The Sea Urchin Embryo as a Model for Mammalian Developmental Neurotoxicity: Ontogenesis of the High-Affinity Choline Transporter and Its Role in Cholinergic Trophic Activity

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Embryonic development in the sea urchin requires trophic actions of the same neurotransmitters that participate in mammalian brain assembly. We evaluated the development of the high-affinity choline transporter, which controls acetylcholine synthesis. A variety of developmental neurotoxicants affect this transporter in mammalian brain. [^{3}H]Hemicholinium-3 binding to the transporter was found in the cell membrane fraction at stages from the unfertilized egg to pluteus, with a binding affinity comparable with that seen in mammalian brain. Over the course of development, the concentration of transporter sites rose more than 3-fold, achieving concentrations comparable with those of cholinergically enriched mammalian brain regions. Dimethylaminoethanol (DMAE), a competitive inhibitor of choline transport, elicited dysmorphology beginning at the mid-blastula stage, with anomalies beginning progressively later as the concentration of DMAE was lowered. Pretreatment, cotreatment, or delayed treatment with acetylcholine or choline prevented the adverse effects of DMAE. Because acetylcholine was protective at a lower threshold, the DMAE-induced defects were most likely mediated by its effects on acetylcholine synthesis. Transient removal of the hyaline layer enabled a charged transport inhibitor, hemicholinium-3, to penetrate sufficiently to elicit similar anomalies, which were again prevented by acetylcholine or choline. These results indicate that the developing sea urchin possesses a high-affinity choline transporter analogous to that found in the mammalian brain, and, as in mammals, the functioning of this transporter plays a key role in the developmental, trophic activity of acetylcholine. The sea urchin model may thus be useful in high-throughput screening of suspected developmental neurotoxicants.
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Of the vast number of new chemical entities produced each year, only a handful ever undergo assessment for developmental neurotoxicity, despite the fact that it is well recognized that the immature brain is especially vulnerable to toxicants (Barone et al. 2000; Claudio et al. 2000). Accordingly, there is a need for high-throughput screens that might help focus and limit testing in mammalian models to those compounds most likely to elicit adverse effects. In this regard, the embryos and larvae of lower organisms, notably the sea urchin, appear to be promising candidates (Buznikov 1983; Buznikov et al. 1997, 2001b, 2003; Hagstrom and Lonning 1973; Pesando et al. 2003). In these species, just as in the developing mammalian brain, neurotransmitters such as acetylcholine (ACh), serotonin, norepinephrine, and dopamine serve as developmental (trophic) signals controlling the timing of cell replication and differentiation, cell death, and architectural organization (Buznikov et al. 1964, 2001a; Buznikov and Podmarev 1990; Gustafson and Toneby 1970; Hohmann and Berger-Sweeney 1998; Lauder 1985; Lauder and Schambra 1999; Weiss et al. 1998). Accordingly, substances that alter mammalian brain development, through their promotion or antagonism of neurotransmitter actions, elicit gross dysmorphology in sea urchin embryos, with critical periods corresponding to developmental surges in transmitter levels and the appearance of the appropriate receptors for the transmitters.

A number of articles have detailed the specific role played by ACh (for review, see Hohmann and Berger-Sweeney 1998; Lauder and Schambra 1999). In the case of mammalian brain development, this transmitter provides a focus for understanding the adverse effects of two major classes of insecticides, the organophosphates and the carbamates, which operate through inhibition of cholinesterase, as well as the effects of nicotine, the major developmental neurotoxicant in tobacco smoke (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1998, 1999). In addition, a wide variety of neurotoxicants appear to converge secondarily on cholinergic function as a contributory end point to their actions on brain development (Yanai et al. 2002). As reviewed a number of years ago (Buznikov 1990), ACh, along with its relevant biosynthetic/biodegradative enzymes and receptors, is present even in unfertilized sea urchin eggs, as well as in zygotes and other "pre-nervous" developmental stages. The concentration of ACh exhibits distinct peaks during early cleavage divisions, but the major, sustained increases occur after the beginning of gastrulation, in tandem with transcription of zygotic genes and attendant rises in choline acetyltransferase, the enzyme that synthesizes ACh (Buznikov et al. 1968; Buznikov and Podmarev 1990; Falugi et al. 2002). In accord with the trophic role of ACh, both ACh antagonists and agonists that are known to exert developmental neurotoxicant actions in mammals perturb sea urchin development, with periods of sensitivity corresponding to the surges in ACh levels (Buznikov 1990; Buznikov et al. 1968, 1996, 1997, 2001a, 2001b; Buznikov and Podmarev 1990; Buznikov and Rakic 2000; Gustafson and Toneby 1970; Pesando et al. 2003).

