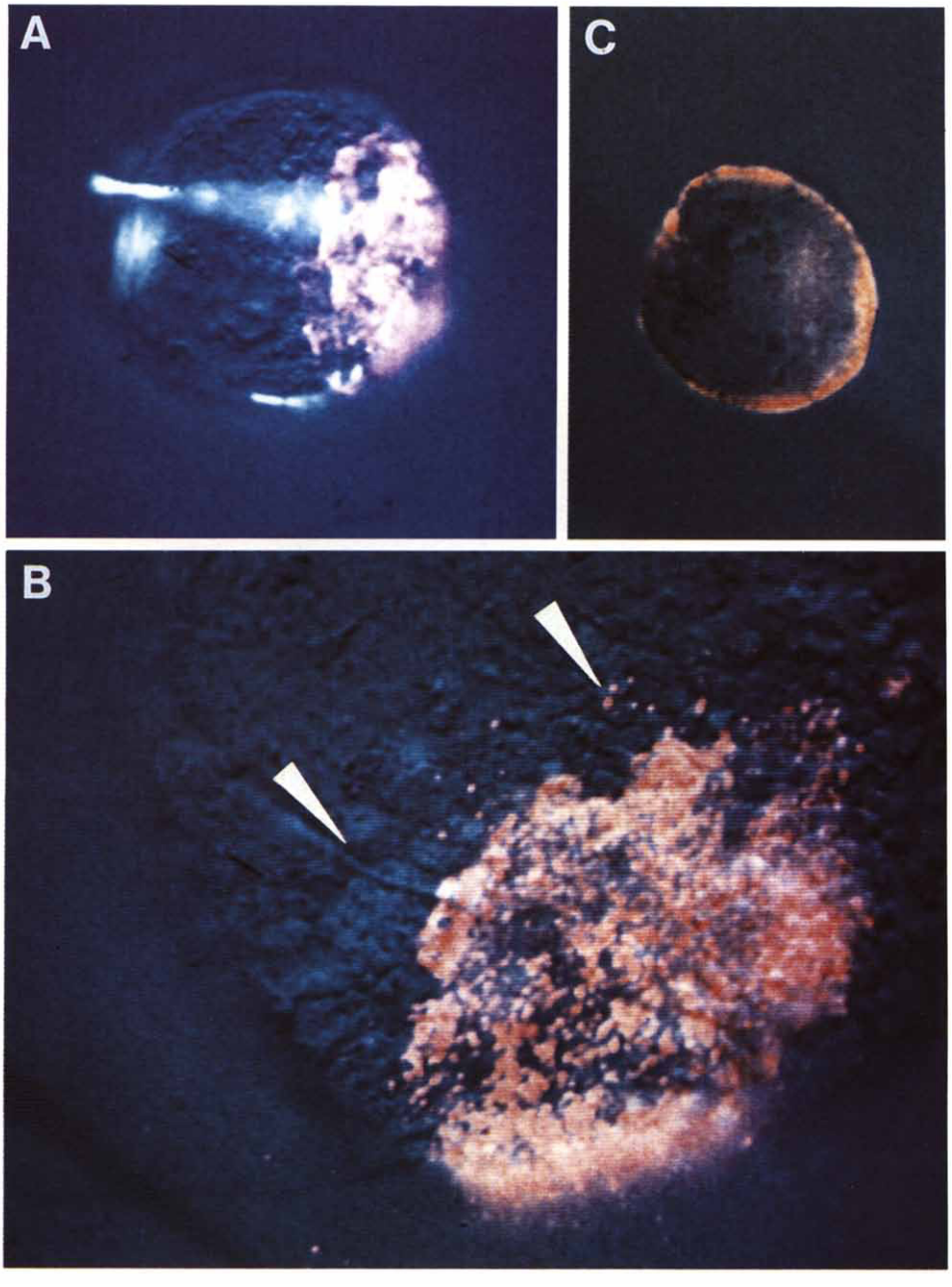

Fig. 11. Ecto V expression in RSE and GA embryos, displayed in false-color composite video images. 72 h embryos of the desired phenotypes that had developed from eggs injected with 8×10^4 molecules of 5HT-R mRNA, and exposed to 30 μM 5HT were processed for indirect immunofluorescence to reveal the location of the Ecto V antigen, as described in Experimental procedures. DIC images are shown in blue, and superimposed Ecto V fluorescent images are in red. In (A) three spicules, displayed by polarized light, are shown in a third color superimposition in green. Ecto V staining is limited to a high nuclear density patch similar in extent to the oral ectoderm of a normal embryo. (B) Higher magnification view of the Ecto V positive region of a different RSE embryo. A narrow high nuclear density strip of unlabeled cells can be seen to surround the cells expressing the Ecto V antigen (arrowheads). (C) A GA embryo in which the entire spherical ectodermal wall expresses Ecto V, while the mesenchyme cells within do not.

