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Original Article

Autoradiographic localization in polychaete embryos of tritiated mesulergine, a selective antagonist of serotonin receptors that inhibits early polychaete development

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ABSTRACT **Developing embryos of the polychaete *Ophryotrocha labronica* were exposed to tritiated mesulergine, a selective antagonist of the serotonin receptors 5-HT1c and 5-HT2, that also has significant affinity to dopamine D-2 sites, and the labeling was analyzed by autoradiography. Already at the earliest developmental stages (1-4 cells), numerous silver grains visualizing ^{3}H-mesulergine binding sites and possibly also serotonin receptors were recorded over the cytoplasm, mostly in association with decomposing yolk granules, but few grains were detected over the nuclear region. In advanced pregastrular embryos (3 days) the number of silver grains was greatly increased over nuclei, cell borders and non-yolk cytoplasmic elements, notably in the animal half of the embryos. For newly gastrulated embryos (4 days), more than 90% of the grains appeared over non-yolk cellular structures. Abundant access to serotonin receptors is probably a fundamental condition not only for gastrulation but also for the high mitotic activity of the cleavage period. An indication hereof is the observation that exposure of cleaving polychaete eggs/embryos to unlabeled mesulergine inhibited cytokinesis and chromosome movements, whereas spindle formation and chromosome duplication were unaffected.**

KEY WORDS: *polychaete embryos, ^{3}H-mesulergine, serotonin (5-HT)-receptors, autoradiography, electron microscopy*

Introduction

During the last decade it has become increasingly clear that serotonin (5-hydroxytryptamin, 5-HT) mediates its action by binding to various receptor subtypes which couple to different intracellular systems (Leysen, 1985; Bradley *et al.*, 1986). Since selective antagonists for the various 5-HT receptor subtypes have recently become available, access to such substances has opened up new possibilities for testing and specifying the function of serotonin, inter alia in early embryo development.

For obvious reasons the unexpected occurrence of serotonin in early animal embryos prior to the development of the nervous system (Buznikov *et al.*, 1964) raised speculations about non-transmitter functions of this amine, unique for early embryo development. However, with serotonin later demonstrated in protozoa (Janakidevi *et al.*, 1966) and planarians (Franquinet, 1979) the attitude is rather that during embryonic development serotonin initially acts intracellularly and then progresses to function intercellularly. It has been convincingly demonstrated that in sea urchin embryos serotonin has a decisive role in the accomplishment of the morphogenetic movements during gastrulation (Gustafson and Toneby, 1970), but its function during the cleavage period is more difficult to define. In the polychaete *Ophryotrocha labronica*, synthesis of serotonin was demonstrated in the disintegrating yolk granules of cleaving embryos (Emanuelsson, 1974). That study also showed that such endogenous serotonin is regularly associated with microfilaments in the cleavage furrow in dividing eggs/blastomeres, which suggested a connection between serotonin and microfilament action in these early cells. Buznikov *et al.* (1970) and Renaud *et al.* (1983) found that serotonin antagonists inhibit cell proliferation in cleaving sea urchin embryos, indicating an important role for the bioamine in the regulation of this process, but there is still much uncertainty about the appearance and location of serotonin receptors at this early stage of development. Therefore, investigations to clarify this problem appear well motivated. The present study reports autoradiographic localization of ^{3}H-mesulergine binding sites and therefore possibly serotonin receptors in polychaete cleavage embryos up to gastrulation. Mesulergine is a selective antagonist for 5-HT1c and 5-HT2 receptors (Closse, 1983; Hoyer *et al.*, 1985), and has also significant affinity to dopamine D-2 sites. There is striking structural conservation between the two 5-HT-receptors in question (Julius *et al.*, 1990). In neurons that express them, receptor activation by serotonin appears to activate phospholipase C and generate inositol polyphosphates, which release intracellular Ca^{2+} (Conn *et al.*, 1986; Julius *et al.*, 1988).

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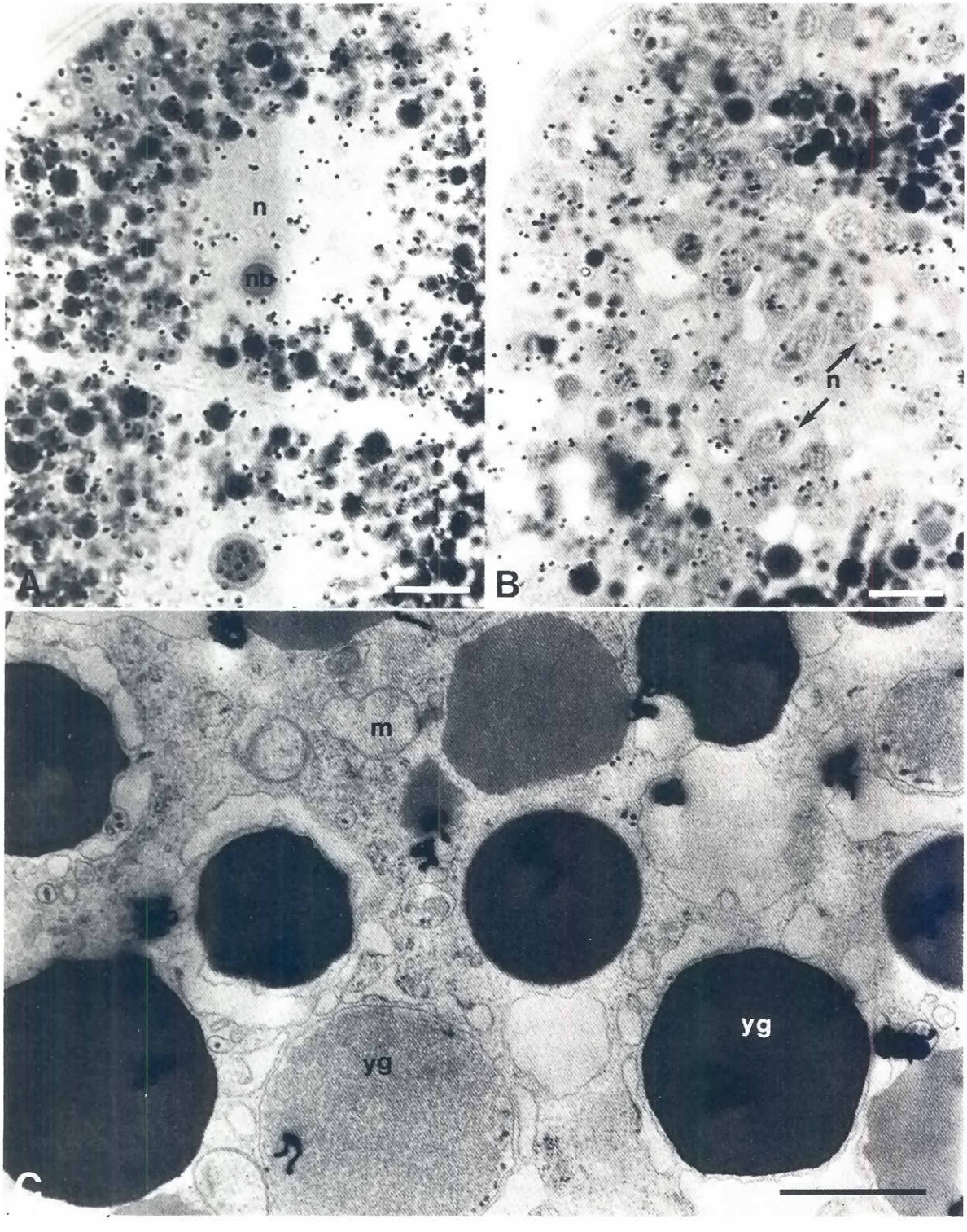