To our knowledge, no studies have appeared on the ontogeny and trophic role of the high-affinity choline transporter in the sea urchin. In the mammalian brain, choline transport, and not the activity of choline acetyltransferase, is the rate-limiting step in ACh biosynthesis and the locus of regulation of ACh levels (Klemm and Kuhar 1979; Simon et al. 1976). Inhibition of choline transport by hemicholinium-3 (HC-3) or its analogs, such as dimethylaminoethanol (DMAE), elicits neural tube defects in mammalian embryos (Fisher et al. 2002); later in development, toxicant effects on choline transport make major contributions to neurobehavioral perturbations (Dam et al. 1999; Happe and Murrin 1992; Sawin et al. 1998; Slotkin et al. 2001; Steingart et al. 1998, 2000; Zahalka et al. 1992, 1993; Zhu et al. 2000). In the present study, we demonstrate the presence of the high-affinity choline transporter in sea urchin embryos and larvae, the development of the transporter over the period in which this organism uses ACh as a

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trophic signal, anomalies associated with inhibition of choline transport, and proof that these adverse effects are related to inhibition of ACh synthesis, rather than other aspects of choline availability.

Materials and Methods

Adult specimens of *Lytechinus variegatus* were maintained in tanks with continuously filtered, circulating artificial seawater (ASW). Gametes were harvested and the eggs were fertilized and incubated as described previously (Buznikov et al. 2001b, 2003; Buznikov and Podmarev 1990). Embryos and larvae were cultured, and all experiments conducted, at approximately 22°C.

Development of the high-affinity choline transporter. For biochemical analyses, we used the material from 22 ripe females at the following stages: unfertilized eggs; embryos at 2–4 blastomeres (2 hr postfertilization), 16 blastomeres (3 hr), early blastula 1 (4.5 hr), and mid-blastula 2 (9.5 hr); and larvae at late blastula 1 (12 hr), prism 2 (25.7 hr), early pluteus 2 (34.3 hr), and mid-pluteus 1 (47.5 hr). The suspensions of eggs, embryos, or larvae were washed with 0.55 M KCl and sedimented, whereupon the precipitates were frozen and kept at –80°C. All embryonic stages and the first larval stage are pre-nervous, with the first neurons arising at the prism 2 stage and the plutei possessing the larval nervous system. The normal developmental stages of sea urchins, including *L. variegatus* embryos and larvae, have been published previously (Buznikov 1990; Buznikov and Podmarev 1990; Buznikov et al. 2003) and also are available on the Internet (Leland Stanford Junior University 2003).

Samples were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, USA) in ice-cold 10 mM sodium–potassium phosphate buffer (pH 7.4) and sedimented at 40,000 × g for 15 min. The supernatant solution was discarded, and the membrane pellet was resuspended (Polytron) and resedimented in the same buffer, after which the resultant pellet was resuspended using a glass homogenizer fitted with a Teflon pestle, in 10 mM sodium–potassium phosphate and 150 mM NaCl (pH 7.4). Radioligand binding to the transporter was evaluated using 2 nM [^{3}H]HC-3 (Vickroy et al. 1984; Zahalka et al. 1992), with incubation for 20 min at room temperature, followed by rapid vacuum filtration onto glass fiber filters that were presoaked for 30 min in 0.1% polyethyleneimine. The nonspecific component was defined as radioligand binding in the presence of an excess concentration (10 µM) of unlabeled HC-3. Binding values were expressed relative to membrane protein (Smith et al. 1985). A standard preparation of adult rat midbrain was used as a control to enable standardization across batches of assays required to assess different developmental stages.

To verify that HC-3 binding involved the high-affinity transporter, we performed Scatchard analysis at two developmental stages corresponding to low and high levels of binding: mid-blastula 2 and mid-pluteus 1. Radioligand binding was evaluated over a range of [^{3}H]HC-3 concentrations (0.1–3.2 nM) chosen so as to bracket the K_d value found for the transporter in the mammalian brain (Vickroy et al. 1984; Zahalka et al. 1992).

Effects of transport inhibitors on development. Embryos and larvae were obtained from 12 females, one for each series of experiments. Fertilized eggs, suspended in ASW, were placed in multiwell cell culture clusters, at approximately 150 eggs per well in a volume of 2 mL, and were incubated at approximately 22°C throughout the experiment up to the final, digitally captured images. Test substances (HC-3, DMAE, ACh, choline) were introduced at the one-cell stage (0.5–0.75 hr after fertilization), two-to-four–cell stage (1.3–2 hr), or mid-blastula 1 stage (7–8 hr). Embryos and larvae in control wells were incubated in pure ASW or in ASW with corresponding volumes of vehicles used for dissolution of the test chemicals (DMSO for HC-3, distilled water for other stock solutions). The addition of vehicle alone had no impact on embryonic or larval development. In some experiments, the eggs were preincubated briefly (5–10 min immediately after fertilization) with 1 M glycine to remove the hyaline layer so as to permit permeation of highly charged quaternary ammonium compounds such as HC-3 (Kane 1973). Freshly formed interblastomere contacts are transiently permeable to hydrophilic substances during the first third of the ensuing cell cycle (Dale et al. 1982; Korobtzov and Sorokin 1974; Sanger et al. 1985), so we anticipated that we could achieve entry of highly charged molecules into the embryo during the first few cell cycles after temporary dehyalination.