These embryos have been stained with a monoclonal antibody for the Ecto V oral ectoderm cell surface marker. Eggs were injected with 8×10^4 molecules of 5HT-R mRNA and exposed to 30 μM 5HT 0–72 h pf. Embryos that developed the GA or RSE phenotype were then collected and processed for display by indirect immunofluorescence. In the example reproduced in Fig. 11A an RSE embryo is seen as a three-color video composite, with the polarized light image of the spicules in green, the DIC image in blue and the epifluorescent Ecto V image in red. The embryo has three spicules, and the Ecto V epitope is localized to a region of the embryo between the branched spicule ends. This region has the high nuclear density typical of the oral ectoderm. At higher magnification (Fig. 11B), the Ecto V labeling can be seen to be localized to a central subregion of the area of high nuclear density. The remainder of the embryo shows no Ecto V staining whatsoever. The Ecto V experiments thus prove that RSE embryos possess oral ectodermal regions of essentially normal extent and disposition, just as they contain an essentially normal aboral ectoderm territory. For contrast, a GA embryo from the same batch is shown in Fig. 11C. This embryo is spherical, and the blastocoel contains various mesenchyme cells, but no archenteron invagination has occurred. The two-color video image shows that the *entire ectoderm* stains for Ecto V. The antigen for Ecto V becomes localized to the oral ectoderm at the late gastrula stage (Coffman and McClay, 1990). Thus the pattern of expression could result from either arrested development or ectopic expression. However the GA and RSE phenotypes differ fundamentally. While the GA embryo appears to consist of mesenchyme cells, vegetal plate and ectoderm, the RSE embryo retains all of the territories of the normal embryo, correctly arranged.

3. Discussion

3.1. Ectopic stimulation of foreign receptors affects embryonic development

It is informative in a general sense that alteration of morphological phenotype can be induced by stimulation of the two foreign receptors (5HT-R and MAChR) we used. These receptors couple to the universal PI signal transduction system, which have been suggested to be involved in morphogenetic processes in both *Xenopus* and sea urchin embryos (see Introduction). The GA and RSE phenotypes are both essentially *morphological* abnormalities, in that all of the detectable major differentiated cell types of the normal gastrula stage embryo undoubtedly remain present (except that the oral ectoderm cannot be distinguished from the aboral in the GA). The implication is that in normal embryos the affected morphogenetic processes depend on appropriate utilization of those signal transduction systems to which the foreign receptor we introduced are coupled.

We did not in this work show directly that stimulation of the 5HT-R or MAChR by their respective ligands produces Ca^{2+} transients or elevations of PI cycle intermediates in the sea urchin embryos expressing these receptors. Sea urchin eggs generate a dynamic series of endogenous Ca^{2+} transients at each cleavage cycle, and in preliminary studies we found these too complex a background to permit such measurements. In any case, it has already been shown that the same rat 5HT-R effectively couples to the activation system of starfish oocytes. Thus Shilling et al. (1990) demonstrated that if mRNA encoding the rat 5HT-R is injected into these oocytes, membrane elevation occurs when 5HT is added to the medium. Arguments that the developmental phenotypes we report in this work do indeed result specifically and exclusively from stimulation of the foreign receptors may be summarized as follows:

(i) Serotonin binding to endogenous receptors does not occur in sea urchin embryos until postgastrular stages (Brown and Shaver, 1989), nor is endogenous serotonin detected until this time, when it is found in neuroblasts of the oral hood (Bisgrove and Burke, 1986) and possibly in pigment cells, ciliated band cells, and digestive tract neurons (Buznikov et al., 1972; Ryberg and Lundgren, 1977). The effects we report here long precede the appearance of these cell types.

(ii) We routinely exposed uninjected control embryos to 30 μM 5HT, and observed no visible effects or abnormal phenotypes; and to 100 μM 5HT with no effect except for a subtle alteration in some ectodermal cell shapes (which does not affect developmental morphogenesis).

(iii) The same phenotypes were obtained in our experiments with two different mammalian brain receptors, one, the 5HT-R, of glial cell origin, and the other, the MAChR, of neuronal origin. These receptors utilize different ligands, but have in common that they are coupled to the same signal transduction pathway(s) (see above for references).

