REVIEW / SYNTHÈSE

Eutely, cell lineage, and fate within the ascidian larval nervous system: determinacy or to be determined?[1]

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Abstract: The larval central nervous system (CNS) of the ascidian *Ciona intestinalis* (L., 1767) arises from an embryonic neural plate and contains sufficiently few cells, about 330, to enable definitive counts. On the basis of such counts, there is evidence both for cell constancy (eutely) in the larval CNS and for small variations in the overall numbers of cells and among defined cell types within this total. However, evidence for the range of such deviations and the existence of a true phenotypic wild type are lacking. The record of cell lineage, i.e., the mitotic ancestry of each cell, and the fates of some of these cells have recently received increased documentation in both the genus *Ciona* and *Halocynthia roretzi* (von Drasche, 1884). Relatively few generations of cells, between 10 and 14, form the entire CNS in *C. intestinalis*, and cell death does not occur prior to larval hatching. The tiny complement of larval CNS cells can therefore be seen as the product of a small fixed number of determinate cleavages, and variations in cell number as the product of minor deviations in this mitotic ancestry. Within these lineage records, some cell fates have already been identified, but knowledge of most is lacking because the cells lack markers or other identifying features. Nevertheless, this tiny nervous system offers the prospect that all its cells can one day be identified, and their developmental histories and larval functions analyzed, cell by cell.

Résumé : Le système nerveux central (CNS) des larves de l'ascidie *Ciona intestinalis* (L., 1767) se développe à partir d'une plaque neurale embryonnaire et contient un nombre suffisamment petit de cellules, de l'ordre de 330, pour permettre des décomptes précis. D'après ces décomptes, il y a des indications que le nombre de cellules est constant (eutélie) dans le CNS et qu'il y a dans cet ensemble de faibles variations du nombre total de cellules et de certains types définis de cellules. Il n'y a pas d'information cependant sur l'étendue de ces déviations, ni de preuve de l'existence d'un vrai phénotype sauvage. On a récemment accumulé de l'information nouvelle sur les lignées cellulaires, l'ascendance mitotique de chaque cellule et le sort de certaines cellules chez *C. intestinalis* et *Halocynthia roretzi* (von Drasche, 1884). Un nombre relativement restreint de générations cellulaires, entre 10 et 14, forme le CNS complet de genre *Ciona* et il n'y a pas de mort cellulaire avant l'éclosion de la larve. Le petit complément de cellules du CNS des larves peut donc être interprété comme le produit d'un nombre réduit et fixe de clivages déterminés et les variations du nombre de cellules comme le résultat de déviations mineures de l'ascendance mitotique. Dans ces lignées, le sort de certaines cellules a déjà été identifié, mais il y a peu d'information sur la plupart des cellules, car elles ne possèdent ni marqueurs ni autres caractéristiques qui permettent leur identification. Néanmoins, le système nerveux de petite taille offre la possibilité que toutes ses cellules soient un jour identifiées et que le parcours de leur développement et leurs fonctions chez les larves soient analysés, cellule par cellule.

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Introduction

Sea squirts, or ascidians, have a development that is metamorphic, the adult stage of the life-cycle arising from a transient free-swimming tadpole larva. Studies of the larvae of ascidians, since their many forms have differing anatomies and habits (Berrill 1950; Burighel and Cloney 1997), are enjoying a particular renaissance. Ascidian larvae have been promoted by enthusiasts as a virtual chordate *Caenorhabditis elegans* (Maupas, 1900) Dougherty, 1955, partly

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because, like the latter nematode, they have few cells. In the larvae of the sea squirts *Ciona intestinalis* (L., 1767) (hereafter simply *Ciona*) and *Halocynthia roretzi* (von Drasche, 1884) (hereafter simply *Halocynthia*), these number only about three times the 959 somatic cells in the nematode *C. elegans* (White et al. 1988). Like the latter, too, the cells of the ascidian larva have a pattern of cell lineage that is claimed to be invariant (Satoh 1994). These features are especially well established in the common sea squirt *C. intestinalis*. Understandable as these rather promotional aspects of such comparisons are, however, there seems little need to dignify the ascidian tadpole larva, certainly not within the context of this issue on protochordate deuterostomes, nor for that matter does *Ciona* need to ride on the coattails of any other organism, simple or complex. There is, in fact, already sufficient interest in the ascidian tadpole larva and its tiny central nervous system (CNS), especially in *Ciona*. The reasons for this are simple enough to see. The larva in *Ciona* is endowed with what has been characterized rather loosely as a chordate brain in miniature (Meinertzhagen and Okamura 2001; Meinertzhagen et al. 2004). This feature alone does not distinguish the genus *Ciona* from the larvae of other ascidians, most notably its close rival in experimental embryology, *H. roretzi*, but analysis of the CNS in *Ciona* is facilitated by two additional features. First, the draft sequence of the *Ciona* genome has been released (Dehal et al. 2002). Second, *Ciona* embryos can be transfected by means of electroporasis (Corbo et al. 1997), providing a way to analyze gene action in the developing nervous system. In the bigger picture, these developments coincide with a reawakening of interest in evolutionary questions for which the basal position of ascidians and their larvae are critically placed in the evolution of the chordate phylum. Despite the existing body of knowledge about the larval CNS (Katz 1983; Nicol and Meinertzhagen 1991), many questions remain concerning the number and lineage of its cells, which this article will seek to discuss.

Cell number and the question of eutely

The larva of *Ciona* contains an estimated 2600 cells (Satoh 1999), while that of *Halocynthia* has somewhat more, about 3000 cells (Yamada and Nishida 1999). In *Ciona*, the cell total includes exactly 40 notochord cells (Cloney 1990) and a similar number of muscle cells, either 36 in *Ciona* (Katz 1983) or 42 in *Halocynthia* (Satoh 1994) in all normal larvae. Burighel and Cloney (1997) provide numbers for yet other species. The constancy itself is a condition referred to in the early literature as eutely (Martini 1909*a*, 1909*b*) and is widely found in many animal groups (Van Cleave 1932). The certainty of such clear cell constancy between individuals is based partly on the relative ease with which small numbers of cells can be counted accurately. By contrast, our best estimate for the CNS is that it has a little more than 330 cells (Nicol and Meinertzhagen 1991), but the constancy of this number is less clear than for other tissues. An additional 40 or so cells form the primordium of the adult CNS, the anteriorly situated neurohypophysis, but constancy in the number of these has so far not been claimed. Even though a nervous system with such a small numerical constituency, tiny by comparison with any other chordate nervous system, might be thought finite, numbers of this order are nevertheless sufficient to introduce indeterminacy simply by the methods used to count them. As a result, it is still hard to distinguish biological variation from variation for methodological reasons. More than this, it is entirely possible that just as some tissues such as notochord and muscle are eutelic and others such as epidermis probably are not, so some cell classes in the brain are strictly eutelic, while others are not, giving rise to variable numbers of CNS cells overall. This would be the special case of incomplete cell constancy seen in many species in different animal groups (Van Cleave 1932). Alternatively, it could still be that CNS cell number in ascidian brains is no more closely determined than it is in much larger vertebrate brains, only that because the total number is much smaller so too is the variation in that number.