Fig. 1. Autoradiographs of polychaete eggs/embryos, exposed to ^{3}H-mesulergine. (A) *Light microscope autoradiograph of 2-cell polychaete egg exposed for 1 h immediately before the first cleavage division. Numerous silver grains appear over the cytoplasm, mostly associated with yolk granules, but grains also occur over the nucleoplasm (n) and the nucleolus-like bodies (nb).* **(B)** *Light microscope autoradiograph of 3-day-old embryo exposed for 1 h. The silver grains — indicating ^{3}H-mesulergine binding sites — are at this stage preferentially associated with cell nuclei (n) and cytoplasmic structures other than yolk granules. Bar, 10 μm.* **(C)** *Electron microscope autoradiograph from the cytoplasm of a polychaete egg, exposed to ^{3}H-mesulergine for 1 h immediately before the first cleavage division. yg, yolk granule; m, mitochondrion. Bar, 1μm.*

Results

Distribution of ^3H-mesulergine binding sites

For autoradiographic localization of 5-HT receptors (or peptide receptors) treatment with the radiolabeled ligand is usually performed on sections from the unfixed, freeze-sectioned material in question (Palacios and Dietl, 1987; Schotte and Leysen, 1988). In the present case, however, the small size of the eggs/embryos and the wish to obtain a precise intracellular location of the binding sites clearly precluded such a technique, and instead *intact* eggs/embryos, i.e., individuals still within their jelly capsules, but freed from the egg pack jelly, were exposed to the radiolabeled ligand (2.96 MBq/ml, 1 μmol/l). For newly laid eggs the pulse covered either the whole or part of the period from laying until the first cleavage division of the control eggs; for older stages it was 1 h. After aldehyde fixation, rinsing and embedding in Epon, the material was sectioned for autoradiography. Eggs/embryos treated in this way showed no tendency to leak radioactivity, and a characteristic, non-uniform distribution of label in them indicates specific, stable receptor-binding.

Table 1 shows the number of silver grains per standard area as observed in the light microscope autoradiographs of the polychaete embryos during the first 4 days of development. Since the embryos were labeled and autoradiographed under identical conditions, and since the embryo area analyzed in the meridionally cut sections is approximately the same in all the stages, it is possible that these values also reflect the frequency of 5-HT receptors in the embryos. As it now appears, the table points to an initial peak number at the onset of the cleavages and another during day 2, i.e., just before gastrulation. The counts were not extended beyond day 4 because of the increasing differentiation and asymmetry in the embryo thereafter.

Fig. 1A, which shows part of a 2-cell embryo, exemplifies the silver grain pattern seen in polychaete eggs, exposed to ^3H-mesulergine during the first two divisions (1-4 cells). One can discern: 1) the peripheral protective jelly coat of the egg/embryo with no or very few silver grains over it. 2) The cytoplasmic, manifestly labeled zone that comprises most of the egg/blastomeres, and which includes the yolk mass. Here about 40% of all silver grains were observed over — or in association with — yolk granules in the yolk region, cf. Table 1. The silver grains were found over or attached to all types/sizes of yolk granules, except the cortical granules, and they had particularly accumulated over medium-sized (2-3 μm) yolk granules. Some of the silver grains in this zone were apparently associated with cytoskeletal elements, notably in the egg periphery. 3) The central, moderately labeled zone containing the nuclear material and with silver grains localized to the nuclear membrane, the nucleoplasm and the nucleolar-like bodies in the karyomeres/nuclei.

Also in the 1-3-day embryos exposed to ^3H-mesulergine only an insignificant number of silver grains was observed over the enclosing jelly capsules, whereas labeling of the embryo cells was highly significant. Up to day 3 (gastrulation) there is normally a considerable reduction, i.e., utilization, of the yolk material in polychaete embryos, particularly in their animal half, and this change was reflected in the labeling pattern. Thus, in 1-day-old embryos about 40% of the silver grains were still found connected with decomposing yolk granules, but in 3-day-old embryos (Fig. 1B), and particularly in their ectoderm cells, most of the grains were observed over nuclei, cell borders and non-yolk cytoplasmic elements, and a minor part only associated with the remaining intracellular yolk material, cf. Table 1. In 4-day-old old embryos (which have just completed gastrulation) the still identifiable yolk material accounted for only about 9% of all silver grains, additional grains were as in 3-day-old embryos, that is, located over other cytoplasmic constituents, nuclei and cell borders.

TABLE 1

DISTRIBUTION OF SILVER GRAINS, OBSERVED IN LIGHT MICROSCOPE AUTORADIOGRAPHS OF DEVELOPING POLYCHAETE EMBRYOS, EXPOSED TO ^3H-MESULERGINE FOR 1 H AND, AFTER FIXATION AND SECTIONING, AUTORADIOGRAPHED FOR 14 DAYS

Embryo age	Part of embryo	Grains per standard area (13 μm^2)	% silver grains observed over yolk granules	% silver grains observed over nuclei and non-yolk cytoplasm
2 cells	Whole	1.64 ± 0.24	40.5	59.5
1 day	Whole	0.71 ± 0.22	43.0	57.0
2 days	Whole	2.51 ± 0.38	41.9	58.1
	Animal half		35.1	64.9
	Vegetal half		60.2	39.8
3 days	Whole	0.95 ± 0.31	34.2	65.8
	Animal half		15.5	84.5
	Vegetal half		50.8	49.2
4 days	Whole	0.43 ± 0.15	8.9	91.1

For each developmental stage 1200-1600 silver grains were counted, and the counts covered the whole embryo area in representative meridional sections passing through the embryo center.