Embryonic and larval development were viewed continuously in the live organisms without any fixation, using a Leitz Wetzlar microscope in brightfield transmission mode, with a color digital video camera (Spot; Diagnostic Instruments, Sterling Heights, MI, USA) connected to a computer to capture the images. As described previously (Buznikov et al. 2001b), all embryos or larvae in a given well were digitized at low magnification (40×). As a rule, all (100%) organisms receiving a specific treatment showed the same phenotype, so representative specimens were then digitized at higher magnifications. We recorded the ages of embryos and larvae (time after fertilization) during digitization of results.

Data analysis. Data for HC-3 binding are presented as means and standard errors, with comparisons across developmental stages carried out by analysis of variance (ANOVA), followed by intergroup comparisons using Fisher's protected least significant difference. Scatchard plots were fitted by linear regression analysis and compared using analysis of covariance (ANCOVA). Significance was assumed at $p < 0.05$. Malformations caused by transporter inhibition are shown qualitatively; however, as described previously (Buznikov et al. 2001b), these effects are uniform over the entire population and represent observations made in > 3,000 embryos under each condition. In no case did control embryos or larvae show any malformations.

Materials. Sea urchins were obtained from Jennifer Keller (Duke University Marine Lab, Beaufort, NC, USA) and Susan Decker (Miami, FL, USA). [^{3}H]HC-3 (specific activity, 161 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA, USA). All other compounds were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA).

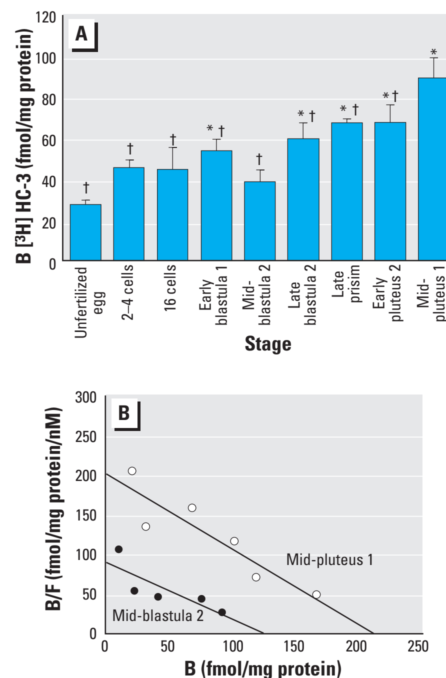

Figure 1. (*A*) Development of [^{3}H]HC-3 binding sites (mean ± SE) in the sea urchin embryo at a single, subsaturating ligand concentration (2 nM); these values allow for detection of effects on either the concentration of binding sites or the affinity of the site for the ligand. ANOVA: $p < 0.0006$ (n = 3–5 per stage). (*B*) Scatchard determinations of binding affinity and the concentration of transporter sites at two stages; results indicate a significant increase in the total concentration of sites (B_{max}) but not in the dissociation constant for binding of the ligand to the site (K_d). Abbreviations: B, bound ligand; F, free ligand. The K_d (nM) and B_{max} (fmol/mg/mg protein) are, respectively, 1.4 ± 0.6 and 126 ± 24 for mid-blastula 2 and 1.1 ± 0.2 and 212 ± 22 for mid-pluteus 1; ANCOVA: $p < 0.005$.
*Significant compared to unfertilized egg. †Significant compared to mid-pluteus 1.

Results

Comparisons of [^{3}H]HC-3 binding across all developmental stages were first conducted at a single, subsaturating ligand concentration (2 nM), which allows differences to be detected regardless of whether there are shifts in the concentration of transporter sites or in their affinity for the ligand. Even in the unfertilized egg, there was significant [^{3}H]HC-3 binding associated with the cell membrane fraction (Figure 1A). The amount of binding doubled by the stage and then rose more substantially at late blastula 2 stage and beyond. To verify that the HC-3 binding represented its association with the high-affinity choline transporter, and to determine whether the differences reflected alterations in the concentration of sites, we performed Scatchard analysis at mid-blastula 2 and mid-pluteus 1 stages (Figure 1B). At both stages, the K_d was in the low nanomolar range, comparing favorably with the affinity of HC-3 for the high-affinity transporter in mammalian brain (Vickroy et al. 1984; Zahalka et al. 1992). The developmental increase between these two stages represented primarily an increase in the concentration of transporter sites, as evidenced by a significant main effect of age (ANCOVA), reflecting an increase in the B_{max} of binding, without significant changes in slope that would indicate a shift in K_d.