More important than the number of cells is the number of their types or classes, each of which requires developmental and genetic specification. Among various examples considered by Williams and Herrup (1988), variation in total cell number is greatest among populations of neurones with parallel arrays, whereas there are numerous cases of identified neurones, mostly in invertebrates, in which there is typically no variation. To some extent, the relative blend between these two extremes is a function of total cell number in the nervous system. Thus, an analysis attempting to define the numbers of equivalent neurones in any one class — those among which it is not possible to recognize phenotypic differences — indicates that as brain number increases so too does the average number in each cell class (Bullock 1978).

With this evidence as a background, we should therefore examine the evidence in the CNS of larval *Ciona* critically.

Cell types and numbers

The most authoritative cell counts still appear to be those of Nicol and Meinertzhagen (1991) on *Ciona intestinalis*. Constancy of cell number in the CNS is indicated from counts in three larvae, with CNS complements of 331, 335 and 339 cells (Nicol and Meinertzhagen 1991). It is important to note that these counts all derive from sibling larvae, which could account for some of their uniformity. For example, counts from a much older report give more widely divergent totals of 354 and 275 cells for two larvae (Balinsky 1931). These numbers bracket the counts reported from semithin sections (Nicol and Meinertzhagen 1991), thus making them at least plausible, but with a difference that is too great to attribute to minor deviations from eutely in a few cell types. Future analysis of possible deviations will depend on being able to identify such cell classes.

Are ascidian larval neurones in fact identifiable? Structure is an exquisite phenotype, and the long history of microscopy has endowed us with good instruments to examine structure. Molecular markers may provide more categorical information on phenotype, for which a genetic rationale is clear, but generally have not been the subject of careful anatomical expression studies and therefore can still incorporate ambiguities. While total cell numbers may still harbor some residual uncertainties, assignment of most cells to identifiable classes is far less certain. On the grounds of their cytological appearance in semithin sections, about 100 larval CNS cells were previously considered to be neurones that

were distinct from ependymal cells. No matter how carefully this evidence is stated, it has apparently been hard to avoid declarative numerical statements in secondary reports, and caution is again urged in the restatement of this evidence. To claim eutely for a particular cell class in the larval brain, we need, in ascending order, first to recognize a unique morphological or other phenotypic signature for each particular neurone; then to recognize neurones as individuals or small groups; before, finally, to confirm whether neurones and their numbers are the same from larva to larva.

The preceding problems are mostly a question of how to characterize neurones and their connections. While that characterization may be easier for the relatively few ascidian larval neurones than it is for any other chordate brain, it still requires firm criteria. These may appear straightforward for structurally discrete sensory neurones (e.g., Eakin and Kuda 1971), but for anonymous interneurones, the task requires adequate markers, possibly among those recently reported (Mochizuki et al. 2003). Nicol and Meinertzhagen (1991) recognized 13 cell types within the larval CNS on purely cytological and positional grounds, with between 1 and 19 member cells in each of the classes that could be identified by structural criteria. However, evidence for some of these classes is weak, and simple arithmetic dictates that the remaining classes must be either more populous or not, in fact, uniform. By contrast, several cell types are structurally quite distinct, or have specific molecular markers, making them easily recognizable. These include motor neurones and some neurones of the sensory vesicle (e.g., photoreceptors and coronet cells). Likewise, pigment and accessory cells are also quite distinct in some cases (e.g., lens cells in the ocellus, or the otolith).

Motor neurones

Several estimates exist for the numbers of motor neurones. Serial electron microscopy of two larvae indicates the existence of five pairs in *Ciona* (Stanley MacIsaac 1999), and there can be little doubt of this number, at least for those larvae. Four cell pairs seen in UA301-labeled *Ciona* larval whole mounts are suggested as being motor neurones (Takamura 1998). In *Halocynthia*, on the other hand, driving expression of GFP under a neurone-specific promoter has revealed three pairs of motor neurones (Okada et al. 2002). Compatible with acetylcholine (ACh) as the neurotransmitter (Ohmori and Sasaki 1977), cells in the visceral ganglion are labeled with probes against either the synthetic enzyme for ACh, choline acetyltransferase (ChAT), or a specific vesicular transporter (VAChTr). However, the numbers of such cells vary between 1 and 4, depending on the larva, with evidence of left–right asymmetry in the numbers of in situ labeled cells. Moreover, the numbers of such cells also differ for the two probes (Takamura et al. 2002). The last two pieces of evidence suggest that these probes do not identify all cells in whole mounts, probably because of technical reasons and (or) the labeled larvae differed possibly in the stage at which they were labeled.

Photoreceptors

For photoreceptors, the problems of numerical identification are reversed. By structural criteria, there are 17–18 cells on the right-hand ocellus (Nicol and Meinertzhagen 1991), whereas two different photoreceptor-specific visual cycle protein antibodies label larger numbers of cells than these. An antibody against Ci-Opsin1 immunolabels about 20 cells inside the ocellar pigment cup and several cells outside it, for example, whereas immunoreactivity against Ci-arrestin (Nakagawa et al. 2002) co-localizes in the ocellus to cells that also express the immunoreactivity against Ci-Opsin1, but it also extends considerably beyond those cells (Tsuda et al. 2003*a*).

These comparisons sound a cautionary tone and raise important questions over which criterion, or combination of criteria, should arbitrate over cell identity. For, while we are still justifiably optimistic that the CNS in *Ciona* is essentially eutelic, the evidence is still far from complete and we should therefore keep an open mind.

Some may see the issue of eutely as a trivial problem in accountancy, but cell constancy is the underlying basis of the identified-neurone approach to invertebrate nervous systems (Wiersma 1974; Hoyle 1983), the backbone to studies on animals without backbones. It offers the eventual prospect that all cells in the larval CNS can be identified, and their developmental histories and larval functions analyzed, cell by cell.