The electron microscope autoradiographs confirmed the light microscopic observations. They demonstrated that in eggs before or immediately after the first cleavage division, silver grains indicating ^3H-mesulergine binding sites were especially associated with yolk granules of medium size, and characterized by high electron density (Fig. 1C). Grains appeared both over central and peripheral regions of such granules — in the latter case frequently over adhering vesicles/loops — and consistently the granules showed either vacuoles or indentations which indicated their progressing decomposition. There were no grains over the full-sized and still visibly intact yolk granules, but sometimes silver grains were found adhering to them. Such grains, as well as part of those located between yolk granules and without contact with any clearly visible cell structure, might, however, be associated with the cytoplasmic network that organizes the congregation of yolk granules in oocytes and early embryos. In the cell periphery silver grains were indeed found located over cytoskeletal elements.

The electron microscope autoradiographs also thoroughly support the light microscopic observations on the multicellular embryos (1-4 days old). Fig. 3 shows silver grains indicating binding sites over an ectoderm cell nucleus in a 3-day-old embryo. At this stage about 20% of all grains over cell nuclei in the animal half of the embryo were found over the nuclear envelope, and the remainder over the nucleoplasm and (when observed) the nucleolus-like bodies. Of the cytoplasmic silver grains about 25% were seen along the cell borders (Fig. 2B) and about as many over the remaining yolk

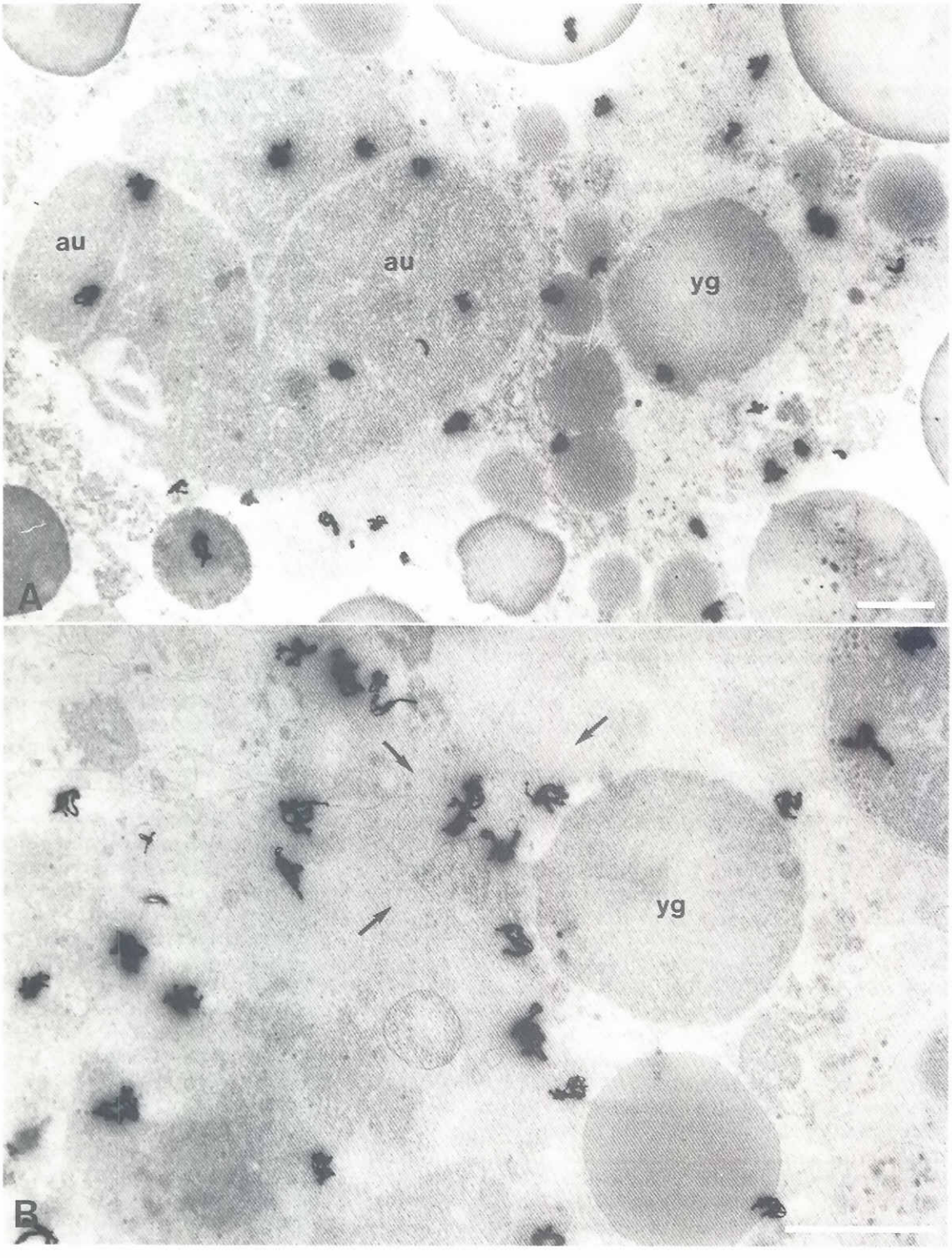

Fig. 2. Electron microscope autoradiographs (A) *from a 1 day-old polychaete embryo, exposed to ^{3}H-mesulergine for 1 h. To the left the autoradiograph shows a typical silver grain pattern for a full-size yolk granule in decomposition. Part of the granule content has already leaked out, forming smaller granules outside the remnants of the original yolk granule. Notice that within the latter, silver grains appear along the surface of the still aggregated units (au) that originally formed the granule during oogenesis, whereas grains associated with the leaked material (to the right) have a less defined position. yg, yolk granule. Bar, 1 μm.* **(B)** *From the animal half of a 3-day-old polychaete embryo, exposed to ^{3}H-mesulergine for 1 h. The autoradiograph shows the border area between adjacent ectoderm cells. Notice the many silver grains over the contact point (arrows) between the cells in the middle of the figure. yg, yolk granule. Bar, 1 μm.*

granules, whereas the numerous endoplasmic profiles and also mitochondria accounted for most of the additional 50%. There was no significant difference in nuclear labeling between ectoderm and endoderm cells in 3-day-old and 4-day-old embryos, but the recorded grain numbers over the nuclei (3-day-old embryos: 7.1±2.6 vs 4.3±1.3; 4-day-old embryos: 6.3±1.6 vs 3.3±1.7) show a tendency in that direction.