We next evaluated whether the functioning of the putative choline transporter played a role in trophic control of embryonic development. Because of limitations of permeability, we initially studied DMAE, a membrane-permeable analog of HC-3 (Alkadhi 1986; Fisher et al. 2002). DMAE added to the embryos at the two-cell stage in concentrations of 100–400 µM did not alter cleavage divisions or blastulation, but at very high concentrations (600–800 µM), cleavages were inhibited (Figures 2 and 3). If the latter effect represents interference with high-affinity choline transport and the resultant deficiency in ACh, then the introduction of ACh or choline should prevent the inhibition. However, this was not the case: concentrations of either agent up to 800 µM failed to restore the ability to divide (Figure 2).

Although cleavage divisions were affected only at very high DMAE concentrations, embryos treated with lower concentrations at the two-cell stage subsequently developed malformations (Figure 3): overabundance of mesenchyme-like cells that accumulated first in the vegetal half of the blastocoel, and later occupied all of the blastocoel, blocking or inhibiting gastrulation; absence of a second pair of arms in mid-pluteus, or sometimes shortening or absence of one arm of the first pair (Figure 3H). At the highest concentrations (300–400 µM), dysmorphology was evident at the late blastula stage, whereas at 200–400 µM, anomalies emerged at gastrulation, followed at the lowest concentrations (100–200 µM) by

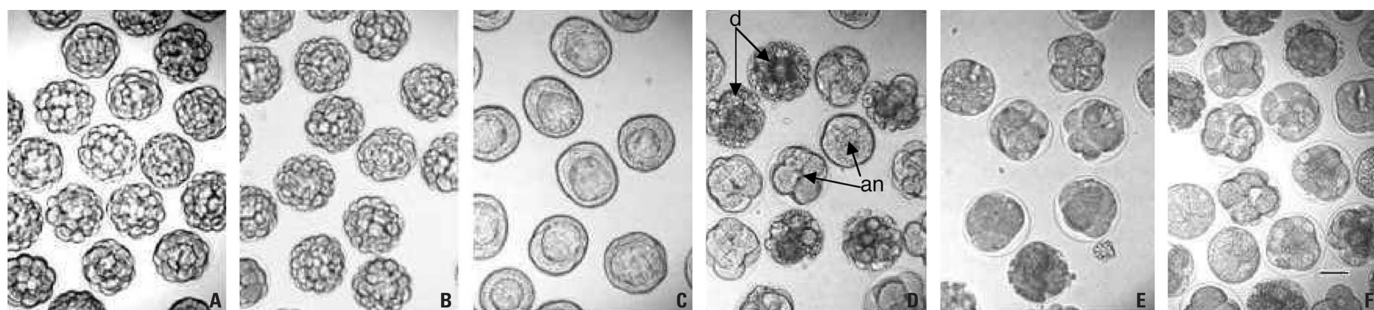

Figure 2. Effects of DMAE, introduced at the two-cell stage, on early cleavage divisions. At concentrations up to 200 µM, there were no effects of DMAE: (*A*) control, 5 hr 18 min after fertilization, early blastula 2 stage; (*B*) 200 µM DMAE, showing normal embryonic development at this stage. At 600–800 µM, cleavage divisions are inhibited: (*C*) control, 11 hr 20 min after fertilization, late blastula 2 stage; (*D*) 800 µM DMAE, showing abnormal (an) or dead (d) embryos, with development blocked at the 4-, 8-, or 16-cell stage. Treatment with 800 µM DMAE and (*E*) 800 µM ACh or (*F*) choline fails to prevent the effects of DMAE (late blastula 2 stage). Bar = 50 µm.