(iv) We showed that the 5HT-R mRNA is present after injection in all regions of the embryo (Fig. 1) and that it begins to be translated within a short time after injection (Fig. 2).

(v) We demonstrated that the abnormal phenotypes can be obtained within a certain range of 5HT-R mRNA inocula, and at certain concentrations of ligand added to the sea water (Fig. 5). The most severe phenotype, CA, occurs at highest levels of stimulation, while GA and RSE phenotypes are seen only at much lower levels of 5HT-R mRNA. Furthermore, except at the highest levels of 5HT-R mRNA, all embryos ex-

pressing the receptor developed perfectly normally, unless the ligand was introduced.

(vi) The three phenotypes we observed, as illustrated in Figs. 3 and 4, are distinct and reproducible, and to our knowledge they are unique in that these phenotypes are not precisely the same as those observed as a result of any other form of treatment.

3.2. The RSE phenotype

The least severe, most precisely defined, and at present the most interesting of the three phenotypes is the RSE phenotype. RSE embryos retain a complete, bilateral, oral/aboral axial ectodermal differentiation; they develop a normal gut; and they produce skeletal elements. Thus they have correctly carried out (and maintained) all of the initial territorial specification of the normal embryo. This observation is consistent with evidence presented in Fig. 7, which shows that the *onset* of the period of sensitivity to receptor stimulation for generation of the RSE phenotype is as late as 12 h pf. This is well after completion of the initial territorial specification processes, which occur during cleavage (Davidson, 1989).

The definitive abnormality of RSE embryos is the presence of radially arranged, supernumerary spiculogenic foci. These are correctly positioned along the primordial animal/vegetal axis of the egg, being located just above the base of the archenteron in the adjacent ectoderm, but they are incorrectly positioned in the oral/aboral axis. In normal embryos, the two spiculogenic foci are bilaterally positioned on the oral side, in the supra-anal ectoderm which is the product of the *VO* cell (Cameron et al., 1987). We know from the experiments of Hardin et al. (1992) and Armstrong et al. (1993) that the positioning of the skeletogenic foci depends not on the skeletogenic cells themselves but on the cells of the ectodermal wall with which the skeletogenic mesenchyme cells are in contact at focus formation. As noted above, in embryos radialized by $NiCl_2$ treatment, the whole ectodermal circumference expresses the Ecto V oral ectoderm marker, such that only a small ring of aboral ectoderm persists (Hardin et al., 1992). These embryos also display radialized rings of supernumerary spicule foci. Armstrong et al. (1993) showed that when skeletogenic mesenchyme cells from normal embryos are implanted in embryos radialized with $NiCl_2$ they generate a radialized set of spiculogenic foci; thus the normal skeletogenic mesenchyme cells evidently obey the spatial instructions of the radialized host ectoderm. It follows that in normal embryos, the positions of the pair of skeletogenic foci on the oral side must depend either on positive signals from the *VO* clone of cells where the foci form, or on negative signals from the lateral *VL* progeny and the *VA* aboral ectoderm cell clones that abut the vegetal plate, or on a downward signal emanating from the overlying oral ectoderm per se, or on some combination thereof. We know from recent experiments of Ransick and Davidson (1993) that there is an additional intercellular signal involved in the placement of the skeletogenic foci as well, which emanates from the vegetal plate. In these experiments second sets of micromeres transplanted to the animal poles of early host embryos were shown to induce the development of secondary vegetal plates from blastomeres that in undisturbed embryos are normally fated to produce only ectoderm. Such secondary vegetal plates then produce fully differentiated secondary archenterons, which fuse with the primary archenteron at the anterior end, sharing the same stomodaeum in the oral ectoderm. The double archenteron embryos also generate a *second set of skeletal elements*. These develop from a new pair of spiculogenic foci that is located 'normally', i.e., between the oral ectoderm and the second anus formed after invagination of the ectopic vegetal plate, and parallel to the host spicule pair. It follows that the location of the spiculogenic foci depends on signals from the vegetal plate, as well as from the oral (and/or aboral) ectoderm. In RSE embryos these intercellular locational mechanisms are deranged. This abnormality could be due to the presentation of ectopic intracellular signals in aboral ectoderm cells, generated as a result of stimulation of the foreign receptors. These signals would mimic those normally received only in the *VO* progeny where the skeletogenic foci are normally located. Or the ectopic intracellular signals might cancel negative signals normally presented in aboral ectoderm cells where spiculogenic foci are not supposed to form. In any case, it is remarkable that a window of sensitivity to relatively low levels of ectopic receptor stimulation exists such that the embryos develop almost normally up to the early gastrula stage, so that only the location of the skeletogenic foci is affected.