To check the specificity of the autoradiographic recordings various controls were made. In the first one, the effect on the autoradiographic response of a simultaneous, or an immediately preceding, exposure to unlabeled mesulergine was analyzed. It was found that silver grain counts on eggs which at the time for the first cleavage division were simultaneously pulsed for 1 h with labeled (1 µmol/l) + unlabeled (20 µmol/l) mesulergine, showed a labeling which was only 6-9% of that in eggs from the same egg pack, pulsed with the labeled substance alone. A pulse under similar conditions with 1 µmol/l labeled + 10 µmol/l unlabeled mesulergine resulted in a labeling of 12-15%. Other eggs were from laying kept in 20 µmol/l mesulergine (2.5 h) and after a rapid rinsing pulsed for 1 h with ^{3}H-mesulergine (1 µmol/l) at the time for the first cleavage division. In these eggs labeling amounted to 46% of that in the controls, suggesting a fairly rapid formation/release of new receptors after the pretreatment, and leading to further experimentation with gradually reduced pulsing time. The most striking result was obtained when developing eggs 30 min before the first cleavage division were first exposed to unlabeled mesulergine (20 µmol/l) for 15 min, and then — after rapid rinsing — were pulsed with ^{3}H-mesulergine (1 µmol/l) for another 15 min. When autoradiographed these eggs showed a unique labeling pattern with more than 90% of the grains observed in them located over full-sized yolk granules. This finding suggests that the binding sites presently indicated by the silver grains originate from these granules. Other short-pulse experiments demonstrated a marked number of binding sites in developing eggs within 30 min after fertilization.

To refute speculation that the observed association of silver grains with small/medium yolk granules does not represent a specific labeling but only a general association of label (e.g., with lipid material in these organelles), polychaete oocytes cultivated *in vitro* were exposed to ^{3}H-mesulergine for 1 h and then fixed and autoradiographed identically with the developing polychaete eggs/embryos. During *in vitro* cultivation *Ophryotrocha* oocytes grow and show a normal development (Emanuelsson and Anehus, 1985), and at the selected stage (mid-oogenesis) there is an intense formation of lipid vacuoles and yolk granules in the oocytes, with all types of yolk constituents being represented. Reasonably, any general association of ^{3}H-mesulergine with, for example, lipids or intermediate and final products in the yolk formation process, would have been revealed in these autoradiographs. However, of the sparse silver grains actually observed in such autoradiographed oocytes, only occasional grains showed association with lipid vacuoles or with yolk granules, and there is accordingly no reason to suspect a general association of label with lipid material or with intact yolk granules in the present material.

Since mesulergine also has significant affinity to dopamine D-2 sites, labeling experiments on the eggs/embryos with tritiated raclopride (which binds selectively to dopamine D-2 sites: Köhler and Radesäter, 1986) were performed as a control. When pulsed with 10-100 nmol/l ^{3}H-raclopride for 1 h at the time for the first cleavage division, the eggs/embryos showed weak labeling only. Moreover, the sparse silver grains actually observed were restricted to the cytoplasm and only occasionally found over yolk granules. When tritiated GR 65630, a selective 5-HT$_3$ receptor antagonist (Kilpatrick *et al.*, 1987), was tested in corresponding experiments, the labeling was insignificant.

As decomposing full-sized yolk granules appear to be centers for silver grain accumulation, there was reason to study the grain constellation inside and around such granules more closely. Inspection of a large number of full-sized yolk granules in early stages of decomposition revealed that they all had a labeling pattern of a similar kind, and a representative example of that is shown in Fig. 2A. Apparently this pattern reflects an initial leakage of part of the granule content, which has resulted in the emergence of small/medium sized, labeled granules outside the original one, and access to the interior of the large granule. The silver grains recorded within it are, however, essentially confined to the exposed surfaces of the still aggregated sub-units that originally coalesced and formed the mature yolk granule. The orderly arrangement of the silver grains associated with these granules appears inconsistent with an unspecific labeling.

***Effects of mesulergine on early development in* Ophryotrocha**

In its capacity as a serotonin antagonist mesulergine might be expected to seriously affect polychaete egg development. To check this the labeling experiments were supplemented with an analysis of the adverse effects on eggs from prolonged exposure to mesulergine. It was observed that polychaete eggs which from laying were continuously exposed to 10-20 µmol/l mesulergine showed the usual flattening at the normal time for the first cleavage, but the ensuing equatorial invagination and attempted constriction were delayed and incomplete (unilateral), and had soon reversed. When control eggs had reached an advanced 2-cell stage the treated eggs were therefore still uncleaved, but showed two mitotic poles/asters. They had not accomplished karyokinesis, however, and accordingly the nuclear material remained in a central position. Later on, when the control eggs had attained the 4-cell stage after a second cleavage division, the treated ones were still uncleaved. At the time when the former performed that division (Fig. 4A), video microscopy revealed new weak attempts at furrowing from the latter, but these incomplete invaginations were soon reversed. Although the treated eggs thus had developed four distinct mitotic poles/asters (Fig. 4 B), the nuclear material was still undistributed. Tests showed that 5 µmol/l mesulergine was still effective as mitotic inhibitor, but with varying results, whereas a 1µmol/l solution was ineffective.

No developmental disturbances occurred if the eggs were simultaneously exposed to mesulergine (10 µmol/l) and serotonin (20 µmol/l).