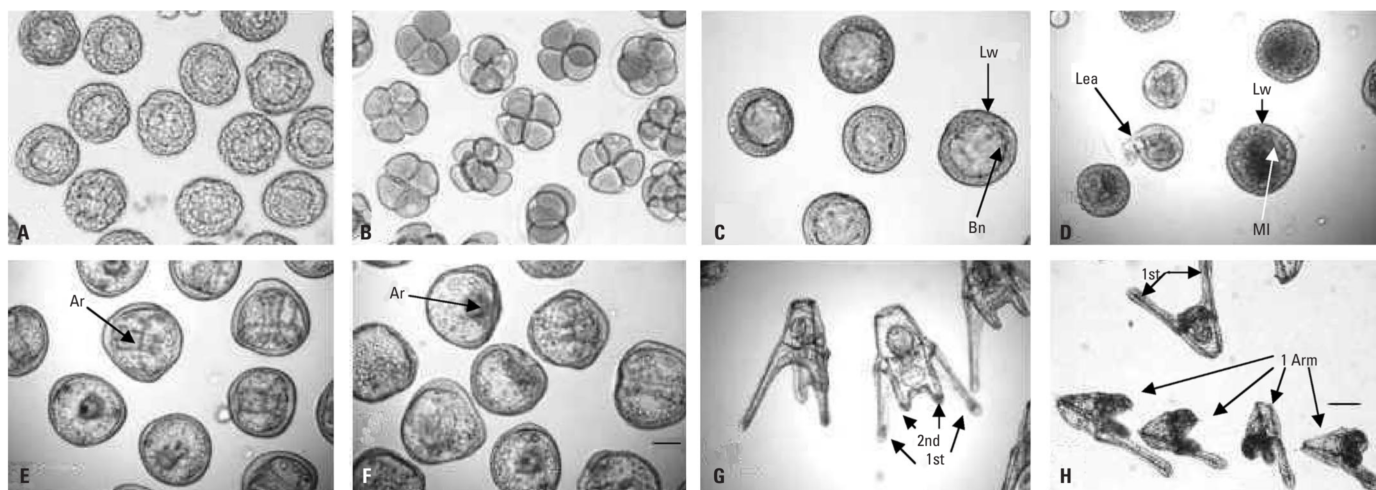

Figure 3. Malformations after introduction of DMAE at the two-cell stage. Abbreviations: Ar, primary gut or archenteron; Lw, larval wall. (*A*) Control, 4 hr after fertilization, early blastula 1 stage. (*B*) 800 µM DMAE, showing abnormal embryos with developmental block at the four-to-eight–cell stage. (*C*) Control, 10 hr 20 min after fertilization, late blastula 1 stage, showing a blastula cavity or blastocoel (Bn) with few mesenchyme cells. (*D*) 400 µM DMAE, showing hyperproduction of mesenchyme-like pigmented cells (Ml) in the blastocoel, with these cells sometimes leaking (Lea) from the blastocoel. (*E*) Control, 23 hr 25 min after fertilization, prism 1 stage. (*F*) 200 µM DMAE, showing underdevelopment or absence of archenterons, that is, inhibition of gastrulation. (*G*) Control, 49 hr after fertilization, early pluteus 2 stage, showing first (1st) and second (2nd) pairs of arms. (*H*) 100 µM DMAE, dwarf larvae, showing absence of a second pair of arms, shortening of the first pair, or absence of one of the arms of the first pair (1 Arm). Bar = 50 µm for *A*–*F*; bar = 100 µm for *G* and *H*.

abnormalities at the pluteus stage. These malformations were all elicited in a similar fashion, regardless of whether DMAE was introduced before cleavage divisions, during the first few divisions or as late as the mid-blastula stage.

In contrast to the lack of protection offered by ACh or choline for the effects of DMAE on cleavage divisions, we found that either agent, introduced 10–15 min before, simultaneously with, or 30–60 min after DMAE, provided protection against the delayed-onset disruption of the later developmental events (Figure 4). For ACh, there was partial protection above a threshold concentration of about 25 µM. Choline was somewhat less effective, requiring a higher threshold concentration for a protective effect: 50 µM choline did not prevent malformations resulting from DMAE treatment but 200 µM (equimolar to DMAE) achieved complete protection.

Permeability factors may limit the protective effect of ACh or choline because both of these contain quaternary ammonium groups, just like HC-3. To characterize this problem, we compared the effects of HC-3 with DMAE in intact embryos and larvae (Figure 5). Concentrations of HC-3 up to 80 µM failed to have any discernible effect on development, despite the fact that this concentration is more than two orders of magnitude above the K_d that we found for the binding of HC-3 to the transporter. Accordingly, we next produced transient elimination of the hyaline layer by a brief incubation with 1 M glycine, a treatment that does not disturb embryonic development but that produces a short-duration permeation to charged molecules. This procedure rendered the embryos sensitive to HC-3 (Figure 6). With the introduction of 20–80 µM HC-3, embryos developed quite normally up to the mid-blastula stage, at which time they manifested the same archetypal developmental malformations as seen with DMAE (compare Figure 6A with Figure 4A). Similarly, introducing ACh or choline during the permeation period then protected the developing organism from HC-3. In this case, ACh was able to protect the embryo fully at concentrations of 25–800 µM; choline gave only partial protection at concentrations equimolar to HC-3, whereas lower concentrations were less effective or ineffective.