RSE embryos later develop two additional, specific abnormalities. They fail to form a stomodaeum and they fail to form a ciliated band. In normal embryos both of these morphogenetic processes are known to depend on intercellular interactions. The stomodaeum forms by interactions between secondary mesenchyme cells associated with the archenteron tip, and their target site is the oral ectoderm (reviewed by McClay et al., 1992). Fusion of the archenteron tip and the oral ectoderm follows. The ciliated band is the first ectodermal structure to develop that does not have an invariant cell lineage, and its morphogenesis requires interactions between cells of oral and aboral ectodermal origin (Cameron et al., 1993). Thus, in the cases of these developmental abnormalities as well, the failures of RSE phenotype embryos can be interpreted as the consequence of interference with the normal spatial

pattern of morphogenetic intercellular signaling. In RSE embryos these consequences are negative with respect to the ciliated band and stomodaeum, which fail to form, while whatever the sign of the underlying signal, they result in extra ectopic structures in the case of the spiculogenic foci.

3.3. The ectopic introduction of foreign receptors: new approaches to the study of signaling interactions in sea urchin embryogenesis

These results open the way to a new series of experimental approaches (that of course may be applied to receptors of other families as well). Ectopic stimulation of the 5HT-R causes a precise perturbation of spiculogenic focus formation in RSE embryos. It should now be possible to utilize similar methods to determine just which clones of cells generate the signals by which the foci are positioned, by introducing the receptor into specific lineage founder cells rather than into the whole egg. Stomodaeum and ciliated band formation might be approached in a similar way. In addition, the CA and GA phenotypes remain almost entirely unexplored. Conceivably, the CA phenotype results from a severe interference with the intercell signaling processes by which many of the initial specifications of the embryo are initially established, while the GA phenotype may imply that *continuing* cell signaling is required to maintain the boundaries, and the functions, of at least some territories during the blastula stage. This is at least an implication of the observation (Fig. 11C) that in GA embryos the basic spatial restriction of oral ectoderm marker expression is abolished.

In conclusion, we believe that this work demonstrates the feasibility of using foreign receptors of phylogenetically distant origin, to specifically perturb endogenous signaling interactions. Since such interactions underlie most of the processes of early sea urchin development, this experimental approach is likely to have widespread applications.

4. Experimental procedures

4.1. Embryo culture and preparation for injection

Sea urchin embryos were prepared for injection using previously reported methods (McMahon et al., 1985). Briefly, *Strongylocentrotus purpuratus* gametes were obtained by electric shock or injection of 0.5 M KCl into the perivisceral coelom. Eggs were collected in 0.45 μm filtered natural sea water (FSW) with 2 mM para-aminobenzoic acid (PABA, Sigma, Inc.) and dejellied in pH 5.0 sea water. Eggs were electrostatically fixed to 60 mm Petri dish lids treated with 1% protamine sulfate (McMahon et al., 1985). Semen was kept undiluted on ice. The eggs were fertilized with a freshly diluted sperm suspension and immediately injected. Typically 50–200 eggs were attached to a single dish and about 10–20 dishes were prepared in a single experiment. All sea water used throughout the incubation contained 2 mM para-aminobenzoic acid (PABA) in order to prevent hardening of the fertilization envelope. Following injection, the sea water was brought to 20 U/ml penicillin and 50 μg/ml streptomycin.

4.2. RNA preparations

Capped mRNAs were prepared by in vitro transcription from various templates using T7 or Sp6 polymerases under standard conditions. The physical integrity of the RNA preparations was assessed before and after injection by denaturing formaldehyde agarose gel electrophoresis. Gels were stained with ethidium bromide or autoradiographed. The functional ability of the RNAs synthesized was tested by bioassay in *Xenopus* oocytes. 10–100 ng of RNA were injected into the oocyte, which was then challenged with 5HT after overnight incubation, and calcium induced chloride currents were measured (data not shown).

For transcription of mouse 5HT-R mRNA we utilized a pGEM3 (Promega) cDNA clone (Lübbert et al., 1987) that was the kind gift of Dr. Henry Lester of this Institute. To prepare the injected mRNA the plasmid was cut with *Eco*RI at the 3′ end of the 2.3 kb insert, and transcribed with Sp6 polymerase. The antisense mRNA was prepared from plasmid cut with *Sal*I and transcribed with T7 polymerase. mRNA encoding the M1 muscarinic acetylcholine receptor was transcribed from a pGEM1 cDNA clone (Bonner et al., 1987), also provided to us by Dr. Henry Lester. This mRNA was also transcribed from the T7 promoter, after truncation with *Hin*dIII.