It was a puzzling experience to find that the mesulergine concentration required for complete inhibition of the cleavage divisions had to be at least 5 µmol/l — i.e., much higher than expected from available data about affinity constants for the 5-HT receptors of interest. (For pharmacological characteristics, pK_d-values, of the binding of ^{3}H-mesulergine to 5-HT1 and 5-HT2 recognition sites, see Hoyer *et al.*, [1985]). One possibility might of course be that the embryonic forms of 5-HT receptors require much higher concentrations of serotonin for their activation, but this is so far pure speculation. Immediately it might seem logical to identify the intact jelly capsules around the eggs as responsible for this discrepancy, but our previous experience from labeling experiments on intact polychaete eggs, using amino acids and nucleosides, have

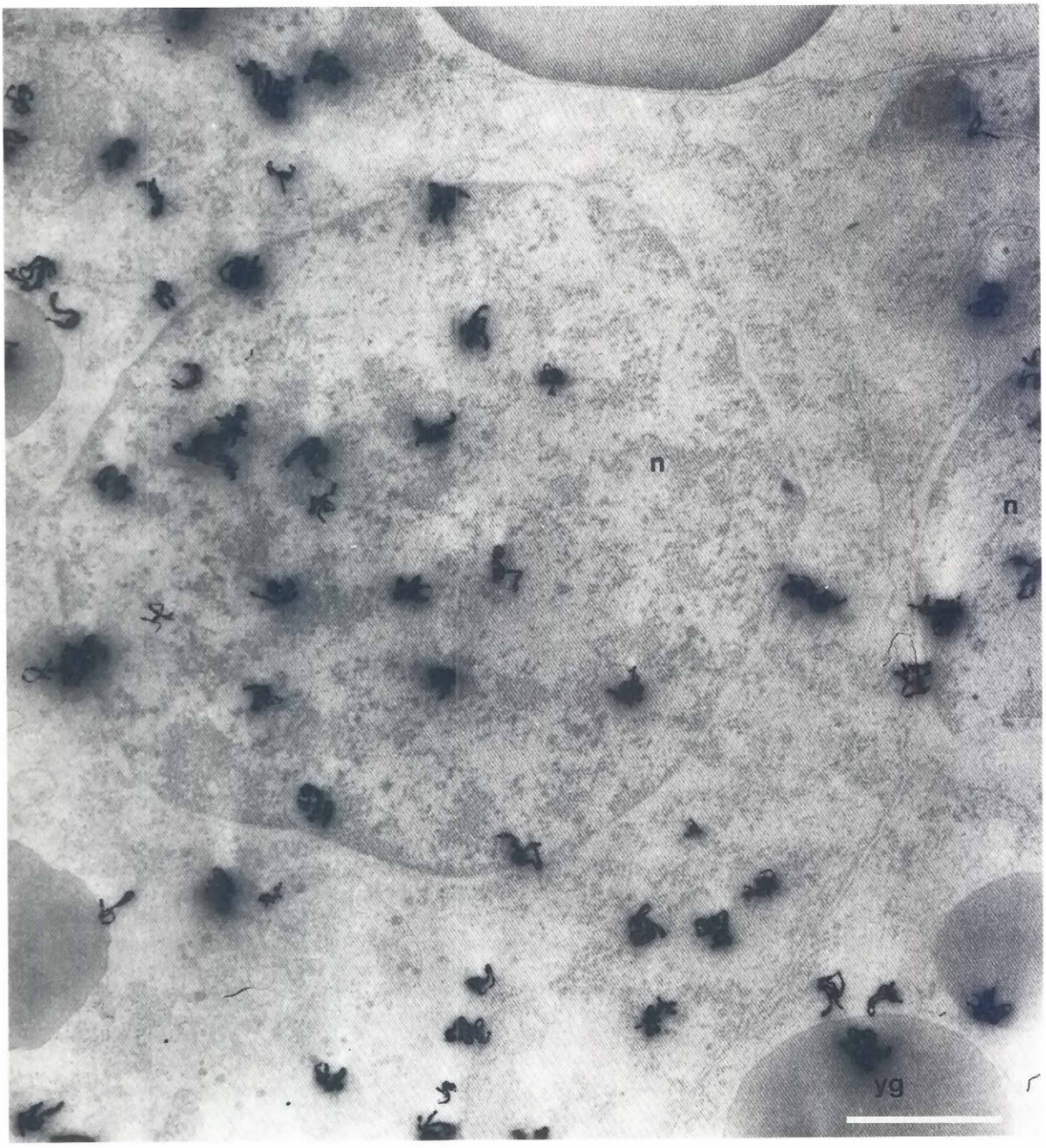

Fig. 3. Electron microscope autoradiograph *showing the location of ^{3}H-mesulergine binding sites in an ectoderm cell from the animal half of a 3-day-old polychaete embryo, exposed to ^{3}H-mesulergine for 1 h. n, nucleus; yg, yolk granule. Bar, 1 µm.*

shown that for water-soluble, low-molecular substances the barrier effect of the capsule is negligible. Any removal of the capsules, mechanically or chemically, was for that matter not attempted, since earlier experiments had shown that development and survival of dejellied eggs is poor. Much points, however, to the endogenously-produced serotonin as the crucial factor in this connection, as there most likely starts a continuous production of it in polychaete eggs with the onset of development. As stated above, administration of serotonin actually protected the eggs from cleavage disturbances, if added simultaneously with the mesulergine. Therefore, with endogenous serotonin present in the eggs the concentration of mesulergine required to produce mitotic inhibition probably has to be substantially above the level normally calculated for effective receptor blocking.

In normal development 1-day-old polychaete embryos have reached a multicellular stage with rather closely packed blastomeres.

Eggs that had been exposed to 10-20 µmol/l mesulergine from the onset of development were still uncleaved after one or two days, but frequently their peripheral cytoplasm had started to fragment into small irregular cytoplasmic drops lacking a nucleus (Fig. 4C). In exceptional cases these appeared to be nucleated, but the bulk of the nuclear material that remained centrally in the egg was not split up. These eggs were disintegrating soon afterwards.

Eggs for which the continuous treatment did not start until 30-60 min before the first cleavage all showed cleavage disturbances, but these were sometimes less spectacular than those described above. Thus in some cases the first cleavage could be completed, but the larger of the two blastomeres did not cleave any more. The resulting embryos, which stopped cleaving at an early stage, were characterized by few blastomeres of very different sizes.

Ultrastructural changes in eggs exposed to 10-20 µmol\l mesulergine

During the early period of development — at least up to the 8-cell stage in the controls — aster formation in the treated eggs occurred synchronously with that in the controls. As the eggs did not cleave but only showed weak attempts at invagination, one could suspect they lacked an organized contractile ring of microfilaments, a condition that was confirmed in the electronmicroscopical analysis of the material. The analysis also showed that in the absence of cytokinesis the first mitotic spindle had not disappeared at the time when the new ones were formed, leading to a manifest presence of variously oriented microtubules in these eggs.

In normal development there is a local accumulation of cortical granules preceding the formation of the cleavage furrow in the egg/blastomere cortex, but most of them have disintegrated at the completion of cytokinesis. In the treated eggs, however, there were still many of them in the furrow region also after the abortive cleavage, and even the central region of these eggs usually displayed a substantial number of cortical granules near the bundles of microtubules (Fig. 4D).