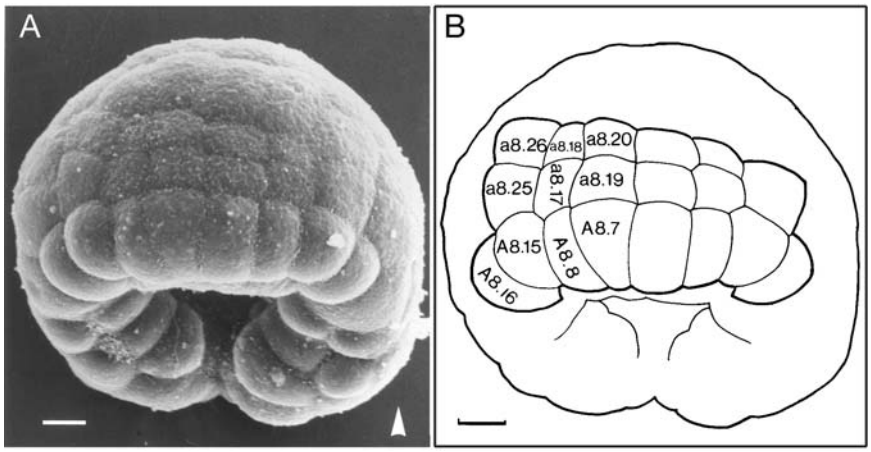
Sources of phenotypic variation

Before we can reach any conclusions about cell constancy, a pressing issue still to resolve is whether there is a true structural wild type for the ascidian larval nervous system, or at least to identify the phenotypic range that exists among wild-type animals. One source of variation among reported structural phenotypes is clearly methodological, arising especially from cell counts taken from whole mounts rather than from sections. Biological sources of variation include the genetic background and developmental stage of the larva, and any possible environmental effects on these of temperature, etc. The developmental stage is particularly important if larval cell death occurs (see below), changing cell complement, even shortly after hatching. It is again worth emphasizing that the only reliable evidence for eutely in the CNS comes from the compositional analysis of sibling larvae of the same age (Nicol and Meinertzhagen 1991).

Cleavage and cell lineage of neural plate progeny

From the moment of fertilization, the embryo undergoes a fixed pattern of rapid cleavages, which are initially synchronous and spaced at intervals of about 30 min (Conklin 1905; Satoh 1994). Along with the short cell cycle, ascidian embryos reach each developmental stage after fewer cleavages than in corresponding chordate embryos. Gastrulation in ascidians takes 7 cleavages, whereas in amphioxus it takes 9–10 and in amphibians 12–15 (Berrill and Karp 1975). Bearing in mind that cell number doubles with each cleavage, differences in cell number can be readily appreciated: 7 cleavages produce 128 cells, whereas 15 produce more than 32 000. Associated with the smaller cell numbers, neurulation in urochordate embryos occurs relatively later than in chordate embryos, with the neural plate remaining with the surrounding ectoderm until more than half way through embryogenesis before internalizing (Cole and

Fig. 1. (A) Scanning electron micrograph (SEM) of a mid-gastrula embryo of *Ciona intestinalis*, in anterodorsal view, at the 112-cell stage, 5.0 h after fertilization at 16 °C. The embryo is at the end of the seventh cleavage and the SEM shows the neural plate of the 8th-generation cells, which corresponds to that in Fig. 2A. Arrowhead points towards the anteroventral direction of the embryo. (B) Neural plate cells are shown in outline. Scale bar = 10 μm. (Modified from Nicol and Meinertzhagen 1988*a*.)



Meinertzhagen 2001). The larva then hatches in less than a day, and a single cross-fertilized brood of embryos can give rise to thousands of larvae.

One way to view the numerical simplicity of the ascidian brain is therefore to consider the number of divisions generating its cells. In the CNS of *Ciona*, all cells arise after 13 or fewer divisions, and the entire CNS is consequently formed from 10th- to 14th-generation cells. In the embryo of *Drosophila melanogaster* Meigen, 1830, by contrast, there are 13 nuclear cleavages just in the syncytium stage of embryogenesis, prior to blastoderm formation (Foe et al. 1993). Of course, this comparison does nothing to explain the numerical simplicity of the ascidian larval brain, it merely re-states the phenomenon: even in the absence of cell death (Chambon et al. 2002; Cole and Meinertzhagen 2004), a small number of cell divisions can only generate a small number of cells. More than this, a small number of determinate cleavages can only generate a fixed, small number of progeny, and thus we should look to the precise regulation of ascidian cleavage for the underlying basis of eutely.

Early cleavages and blastomere nomenclature

Just as in amphioxus and amphibian embryos, the first three cleavages divide the embryo into, respectively, left and right, anterior and posterior, animal and vegetal hemispheres. Blastomere progeny of the animal hemisphere are given lowercase letters (a, b) in Conklin's (1905) nomenclature, while those of the vegetal hemisphere are given uppercase letters (A, B). These progeny are designated by their generation number (1–13) and by the individual blastomere number within that generation. The progeny of each blastomere inherit the individual blastomere numbers that become inflated from that of its progenitor; for example, the daughters of a4.2 are a5.3 and a5.4 (and give rise to anterior neural plate), while those of A4.1 are A5.1 and A5.2 (and give rise to the posterior neural plate). The final larval nervous system arises from some of the progeny of a4.2, A4.1, with smaller dorsal contributions from b4.2 (Nishida 1987; Cole and Meinertzhagen 2004). The progeny of these cells comes to form the neural plate (Fig. 1), which later undergoes neurulation to form the neural tube.