Discussion

Results obtained in the present study indicate that the developing sea urchin embryo possesses a high-affinity choline transporter whose biochemical and pharmacologic properties resemble those found for the transporter present in the mammalian brain, and that this transporter plays a similar role in the trophic functions of ACh. We used differential sedimentation to isolate the cell membrane fraction and found significant, saturable [^{3}H]-labeled HC-3 binding even in the unfertilized egg, with progressive increases through development to the pluteus stage. This site displayed the same characteristic, high-affinity binding (nanomolar K_d) of HC-3 as in the mammalian brain, and by mid-pluteus 1, the concentration of binding sites relative to membrane protein was > 200 fmol/mg, comparable with mammalian brain regions that are enriched in cholinergic nerve terminals (Zahalka et al. 1992). Future studies should be able to identify whether the protein that mediates high-affinity choline transport in the sea urchin is indeed highly homologous to the transporter in mammalian brain, or whether the two proteins represent convergent evolution. Regardless of the actual amino acid sequences of the proteins, the main importance, as studied here, lies in the fact that the transporter plays a similar trophic role in sea urchin development as in mammalian brain, and that it responds to neurotoxicants in the same way.

In cholinergic neurons, high-affinity choline transport provides the control point for ACh synthesis (Klemm and Kuhar 1979; Simon et al. 1976) and is a major site of alterations induced by developmental neurotoxicants that affect cholinergic trophic or synaptic function (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1998, 1999; Yanai et al. 2002). If the transporter plays a similar role in the morphologic assembly of the sea urchin embryo, then pharmacologic interference with the transporter should evoke distinct malformations. We observed such effects for DMAE, an analog of HC-3 that can readily penetrate the hyaline layer and cell-surface membranes of the embryo, but there were two distinct phases of effect. High concentrations of DMAE interfered with early cleavage divisions, an effect that was not antagonized by coadministration of ACh or choline. Accordingly, the actions of DMAE at these stages may involve secondary actions unrelated to inhibition of choline transport. On the other hand, it is clear from previous work that ACh does play a role in triggering cleavage divisions in sea urchins (Buznikov et al. 1970). It is therefore likely that activity of the transporter is relatively unimportant at that stage, perhaps because of high levels of