Also the appearance of mitochondria and yolk granules differed from that in normal eggs. The former were frequently smaller than usual and essentially lacked attachment to endoplasmic profiles. The large (4 µm) yolk granules showed a more widespread vacuolization than in control eggs, and there was a higher number of small yolk granules than in the controls.

Discussion

Mesulergine is a selective antagonist for 5-HT1c and 5-HT2 receptors (Closse 1983; Hoyer *et al.*, 1985), and our conviction that the silver grains in autoradiographed polychaete eggs/embryos exposed to ^{3}H-mesulergine might visualize the location of 5-HT1c or 5-HT2 receptors gains support from the finding that simultaneous exposure of the eggs to labeled + unlabeled mesulergine influenced the ^{3}H-mesulergine labeling as predicted. Moreover, the quantitative estimations of grain numbers during the period indicated peak values at the onset of the cleavage period and immediately before gastrulation, i.e., at the time for developmental events that reasonably would require presence of such receptors. A fully conclusive test of the specificity of the labeling can, however, scarcely be performed without access to purified antibodies against the receptors, but this resource has not been available.

The investigation shows that silver grains, intimating the presence of 5-HT1c or 5-HT2 receptors, were recorded in substantial amounts in the developing eggs/embryos during the whole of the pregastrular period, in fact even before the first cleavage division. Presumably either one or the other of these receptors have been labeled, but further studies will be needed to clarify this matter. The observed grain pattern was characterized by an initial abundance of grains over disintegrating cytoplasmic yolk granules, which then gradually gives way to a state where the vast majority of grains were found over other cytoplasmic elements and over nuclei. The fact that 1) after pretreatment with unlabeled mesulergine short pulses of ^{3}H-mesulergine almost exclusively labeled the full-sized yolk granules in decomposition, and 2) the finding that such granules exhibit a common, specific labeling pattern, suggests that pregastrular 5-HT receptors originate from these large grains.

The controls have consistently shown that visibly intact yolk granules in developing embryos, as well as growing yolk granules in maturing oocytes, are not significantly labeled by ^{3}H-mesulergine, nor is that the case with lipid vacuoles at any stage. The labeling is accordingly confined to yolk granules in decomposition, and the very existence of a common grain pattern over the large ones, with the grains located along the periphery of the discernible yolk sub-units, points to labeling specificity, not to unspecific adherence of label. There is hardly any particular silver grain pattern associated with the small yolk granules, but on the other hand the silver grains associated with them are not so clustered that they suggest any unspecific adherence.

It must be emphasized that release of 5-HT receptors from the yolk material is by no means in opposition to established facts about the pathways for the early production of embryo constituents. Much of that production is accomplished through expression of stored maternal mRNA, bound to the egg-cytoskeleton and/or the yolk mass. The polychaete yolk granules are very complex organelles formed during oogenesis by cooperation of various cellular elements (Emanuelsson, 1969; Pfannenstiel and Grünig, 1982). The existence of maternal mRNA and rRNA in full-sized yolk granules in *Ophryotrocha* has earlier been demonstrated (Emanuelsson, 1985), and it seems highly probable that just receptor-mRNA is among the maternal mRNAs stored there. It has previously been observed that membrane material from the disintegrating polychaete yolk granules under normal conditions is incorporated into the cell membrane of the blastomeres (Emanuelsson, 1974), and in accordance with present observations this process should also reasonably include incorporation of 5-HT receptors. Since the same study also showed that serotonin is synthesized in the disintegrating polychaete yolk granules, it would seem that in developing polychaete eggs the yolk granules actually supply both the receptors and the receptor activator.

With increasing age the developing polychaete eggs/embryos gradually displayed a changing labeling pattern with silver grains appearing in increasing numbers over cell membranes and cell nuclei, so that in gastrulated (4-day-old) embryos only a minimal number of grains were still related to yolk material. Interestingly this transformation was considerably more pronounced in the ectoderm cells than in the endoderm cells of the 3-day-old embryos, leading to a proportionally higher number of indicated receptor sites over nuclei and yolk-free cytoplasm in the ectoderm cells than over corresponding cellular domains in the endoderm cells. This difference might reflect incipient differences between the two cell types to perform serotonin-dependent cell functions and thus announce important physiological differences between them.