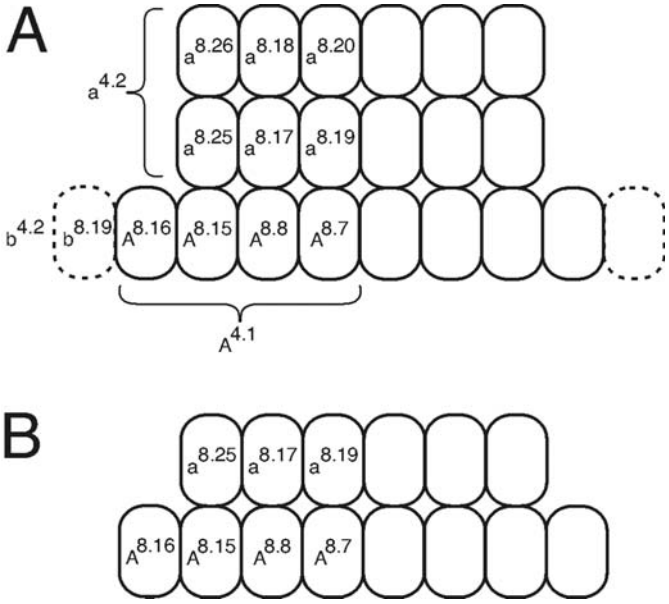
Many details of early cleavage from classical studies, especially by Conklin (1905), have been confirmed by scanning electron microscopy (Satoh 1979; Nishida 1986; Nicol and Meinertzhagen 1988*a*) and are now textbook examples of descriptive embryology (Satoh 1994). For the nervous system, the result of early cleavage gives rise to the ascidian neural plate, a conserved embryonic feature of both urochordate and vertebrate groups, which is illustrated in *Ciona* for cells in their 8th generation (Fig. 2). After one further division in the late *Ciona* gastrula at roughly the 218-cell stage, this epithelium of precisely patterned cells comprises the combined 9th-generation contributions from four rows of animal and two rows of vegetal blastomere progeny (Nicol and Meinertzhagen 1988*a*). On both left and right sides of the embryo, these form three and four files of cells, respectively.

Cell lineage

"With sufficient labor and material I believe that the lineage of every cell could be traced through to the tadpole stage, but I have lacked both the time and the material for such a study" (Conklin 1905). Despite the abundance of material and time since then, and attempts by different methods, this objective has still not been fully attained for the nervous system, and there is sharp division between those who would try and those who would think it not worthwhile to do so. Among the former are the authors of two recent studies on *Ciona* (Cole and Meinertzhagen 2004) and *Halocynthia* (Taniguchi and Nishida 2004).

Despite their critics, studies of cell lineage provide one rational way to trace the history of the larva, cell by cell, and to compare that history with the cells in different species. That lineage seems to be remarkably well conserved among ascidians, even despite the wide phylogenetic separation between *Halocynthia* and *Ciona* (Swalla et al. 2000). This contrasts with the situation in nematodes in which two species in particular illustrate the full range of embryonic determinacy. *Caenorhabditis elegans* provides a textbook case for

Fig. 2. The arrangement of the 8th-generation neural plate cells. (A) The 20-cell neural plate in a 112-cell stage embryo of *Ciona* (after Nicol and Meinertzhagen 1988*a*). (B) The 14 cells with neural fates in the neural plate of a 110-cell stage embryo of *Halocynthia roretzi* (after Taniguchi and Nishida 2004).



determinate cleavage, with which cell lineage in *Ciona* is naturally compared (Cole and Meinertzhagen 2004), whereas in forms such as the marine nematode *Enoplus brevis* Bastian, 1865, for example, embryonic development is regulative and cleavage indeterminate (Goldstein 2001).

Cell migration is generally absent during the development of the ascidian larval brain, and the progeny of each blastomere come to occupy a continuous strip of neural territory. Most such strips are longitudinal, and these have been charted either by tracing the mitotic history of each blastomere of the neural plate from confocal image stacks (Cole and Meinertzhagen 2004) or by identifying the larval progeny of 8th-generation *Halocynthia* neural plate cells injected with horseradish peroxidase (Taniguchi and Nishida 2004). The absence of migration is an important point partly for methodological reasons insofar as undetected cell migration would vitiate observations based on descriptive methods, as in the early cell mapping studies of Nicol and Meinertzhagen (1988*a*, 1988*b*) and the more recent confocal study of Cole and Meinertzhagen (2004), on *Ciona*. Based on confocal images of multiple whole-mount embryos at each stage, the latter study incorporates scrupulous counts of all nuclei in different regions of the neural tube to identify possible losses through migration or cell death. This study also found no evidence for migration, certainly in the progeny of A-line and many a-line blastomeres (Cole and Meinertzhagen 2004), confirming the validity of confocal mapping methods. In fact, there is evidence for cell migration within the neural plate in only one case, a pair of cells in the dorsal neural tube that are the progeny of a8.25 in *Halocynthia*. These cells, probably neurones, migrate posteriorly during the late tail-bud stages (Nishida 1987; Taniguchi and Nishida 2004). In *Ciona*, the progeny of a8.25 populate the posterior sensory vesicle, a region where not all cells could be followed individually through to the hatchling stage by confocal mapping methods (Cole and Meinertzhagen 2004). Migration of these cells in *Ciona* therefore remains a possibility, but one that must await confirmation.

Descriptive reports of cell lineage such as that of Cole and Meinertzhagen (2004) tend to homogenize the patterns of cleavage for the embryo, because they are produced by observing not one but many embryos. Final confirmation of the absence of cell migration in all regions of the *Ciona* neural tube, and the definitive account of cell lineage in its anterior regions, still awaits the development of methods for the in vivo real-time analysis of individual embryos undergoing cleavage. This conclusion is supported by experience from cell-lineage studies in *C. elegans* in which four-dimensional studies of cleavage in individual embryos do in fact report variations among what had become a textbook example of a wholly determinate cell lineage (Schnabel et al. 1997).

Cell lineage in *Ciona*

The cell lineage of *Ciona* has now been followed through the last half of embryonic life, roughly after the neuropore closes, from reconstructed confocal image stacks of a series of whole-mounted embryos at consecutive stages of development (Cole and Meinertzhagen 2004). Within each reconstructed embryo, cells were mapped from the positions of their stained nuclei and from mitotic figures used to link dividing cells to their progeny from their positions in the maps of later embryos and hatchling larvae. Most mitoses cease around 85% of embryonic development and the mitotic record obtained documents the lineage of 226 cells, including all those that derive from the A- and b-line blastomeres (Fig. 3). The lineage pattern exhibits a scrupulous left–right pattern of symmetry, as well as a high level of synchronicity among divisions, especially those of closely related lineages, that is retained right up until cells are post mitotic.

Cell fate in relation to the lineage of neural plate cells

The fates of cells within the larval CNS of *Ciona*

The recent report of Cole and Meinertzhagen (2004) tentatively identifies the territories of blastomere lineages in *Ciona* (Fig. 4). In the anterior (animal) lineage (a), the blastomere pairs a6.5 and a6.7 found the anterior neural plate and rostral sensory vesicle, respectively (Nicol and Meinertzhagen 1988*b*; Cole and Meinertzhagen 2004). The posterior animal b4.2 blastomeres give rise to the dorsal cell row of the tail nerve cord (progeny of b8.17 and b8.19) and the midline epidermis from which the tail sensory neurones are derived (progeny of b6.5, 7.11, 7.14, and 7.16), as is also reported for *Halocynthia* (Nishida 1987). Finally, the daughters of the vegetal blastomeres A7.4 and A7.8 found the tail nerve cord, visceral ganglion, and posterior sensory vesicle in both species (Nishida 1987; Cole and Meinertzhagen 2004).