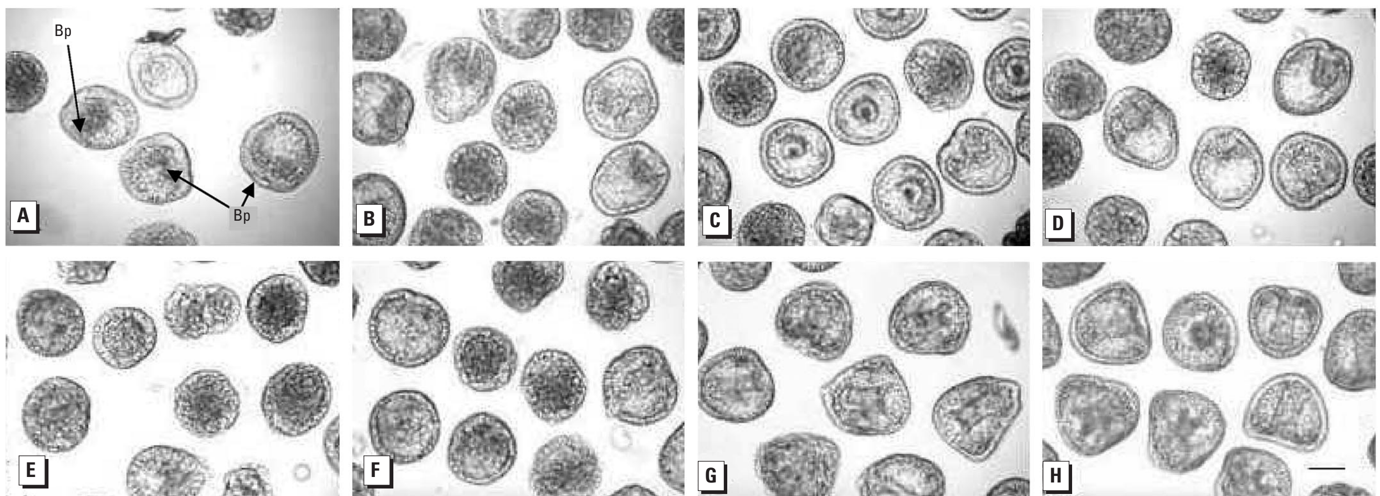

Figure 4. Effects of DMAE (200 µM) on developing sea urchin embryos and the protective effects of ACh and choline. Test substances were added at the two-cell stage (1 hr 25 min after fertilization), and larvae were examined 22 hr 50 min after fertilization (prism 1 stage in the controls). (*A*) DMAE alone; development is blocked at the late blastula 2 to early gastrula 1 stages, with hyperproduction of mesenchyme-like cells near the primary mouth or blastopore (Bp). (*B*) DMAE plus 12.5 µM ACh produces nearly the same effect as with DMAE alone, but for 20–30% of the larvae, development is blocked later, at the early gastrula 2 to mid-gastrula 1 stages, representing slight protection. (*C*) DMAE plus 25 µM ACh; development is blocked at the mid-gastrula 2 stage (moderate protection). (*D*) DMAE plus 50 µM ACh; blockage is postponed to mid-gastrula 2 to late gastrula 1 stages (moderate protection). (*E*) DMAE plus 50 µM choline, showing no protection. (*F*) DMAE plus 100 µM choline, showing slight protection (normal larvae, as in *H*). (*G*) DMAE plus 200 µM choline, showing full protection (normal larvae). (*H*) Controls raised in ASW alone reach the prism 2 stage. Bar = 50 µm.

endogenous choline stored in the yolk granules before fertilization.

We obtained very different results for effects of DMAE arising at the mid-blastula stage and later. The mid-blastula represents a point of rapid transcription of zygotic genes (Buznikov 1983, 1990; Buznikov et al. 1996, 1997; Buznikov and Podmarev 1990); accordingly, this is the stage at which effects of environmental toxicants on the programming of trophin/differentiation interactions, and most especially those of cholinotoxicants, are most likely to emerge (Buznikov 1990; Buznikov et al. 1968, 1996, 1997, 2001a, 2001b, 2003; Buznikov and Podmarev 1990; Buznikov and Rakic 2000; Gustafson and Toneby 1970; Pesando et al. 2003). Here, we found a dose-dependent effect of DMAE that operated with a distinctly lower threshold than for inhibition of cleavage divisions, with the onset of anomalies shifting to later and later stages as the dose was lowered. Equally important, the embryos were protected from the effects of DMAE by inclusion of either ACh or choline, thus indicating a mechanistic connection to the activity of the choline transporter. That finding alone does not identify ACh as the important trophic factor because deficiencies in choline could also affect membrane integrity through interference with phosphatidylcholine synthesis, a mechanism proposed for DMAE's ability to produce neural tube defects in mammals (Fisher et al. 2002). It is therefore critical that we found a lower threshold for protection by ACh compared with choline. Conceivably, ACh could be broken down to choline by the action of cholinesterase, but under no circumstance would ACh then be more effective than equimolar concentrations of choline. Accordingly, our results are consistent with a fundamental role for the high-affinity choline transporter in the synthesis of ACh required by the developing sea urchin for morphologic assembly during and after the rise in zygotic gene transcription at the mid-blastula stage.

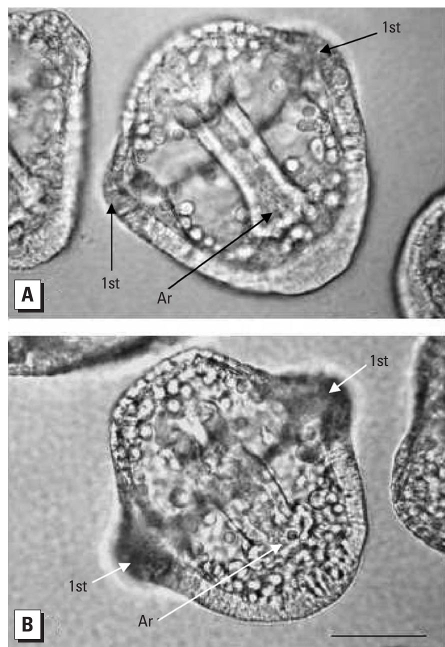

Figure 5. Intact sea urchin larvae are insensitive to 70 μM HC-3. Test substances were added at the one-cell stage, 40 min after fertilization, and larvae were examined at 26–27 hr. (*A*) Controls (0.4% DMSO in ASW) have reached the prism 2 to early pluteus 1 stages. (*B*) The HC-3–exposed group appears normal. Abbreviations: 1st, buds of the first pair of arms; Ar, upper end of archenterons and secondary mouth. Bar = 50 μm.