In the present situation it is natural to connect a release of 5-HT

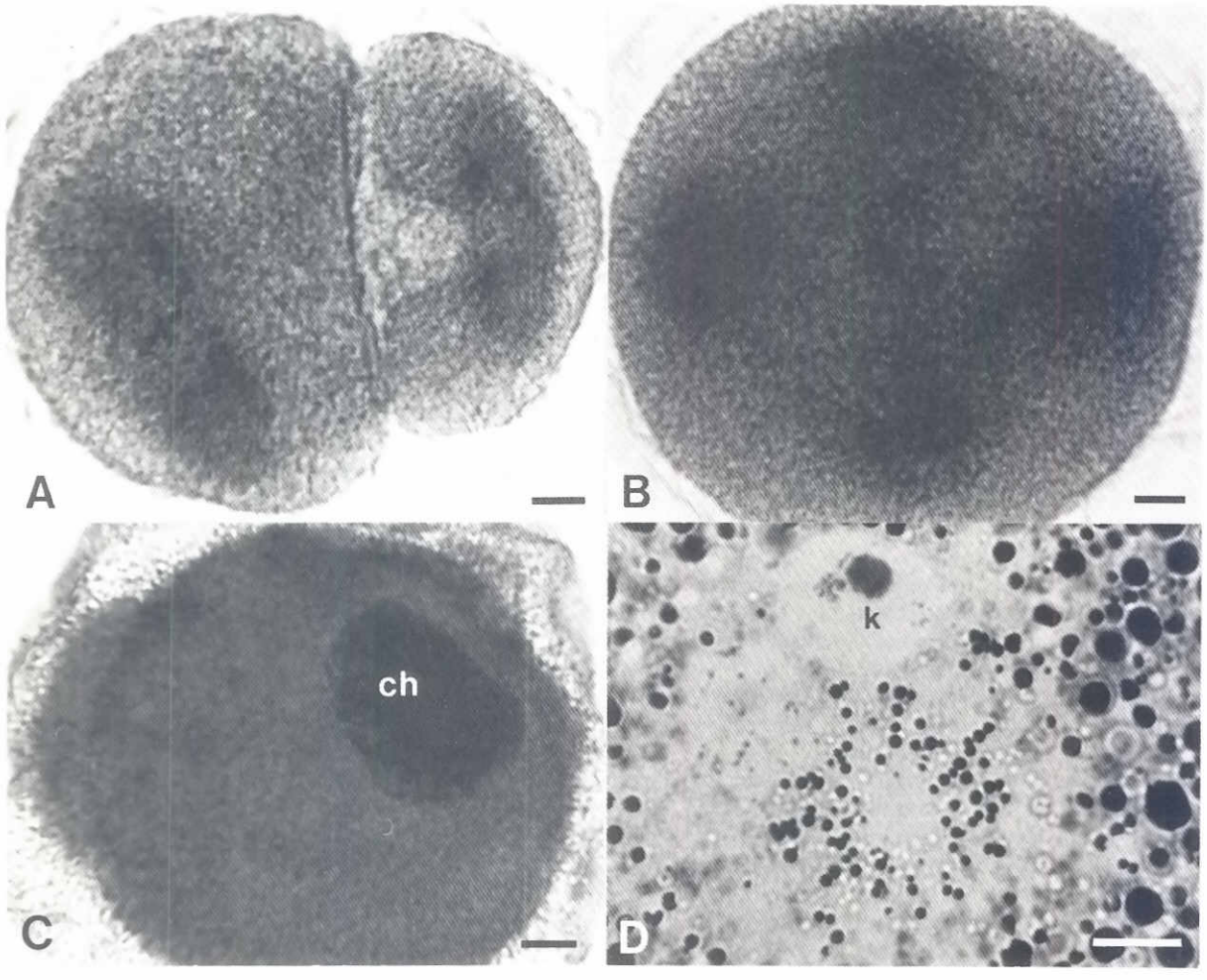

Fig. 4. Effects of mesulergine on early polychaete eggs/embryos. (A) *Normal 2-cell embryo immediately before the second cleavage division.* **(B)** *Embryo of the same age as A, but exposed from laying to 20 μmol/l mesulergine. No cleavage has occurred and there are four symmetrically distributed asters.* **(C)** *One-day-old embryo exposed from laying to 20 μmol/l mesulergine. To the right the chromatin material (ch), which forms one enormous «nucleus», and to the left an unnucleated cytoplasmic protrusion.* **(D)** *Section through a polychaete egg, exposed to 20 μml/l mesulergine. The egg was fixed at the time when the controls had just completed the first cleavage division. The radially arranged granules in the center are cortical granules, appearing along the spindle tubules. The granules in the periphery are yolk granules. k, karyomere. Bar, 10 μm.*

receptors from the yolk granules primarily with the high mitotic activity in polychaete eggs at the onset of development, since eggs, exposed to the receptor-blocker mesulergine, actually were unable to perform mitosis. This opinion is, moreover, in agreement with findings in other cell materials, e.g., cultured smooth muscle cells (Nemecek *et al.*, 1986) and fibroblasts (Seuwen *et al.*, 1988), in which serotonin was observed to be connected with increased mitotic activity. As the microscopical examination of the disturbed eggs revealed both aster formation and furrowing, it is obvious that the intracellular signals triggering these events were not affected. However, the constriction process necessary for cytokinesis did not work, apparently due to a lack of a functioning ring of microfilaments, nor was there any chromosome segregation. The results are similar to those observed in sea urchin eggs, exposed to the serotonin antagonists metergoline and gramine, in which these antagonists caused a delay of cleavage divisions without affecting DNA synthesis and mitosis. In fact, in sea urchin eggs 40 μmol/l metergoline even suppressed cleavage completely (Renaud *et al.*, 1983). It seems reasonably certain that the disturbances produced in the polychaete eggs should be ascribed to impaired access to intracellular Ca^{2+}. Active 5-HT1c and 5-HT2 receptors appear to generate inositol polyphosphates that release intracellular Ca^{2+} (Conn *et al.*, 1986; Julius *et al.*, 1988), and accordingly a blocking of them should therefore interfere with such Ca^{2+} release.

A deficiency of intracellular Ca^{2+} in the treated polychaete eggs is also suggested by the abnormal behavior of their internal cortical granules. These granules are electron-dense bodies about 1μm in diameter which in mature eggs are preferentially located in the egg cortex but also appear distributed in the cytoplasm. Analyses on polychaete cortical granules *in situ* have shown that they contain Ca (Emanuelsson and Odselius, 1985).

In normal polychaete eggs they appear in marked numbers only at fertilization and at the time of the early cleavage divisions, otherwise they are relatively sparse but can be observed in the

blastomeres up to gastrulation. The presence of numerous intact cortical granules in the treated polychaete eggs means that normal degradation of these granules (which should have resulted in release of Ca^{2+}) has not occurred. It is significant that in these eggs the still intact granules are located in the regions with the most obvious structural defects, i.e., the furrow region and the mitotic spindle, where absence of Ca^{2+} prevents microfilament action. In addition, dissolution of the spindle microtubules requires Ca^{2+} (McIntosh and Koonce 1989).

The enhanced decomposition of full-sized yolk granules observed in mesulergine-treated eggs should possibly be conceived as a forced attempt to mobilize more 5-HT receptors in order to compensate for the failing release of Ca^{2+}, and the substantial labeling recorded in eggs even upon pretreatment with unlabeled mesulergine is apparently consistent with that idea.

In the present situation one should finally reflect on the possibility that yolk granule-derived serotonin and serotonin receptors, besides their function in the pregastrular mitotic activity and in the gastrulation process, may also play a decisive role in early embryonic induction. Numerous investigations have shown that during the inductive interaction in early embryos the reaction system needs a stimulus, and enormous efforts have been spent on the search for specific inducers. The latter may be of an unspecific nature, however, and therefore it is rather the changing physiology of the action and the reaction system, and the differences arising between them, that should be investigated (Nieuwkoop, 1985).

Unequal release and distribution of important receptors among the cells in the early embryo — as was in fact observed for the 5-HT receptors in the present material — could obviously be one possible mechanism creating such differences. Interestingly, a key role for 5-HT receptors in cell differentiation was indeed recently indicated in a study which described cellular effects after introducing the 5-HT1c receptor in an unnatural environment, represented by fibroblasts in cell culture (Julius *et al.*, 1989). As the result of expression and activation of 5-HT1c receptors on the cell surface, the transfected fibroblasts in that case actually formed foci in the cell culture, i.e., they exhibited a transformed phenotype.