4.3. Microinjection

mRNAs and mixtures of mRNA and DNA reporter genes were pressure injected into zygotes. Aliquots of mRNA were brought to concentration of 1 μg/μl in 0.2 M KCl and diluted to the desired concentrations in 0.2 M KCl. *CyIIIa · CAT* reporter gene constructs (with carrier DNA; Franks et al., 1988) were mixed with mRNA to achieve a concentration of 750 molecules of reporter gene per picoliter. Microinjection needles were pulled from Omega Dot glass capillaries (1 mm O.D., F. Haer & Co., Brunswick, ME) to make closed tips which were approximately 0.5 μm when broken. The needles were back-filled with injection solution. Pressure injection was accomplished with a 'Picospritzer II' (General Valve Co.). Based on scintillation counting of

embryos injected with [^{35}S]methionine, we estimated that the average injection volume was 10 pl.

4.4. Translation of exogenous message in injected embryos

To measure the translation of the 5HT-R protein, zygotes were injected with about 0.15 nanocuries of [^{35}S]methionine (specific activity 1273 Ci/mmole) or with [^{35}S]methionine and 4×10^7 molecules of 5HT-R mRNA. Embryos injected with RNA and [^{35}S] methionine, or with [^{35}S]methionine only, were separately collected at 4 to 6 h postfertilization (pf), and lysed in protein gel loading buffer (4% SDS, 0.2% glycerol, 20% β-mercaptoethanol, trace of bromphenol blue). Standard SDS-polyacrylamide gels (3% stacking and 10% running) were used to separate the proteins. The gels were run at constant current, 20 mA, for 3 h, soaked in Enhance (NEN Research Products), dried, and autoradiographed. The band containing the 5HT-R polypeptide, which is about 51 kDa in mass, was cut out of the lane, dissolved in 'Safety-Solve' scintillation fluid (Research Products International Corp.), and counted for 5 min.

4.5. Whole mount in situ hybridization

Whole mount in situ hybridizations were performed according to Ransick et al. (1993), modified as described there from Harkey et al. (1992). Briefly, glutaraldehyde fixed embryos were dehydrated to xylene, brought to 70% alcohol and stored. Stored embryos were rehydrated, digested with proteinase K, post-fixed in paraformaldehyde and equilibrated in hybridization solution at 50% formamide and 0.6 M NaCl. Hybridization to a digoxygenin-labeled RNA probe at a concentration of 0.1 ng/ml was carried out at 42°C 16 h or overnight. The probe was washed out at 60°C using 1 × SSC. The probe was detected with commercial Fab fragments of antibody to digoxygenin conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis). The embryos were incubated in alkaline phosphatase buffer containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate until inspection revealed sufficient color reaction. The embryos were dehydrated, transferred to terpeniol and mounted for observation.

4.6. Immunofluorescence

Indirect immunofluorescence observations were carried out using a monoclonal antibody (McAb) to the cell surface protein Ecto V (Coffman and McClay, 1990). Embryos were fixed in 4% formaldehyde in sea water for 15 minutes on ice, then for 15 minutes in acetone at −20°C (D.R. McClay, pers. comm.; Wessel and McClay, 1985). The fixed embryos could be stored at this stage in sea water with 5 mM sodium azide. Fixed embryos were incubated in McAb tissue culture supernatant (1:1 with FSW) for 1 h, washed 3 × with FSW then incubated for 1 h in rabbit anti-mouse-IgG conjugated to the fluorochrome Cy3 (1:100 with FSW) (Jackson Immuno Res.). The preparations were then viewed with an epifluorescent microscope equipped with a low-light level video system described earlier (Cameron et al., 1990). Images were recorded on an optico-magnetic disc recorder, and pseudocolor composite video images were printed on a Sony video printer.

4.7. Nuclear staining

The distribution of embryonic nuclei in the ectoderm was observed with Hoechst 33342 (Aldrich Chemicals) after the method of Hinkley et al. (1986). Live or fixed embryos were stained for 10–30 min in FSW with 10 mM tris-[hydroxymethyl]methylaminopropane sulfonic acid (TAPS) (Sigma Chem. Co. #T-0647), washed briefly and observed under UV epifluorescence. Representative images were recorded on Kodachrome VR400 or the video system described above.

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