The same report on *Ciona* also provides tentative identifications of cell fate within such lineages (Cole and Meinertzhagen 2004). For example, progeny of the following blastomeres are thought mostly to adopt ependymal

Fig. 3. Cell lineage of the larval central nervous system (CNS) presented as a space-filling polar plot that has been extended from Nicol and Meinertzhagen (1988*b*) to incorporate further details (Cole and Meinertzhagen 2004). Note that the CNS incorporates some of the progeny from a4.2 (blue), A4.1 (red), and b4.2 (green) blastomeres. The fates for final progeny are indicated where these are either known or postulated (Cole and Meinertzhagen 2004). A total of 226 cells of the final CNS have an identified lineage shown in this figure. The positional fates are shown for most cells by the region of the CNS to which they contribute. Note that the clockwise arrangement of progeny does not run in a coherent rostrocaudal sequence along the neuraxis. endo, endoderm; ecto, ectoderm; mus, muscle; noto, notochord; cnc, caudal nerve cord; vg, visceral ganglion; sv, sensory vesicle; nh, neurohypophysis.

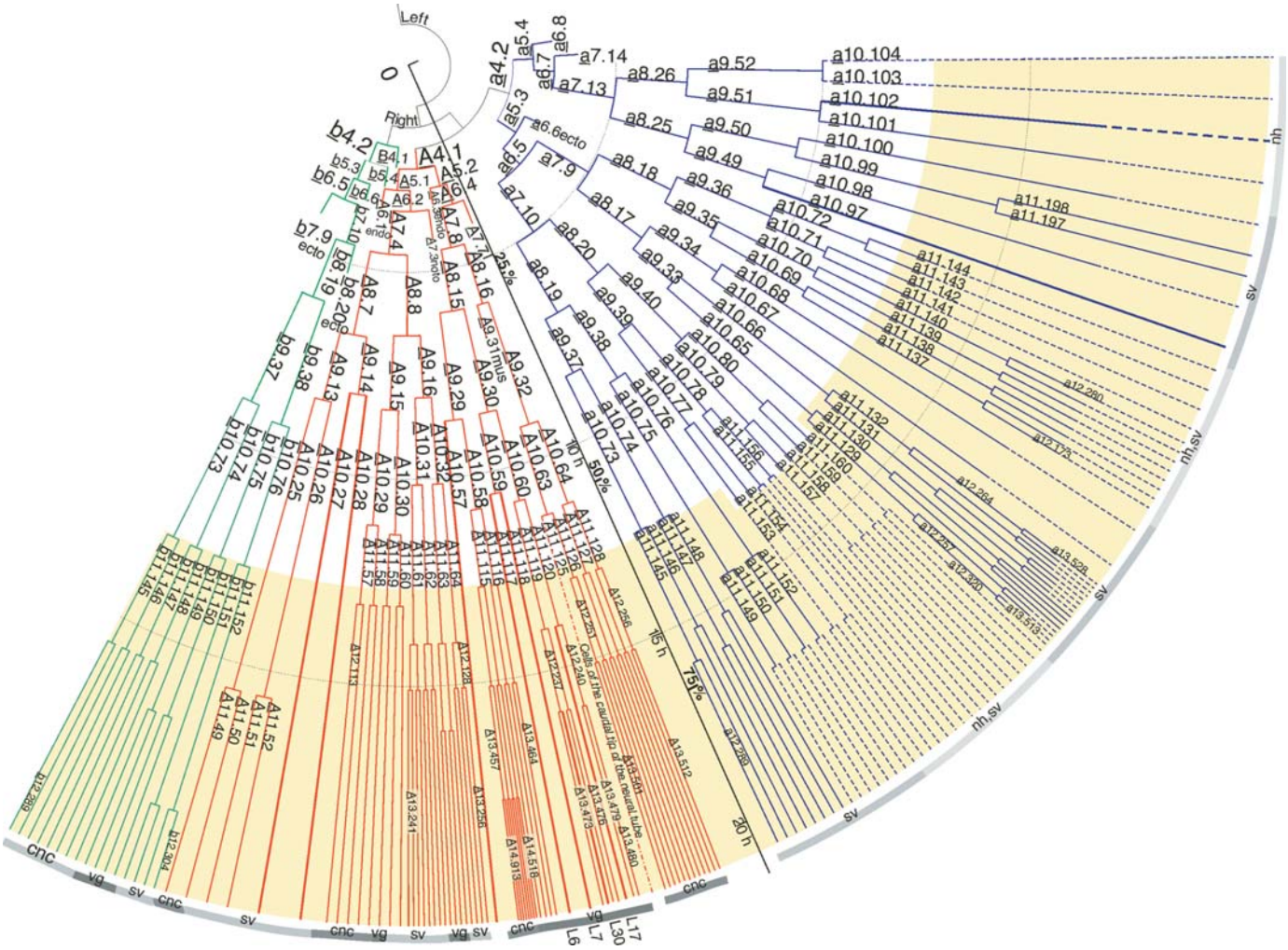
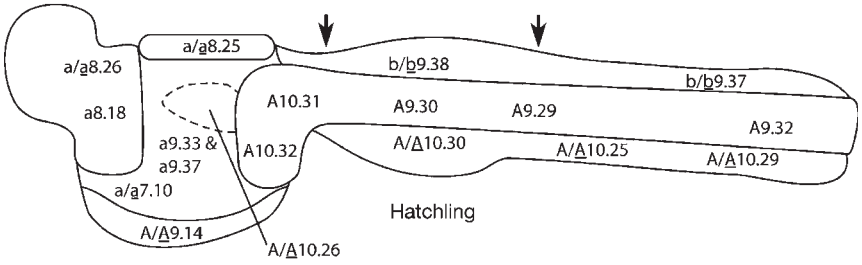


Fig. 4. Diagrammatic lineage territories of neural tube cells in the CNS of a hatched *Ciona* larva. Note that territories are coherent, signifying the lack of mixing of cell progeny from different lineages. Most are also elongate strips, at least in the nerve cord and visceral ganglion, indicating that the prevailing mode of division in those regions is for daughter cells to occupy anteroposterior locations. Arrows designate approximate borders between sensory vesicle, visceral ganglion, and caudal nerve cord (modified from Cole and Meinertzhagen 2004).



fates: A/A10.64 and A/A11.126, the b-line-derived dorsal cells b/b10.73 through b/b10.75, and the ventral cells A/A9.15 and A/A10.25. Likewise, the neck region of six cells between sensory vesicle and visceral ganglion (Nicol and Meinertzhagen 1991) probably corresponds to cells A/A13.241–A/A13.243 or A/A13.242–A/A13.244. The fates of cells within the more heavily populated sensory vesicle are harder to discern, and before dealing with these below it will first be necessary to discuss some differences between *Ciona* and *Halocynthia*. Despite the overall similarity of the neural plates in these two species, it is clear that the cell fates of anterior neural plate blastomeres in fact differ.