Materials and Methods

Egg material

Cultures of the marine polychaete *Ophryotrocha labronica* LaGreca and Bacci from the Mediterranean (Naples) were kept in sea water at room temperature (18°-20°C). The polychaetes breed all through the year and provide a continuous supply of embryo material. When deposited, the fertilized eggs are cemented together as cylindrical egg packs, with each of the approximately 300 eggs provided with a separate, thin jelly capsule, permeable to nucleosides, amino acids, etc. Development of the unpigmented eggs is easily followed in the dissecting microscope, and thanks to their smallness and transparency, they can be directly video filmed *in vivo* or alternatively photographically recorded in stained whole preparations without previous sectioning. Another great advantage is the strict synchronism in development that exists between all eggs of an egg pack; part of the pack can accordingly serve as a very reliable control in experimental work.

Early embryonic development in Ophryotrocha labronica

For newly laid eggs of this species the time interval until the first cleavage division is about 150 min at 20°C. During the early part of that period the meiotic division is completed, after which the pronuclei are formed and the first mitosis occurs. At the first cleavage division some discrete steps in the process can be discerned: first a flattening of the egg in the polar body region, followed by a local invagination in the same place. Then the invagination spreads and deepens along the equator of the egg, and eventually it leads to total constriction and cytokinesis.

The second cleavage division, resulting in the 4-cell-stage occurs about 90 min later, and the 8-cell-stage is attained after another 90 min. The following two cleavage divisions are still reasonably synchronous, but after that synchronism has practically ceased. Gastrulation takes place during day 3, and two days later the free-swimming larva appears.

Autoradiography

Polychaete eggs/embryos were removed from the egg pack jelly and transferred into sea water containing [N^6-methyl-^{3}H] mesulergine (Amersham International, specific activity 2.81-3.11 TBq/mmol; final activity 2.96 MBq/ml,1 µmol/l). The prospects of improved penetration and reduced risks of decomposition of the ^{3}H-mesulergine (the eggs cannot be isolated under sterile conditions) motivated the comparatively high concentration (1 µmol/l) of this substance in the experiments, even though significant labeling was found also at lower concentration (20 nmol/l). For newly laid eggs the exposure to ^{3}H-mesulergine was 1-2.5 h (until the time for the first cleavage of the control eggs); embryos aged 1-4 days were exposed for 1 h. After that they were briefly rinsed in pure sea water, fixed in buffered 2.5% glutaraldehyde according to Karnovsky (1965) (pH 7.4, 1 h, 20°C), rinsed in buffer (1 h, 20°C) and postfixed in buffered 1% osmium tetroxide (pH 7.4, 1 h, 4°C). Following repeated rinsing in buffer they were then dehydrated, stained in ethanol containing 1% phosphotungstic acid and 0.5% uranyl acetate, embedded in Epon, and finally sectioned for light microscopy (1 µm) and electron microscopy.

Sections for light microscope autoradiography (1µm) were covered with Ilford K 2 liquid nuclear emulsion according to the dipping method. Exposure: 6-14 days at 4°C. The autoradiographs were developed in Kodak D 19 (5 min, 18°C), briefly rinsed in distilled water, and fixed in Kodak Unifix (6 min, 18°C). After a final rinsing they were stained through the film in Richardson's azure II and methylene blue and mounted in DePeX.

The silver grain countings were made on light microscope autoradiographs of 1 µm sections (exposure: 14 days), using a 90x oil immersion objective and an eye-piece with a graticule in which each single square delimited an area of 13 µm^2 (the standard area referred to in Table 1).

Ultrathin sections for electron microscope autoradiography were first coated with a protective carbon layer and then covered with a monolayer of Ilford L 4 liquid nuclear emulsion according to the loop method .

Exposure: 1 month at 4°C. The autoradiographs were developed in Kodak D 19 (2 min, 20°C), rinsed in distilled water (30 sec, 20°C), fixed in newly made 15% $Na_2S_2O_3$ (3 min, 20°C), and finally washed in distilled water. They were examined in a Jeol 1200 EX electron microscope at the Unit of Electron Microscopy, Department of Zoology, University of Lund. Both in the light and the electron microscope autoradiographs the background was insignificant.

Since mesulergine has significant affinity to dopamine D-2 sites, labeling experiments with tritiated raclopride, a drug which binds selectively to dopamine D-2 sites (Köhler and Radesäter, 1986), were also performed on the eggs/embryos. Likewise, tritiated GR 65630, a selective 5-HT_3 receptor antagonist (Kilpatrick *et al.*, 1987), was tested in such control experiments.

Exposure to unlabeled mesulergine

Newly laid polychaete eggs as well as pregastrular embryos were removed from the egg pack jelly and rapidly transferred into sea water containing mesulergine (Sandoz A.G., Basel) in a 5-20 µmol/l concentration. The time for exposure, as well as the onset of exposure, was varied as described above. Controls from the same egg pack were kept in pure sea water. The developing eggs/embryos were continuously inspected during the exposure. Separate eggs were selected for video filming and fixation for light- and electron-microscopical analysis.

Another 5-HT2 receptor antagonist, ketanserin, was also tested in these experiments (with similar effects to mesulergine), but its low solubility in sea water, even in the presence of DMSO (1%), precluded more extensive use of it. The low solubility also prevented the use of ^{3}H-ketanserin in autoradiography. At acceptable concentrations of DMSO the resulting labeling — although of a character similar to ^{3}H-mesulergine — still proved too weak to be useful. All attempts to test the antagonist ritanserin failed owing to its extremely low solubility in sea water.

Microscopy

Video microscopy was performed using a Grundig FA 76 video camera mounted on an inverse microscope (40x objective) and connected with a monitor and a Grundig 2000 longplay video recorder. The high light sensitivity of the video camera permitted continuous recording of the eggs/embryos without adverse heat effects.

For light microscopy of whole mounts of eggs/embryos the latter were fixed for 1 h in a solution of 1 part glacial acetic acid + 3 parts absolute ethanol and then stained in toluidine blue or Gomori's hematoxylin (Melander and Wingstrand, 1953).

For light and electron microscopy of sectioned material the eggs/embryos were fixed in buffered 2.5% glutaraldehyde according to Karnovsky (1965) (pH 7.4, 1 h, 20°C) and postfixed in buffered 1% osmium tetroxide (pH 7.4, 1 h, 4°C). They were processed and embedded in Epon as described and then sectioned for light- and electron-microscopy.

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