One limitation of any proposed cell culture system or invertebrate model for developmental neurotoxicity is that the fetal mammalian brain is typically protected by diffusion barriers: the placenta (Sastry 1991) and, depending on the stage of development, the blood–brain barrier (Saunders and Møllgard 1984). Similarly, the sea urchin embryo is protected by a hyaline barrier that, like the placenta and blood–brain barrier, tends to retard the penetration of charged molecules. We were able to distinguish between permeant and nonpermeant inhibitors of the choline transporter by comparing effects of DMAE with the quaternary ammonium–containing analog HC-3. Without removal of the hyaline barrier, HC-3 was totally ineffective in eliciting the delayed-onset defects associated with inhibition of the choline transporter. The hyaline layer specifically protects the highly permeable zones of the freshly formed interblastomere contacts during the first few cell divisions (Dale et al. 1982; Korobtzov and Sorokin 1974; Sanger et al. 1985). Dehyalination opens these diffusion barriers for a brief period during each cell cycle, and even these few episodes of permeation were sufficient to allow HC-3 to penetrate and produce the same anomalies as seen with DMAE. Again, the specific role of ACh was demonstrable in the protective effects of ACh and choline. This means that the sea urchin provides a way for testing different compounds in the same chemical class for the role of permeation in their ability to elicit developmental anomalies, namely, by comparing effects with and without dehyalination. Accordingly, the sea urchin may also afford an appropriate model with which to predict the relative importance of diffusion barriers that operate to protect the fetal mammalian brain.

In conclusion, the developing sea urchin embryo possesses a high-affinity choline transporter whose expression increases greatly over the period in which the embryo uses ACh as a trophic signal. Inhibitors of transport elicit a

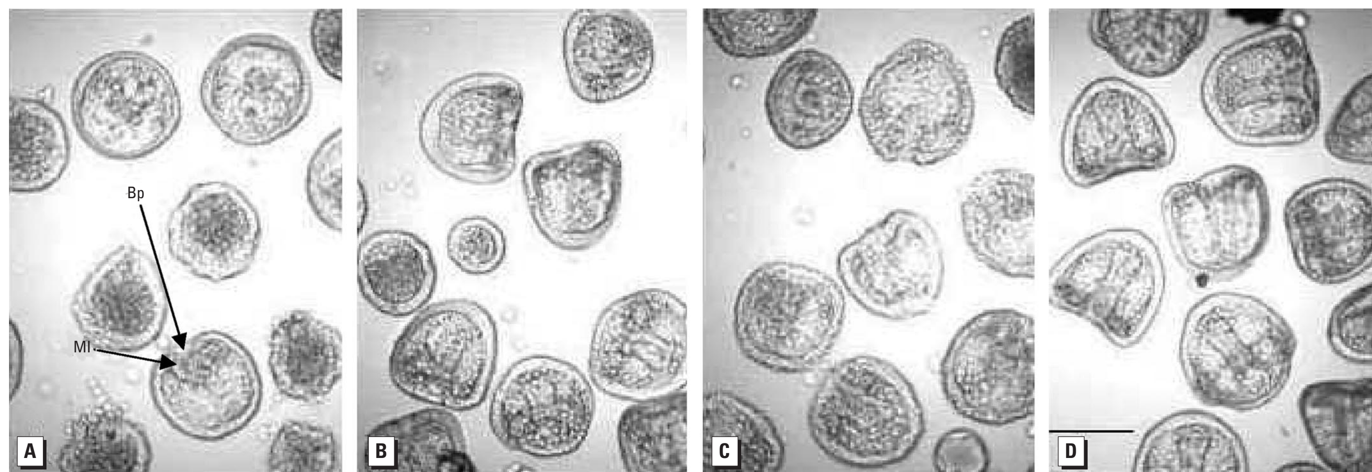

Figure 6. Dehyalination with glycine (see "Materials and Methods" for details) allows HC-3 to penetrate the embryo and elicit malformations. Test substances were added at the one-cell stage (40 min after fertilization); larvae are shown 25 hr after fertilization. (*A*) Effects of 40 μM HC-3; development is blocked at the late blastula 2 to early gastrula 1 stages, again with hyperproduction of mesenchyme-like cells (Ml) near the blastopore (Bp) (see Figure 4A). (*B*) HC-3 plus 200 μM ACh; most larvae are normal (full protection). (*C*) HC-3 plus 200 μM choline, showing clear but incomplete protection. (*D*) Control (0.4% DMSO in ASW) at the prism 1 stage. Bar = 100 μm.

characteristic pattern of delayed-onset malformations that do not arise until the mid-blastula stage, the same period when cholinotoxicants disrupt morphologic development. Adverse effects of DMAE and HC-3 were offset by either ACh or choline, indicating that the transporter plays the same critical role in the control of ACh levels as it does in the mammalian brain. Furthermore, the hyaline barrier provides protection of the embryo from charged molecules, just as the placenta and blood–brain barrier do for the fetal mammalian brain, and the technique of transient dehyalination can therefore be used to model the protective effect of these diffusion barriers against potential toxicants. Each female sea urchin provides many thousands of eggs. After fertilization, these large numbers of embryos can be evaluated quickly with simple light microscopy and video capture, yielding robust, obvious, and uniform dysmorphologies as a result of exposure to compounds that are likely to produce far more subtle neurochemical and behavioral alterations in the developing mammalian brain. The sea urchin embryo may therefore provide a high-throughout screen for developmental neurotoxicants.

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