Some differences in fate: *Ciona* versus *Halocynthia*

Differences in the fates of anterior neural plate blastomeres first came to light in two studies. The one, in *Halocynthia*, examined the fates of progeny from injected blastomeres (Nishida and Satoh 1985; Nishida 1987), while the other, in *Ciona*, mapped the consecutive positions of neural plate cells at consecutive embryonic stages (Nicol and Meinertzhagen 1988*a*, 1988*b*).

The difference can be seen with respect to the composition of the neural plate's 8th-generation cells. The neural plate is recognized on structural grounds, simply as a coherent epithelium that undergoes neurulation as a single structure. In *Halocynthia* embryos at the 110-cell stage, 14 8th-generation neural plate blastomeres generate progeny, some of which give rise to the CNS in this species' larva (Fig. 2B). In *Ciona*, an additional six cells (a8.26, a8.18, and a.8.20 on each side of the neural plate) contribute to the formation of the anterior regions of the sensory vesicle and neurohypophysis (Fig. 2A). However, in *Halocynthia*, the progeny of cells having the same lineage migrate rostrally and assume largely non-neural fates in the palps and epidermis (Nishida 1987). The situation is somewhat reminiscent of the anterior region of the avian neural fold, which also migrates away from the CNS to form tissues having an ectodermal fate (Couly and Le Douarin 1990). As a result of this difference between anterior fates of ascidian neural plate cells, more 8th-generation neural plate cells contribute to the formation of the larval CNS in *Ciona* than in the embryo of *Halocynthia*, although the final number of such progeny is not known in the latter. Despite these differences, the boundary between the a-line and A-line blastomere progeny falls at the same level in the neuraxis in both species, the two populations mixing within the posterior sensory vesicle.

In addition to this major difference in lineage between the two species, there are also minor differences, which we will consider next, in the context of individual cells in the larval CNS.

Developmental life history and differentiation of identified cells

Combining cell-lineage information with other developmental studies, as well as other means to characterize individual neural cells of the larva, means that for the first time it has now become possible to identify some cells in the CNS at all stages of their embryonic development. The otolith, ocellus, and motor neurones provide cases in point that are reconstructed here.

Pigment cells of the otolith and ocellus

Because they incorporate melanin and are conspicuous, otolith and ocellus pigment cells have long provided developmental markers in whole-mount embryos. The otolith, for example, contains a single large pigment granule 15–25 μm in diameter (Dilly 1962), whereas the ocellus has several hundred small granules each of 0.5–2 μm crowded within a pigment cell 18–20 μm wide (Dilly 1964). As a result, both these cells are easily visible at high power in a stereomicroscope. They arise from the a8.25 cell pair (*Halocynthia*: Nishida 1987; Taniguchi and Nishida 2004), from the left and right a10.97 blastomeres in both *Ciona* (Cole and Meinertzhagen 2004) and *Halocynthia* (Sato et al. 1999), and their fate is determined in two early steps. The first occurs during neural induction in the cleavage stage, between the 16- and 64-cell stage, and involves an induction for which the A-line blastomeres are both sufficient and necessary (*Halocynthia*: Nishida and Satoh 1989; Nishida 1991). Basic fibroblast growth factor (bFGF) can substitute for this inducing signal (Inazawa et al. 1998). The second step occurs in the early gastrula between the 110- and 180-cell stages, when BMPb, which is expressed in the A-line but not in the b-line-derived blastomeres that will form the lateral edges of the caudal nerve cord, induces pigment precursor cells among the neuralized a-line blastomeres. The decision as to whether progeny of the left a8.25 cell give rise to ocellus or to otolith is made by left–right interactions between the cell pair (Nishida 1987). These two cells constitute a small equivalence group in which the fate to become an ocellus is dominant and is decided after the neural tube closes (Nishida and Satoh 1989). The final specification of cell fate, otolith or ocellus, occurs by interaction between the pigment cell precursors in the tail-bud embryo. This step involves antagonism between BMPb, which is expressed by the four progeny of left and right a9.49 that at this stage form a single row of dorsal cells, and chordin; chordin is expressed by a cell posterior to this line and suppress an otolith fate in the neighbouring posteriormost a10.97 cell while promoting the ocellus fate in that cell (Darras and Nishida 2001).

Motor neurones of the visceral ganglion

Visceral ganglion motor neurones are also identifiable. They are tentatively identified as A/A11.117 and A/A11.118 (progeny of A/A10.59), and A/A12.239 and A/A13.474 (derived from A/A10.60), and thus all progeny of A9.30; as well as the 11th-generation progeny of A10.57 (Cole and Meinertzhagen 2004). Insofar as the differentiation of these cells is apparently cell autonomous, they apparently do not require diffusible factors such as Sonic Hedgehog to undergo final differentiation, as do vertebrate motor neurones (Ericson et al. 1997), nor are there signs of programmed cell death in ascidian motor-neurone lineages. These are ways in which motor neurones may differ from their vertebrate namesakes, another being the fact that they arise in a territory of *Hox3* gene expression (Locascio et al. 1999), and are thus perhaps more closely comparable to vertebrate hindbrain reticulospinal neurones (Meinertzhagen et al. 2004). An in situ hybridization probe against *TuNa2* reveals that these cells in the presumptive visceral ganglion region of whole-mount embryos in tail-bud embryos of *Halocynthia*,

in which there are three pairs (Nagahora et al. 2000). At this stage they express the orthologue of *Lim* (Wada et al. 1995), incidentally resembling vertebrate motor neurones, which express another member of the LIM homeobox gene family, *Isl-1* (Ericson et al. 1992). In contrast to vertebrate motor neurones, however, there is no sign of programmed cell death in ascidian motor-neurone lineages. This suggests that mechanisms for long-range axonal pathfinding or activity-dependent regulation of cell number mediated by target tissues may both be absent. It also correlates with the lack of tropic factor genes in the *Ciona* genome, although this does not address the needs of the adult nervous system. Lack of apoptosis in the motor-neurone lineage reflects more generally the lack of apoptosis in ascidian embryos until shortly before metamorphosis (Chambon et al. 2002) as discussed below.

The development of neuromuscular transmission in *Halocynthia* includes a rapid increase in sensitivity to ACh at about 63% of embryonic development. The appearance at about 80% of giant excitatory junctional potentials (ejps) is interpreted as random synchronized presynaptic activity followed in a few hours by the miniature ejps typical of the free-swimming larva (Ohmori and Sasaki 1977). In the hatched larva, motor neurones having an axon extending into the caudal nerve cord have been revealed by transient transfection with GFP (Okada et al. 2001), as well as from computer three-dimensional reconstructions (Meinertzhagen et al. 2000)

Photoreceptor and coronet cells

Photoreceptor and coronet cells are also identified cells of the CNS, some of the few we can recognize in the sensory vesicle. Both are structurally differentiated from surrounding cells, and thus constitute two anatomically identifiable groups. The two fans totalling ~18 photoreceptors that constitute the right-hand ocellus express the visual pigment *Ci-opsin1* (Kusakabe et al. 2001) and are the means of light detection in the larva, the required input during downward, negatively phototactic swimming prior to larval settlement in *Ciona* (Tsuda et al. 2003*b*). The pathway for that input is apparently derived from a nerve that arises from the posterior of the ocellus, runs towards the midline, and then caudally (Tsuda et al. 2003*a*).

The protuberances of the left-hand coronet cells bear close resemblance to cells in the saccus vasculosus of the fish hypothalamus, for which they have been explicitly named (Svane 1982). Despite their homology, they are functionally enigmatic and apparently not sensors of hydrostatic pressure (Tsuda et al. 2003*b*) as once proposed (Eakin and Kuda 1971). We will consider them to be neurones, even if definitive evidence on this point is still lacking (Torrence 1983). The lineage of these cells has been explicitly suggested for the sensory vesicle of *Ciona*, in which some of the left lateral cells derived from a9.33 and a9.37 are tentatively proposed to form the coronet cells (Cole and Meinertzhagen 2004).

The fact that the photoreceptor and coronet cell populations appear to be matched left and right, with approximately the same numbers in each group, suggests that they may arise from left and right neural plate blastomeres with the same lineage. This correspondence has been assumed for the corresponding dorsal progeny of the lateral cells of the right side of the neural plate, a̲9.33 and a̲9.37, which have likewise been proposed to form the photoreceptor cells (Cole and Meinertzhagen 2004). The fact that counts for these cells in a single larva identified 19 coronet and 17 photoreceptor cells (Nicol and Meinertzhagen 1991) indicates that if left–right origin for these two populations of cells is correct, then we should anticipate minor variations in the mitotic history of the two sides.

Alternatively, against the interpretation of a left–right origin for these two cell populations, *Ci-opsin1* expression is reported to occur in two roughly bilateral clusters of cells in late-tail-bud embryos of *Ciona* and not a single group, which has been taken to suggest that both left and right lineages contribute photoreceptors (Kusakabe et al. 2001). In that case, some of the left and right progeny of a9.33 and a9.37 could form both the photoreceptors and, presumably, the coronet cells. The situation in *Halocynthia* seems even more complicated. In that case, most photoreceptor cells originate from the right blastomere A8.7, whereas the associated lens cells arise from A8.8 (Taniguchi and Nishida 2004). Although A8.7 does give rise to some cells of the sensory vesicle in *Ciona*, these cells are located ventrally in that species (Cole and Meinertzhagen 2004), which is inconsistent with them having a fate associated with the ocellus.

The coronet cells in *Halocynthia*, by contrast, arise from descendants of a8.19 and a8.17, and thus have the same lineage (i.e., a8.17 and a8.19, progenitors of a9.33 and a9.37) as has been proposed for these cells in *Ciona* (Taniguchi and Nishida 2004). On the other hand, in *Halocynthia*, progeny of right a̲8.19 as well as left a8.19 form coronet cells. Thus, while cleavage is bilaterally symmetrical, there is evidence that the assignment of fate is not and may depend on cell interactions (Taniguchi and Nishida 2004).

The significance of cell lineage

The ultimate significance of cell lineage is not yet clear. The term lineage itself is often used loosely to refer to cells that adopt a particular fate; however, to confuse cell lineage with cell fate is to confuse categories insofar as cell fate is usually controlled independently (Meinertzhagen 2002). The mitotic descent of a cell plays an important role in determining the fate of its progeny, and many examples are available in various metazoan nervous systems (Stent and Weisblat 1985). The problem is to understand the mechanisms whereby cell lineage influences cell fate. The existence of an invariant cell lineage was initially taken to indicate autonomous differentiation of blastomeres, and the ascidian embryo was consequently seen as a typical example of mosaic development (Lemaire and Marcellini 2003). This is now demonstrated to be wrong for many lineages including some that give rise to neural tissue. Cell lineage can be seen as playing both a topographical and a typological role in the assignment of cell fate (Stent 1987, 1998). In any one species' embryo, these incorporate a blend of mechanisms derived during the course of evolution, a blend that may have optimized two sets of developmental demands: those required to organize the spatial distribution of determinants for cell commitment and those for regulating the migration of differentially committed embryonic cells (Stent 1998). For example, the a-lineage from a4.2 requires inductive mechanisms

like those in vertebrates. In this case, the fixed cleavage pattern provides the precise positioning of the induced blastomere with respect to its inducing neighbours. In contrast, in the A-lineage from A4.1, a generic neural identity is achieved cell autonomously, and the role of the fixed cleavage is to partition maternal determinants precisely into different blastomeres (Okada et al. 1997; Minokawa et al. 2001).

Morphogenesis of the neural tube

Elongation of the vegetal hemisphere progeny of the *Ciona* neural plate occurs initially when six files of A-line neural plate cells and two lateral files of caudal ectoderm cells from b4.2 collapse into four files to form the cross section of the caudal nerve cord (Nicol and Meinertzhagen 1988*b*). The rearrangement of file rows is associated with positional changes of left–right cell pairs relative to their neighbors, "the ascidian square dance" (Nicol and Meinertzhagen 1988*b*), which resemble in a qualitative sense those occurring during convergent extension of the amphibian neural tube (Keller et al. 1992, 2000). Dorsal (capstone) and ventral (keel) cell pairs interdigitate their positions to form a single line (Fig. 5). Such changes are some of the first steps in the morphogenesis of a rectangular plate into an elongate neural tube.

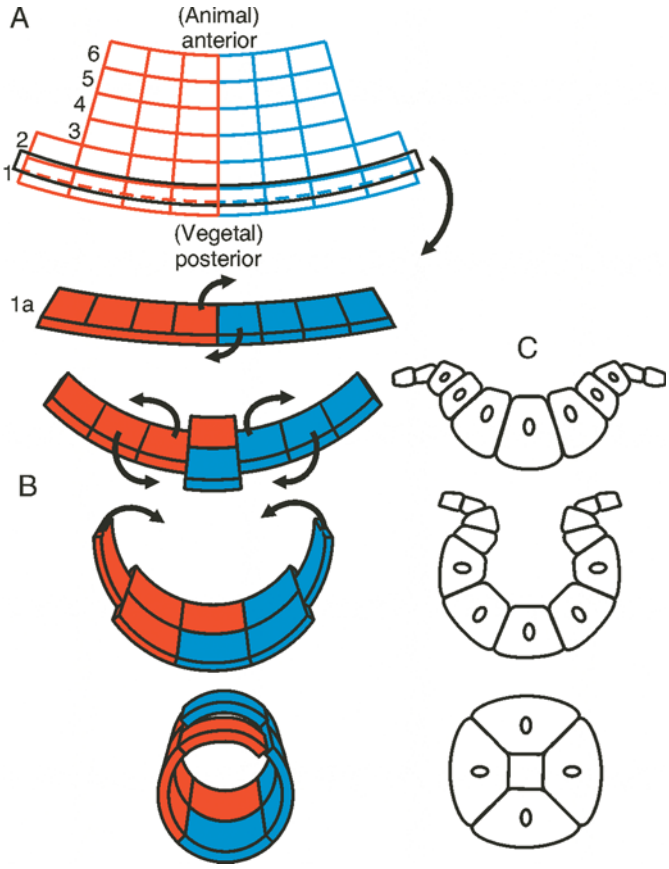
Little detail is known about later stages in the morphogenesis of the CNS. In *Ciona*, most late cell divisions in the CNS are thought to be those of non-neuronal, ependymal cells (Bollner and Meinertzhagen 1993). In *Styela clava* Herdman, 1881, embryos treated with antisense oligonucleotides for proliferating cell nuclear antigen cease DNA synthesis and exhibit nuclear DNA fragmentation typical of programmed cell death (Jeffery 2002). Late application of oligonucleotides in embryos, after 67% or 79% of embryonic development, produces larvae that are boomerang-shaped and swim in circles (Jeffery 2002). This finding indicates that the normal morphogenesis of the larva requires the presence of late-dividing cells, and although the exact cells that produce this effect will need to be ascertained, the finding suggests that ependymal cells are candidates.

In *Halocynthia*, there is an apparent 45° rotation of the anterior CNS in a clockwise direction as viewed from the caudal end, which occurs during the final 5 h of embryonic development (Taniguchi and Nishida 2004). The evidence for this previously undetected shift is based on the asymmetry of fates from symmetrical patterns of cleavage among 8th-generation neural plate cells and the final locations of the progeny of those cells (Taniguchi and Nishida 2004). Possibly related, in *Ciona* an apparent left–right asymmetry results from the outward bulging of the sensory vesicle on the right-hand side (Cole and Meinertzhagen 2004), causing cells of the opposite side to be displaced. Although not reported in detail, this bulging actually occurs in a ventrolateral direction so that other surrounding cells would also appear to be shifted 45° in a clockwise direction as reported for *Halocynthia*.

Conclusion: the significance of cell constancy in the brains of urochordates

What significance, if any, can we attach to the constancy of cell number in the ascidian brain? The same question should really be addressed to eutely of the entire larva, of which the CNS is merely a special case. The small number of cell generations, and the small numbers of cells that necessarily result from these, are associated with rapid development. Embryonic development in *Ciona*, which has a simple larval body, takes only about 22 h at 16 °C (Nicol and Meinertzhagen 1988*a*). However, larvaceans such as *Oikopleura dioica* Fol, 1872, in which the adult has a body form like that of the ascidian larva and which develop directly from an embryo, do even better than this and appear to hold the speed record. They undergo gastrulation 1–2 cell generations even earlier than ascidian embryos, complete embry-

Fig. 5. (A) The neural plate of the 9th-generation cells of *Ciona*, one division after the stage shown in Figs. 1 and 2. The daughter cell products of the previous division come to lie anteroposteriorly so that each of the original three rows, two animal and one vegetal, has become two, thus making rows 1–6. Cell divisions are left (red) and right (blue) symmetrical. (B) The neural plate rolls up to form a neural tube and is shown in four successive stages for row 1*a*, the anterior daughter cell row of the 9th-generation row 1, as follows: the median cells, one on each side, shear their positions to form what will become a ventral row of midline or keel cells; lateral cells shear their positions to double up the rows of wall cells, while the outer cells interdigitate along the dorsal margin to form a line of dorsal or capstone cells. As a result, the files of both dorsal and ventral cells are of alternating left (red) and right (blue) composition. (C) Three successive cross sections showing the transformation of the neural plate into the neural tube, four cells in cross section at this level. For further details, see Nicol and Meinertzhagen (1988*b*).



onic development within 6–7 h, and at 15 °C have the remarkably short generation time of 5–6 days (Thompson et al. 2001). Larvaceans possibly arose from a urochordate ancestor by a process of neoteny (e.g., Garstang 1928; but see Lacalli 2005) with which eutely is a possible correlate (Martini 1909*c*). A recent appraisal of embryonic development in different groups points out that determinate cleavage in embryos seems to be an adaptation for rapid embryonic development and the generation of forms with small cell numbers, and that this rather than phylogenetic relatedness between different groups is the common feature underlying embryonic mosaicism (Lemaire and Marcellini 2003). Thus, it is perhaps to ecological aspects of urochordates that we should turn in seeking a reason for their small cell numbers and eutelic organization: to the ascidian life cycle in which large numbers of embryos are produced rapidly for random larval settlement or to the rapid growth of larvacean populations during seasonal changes of increased marine productivity.

What significance, if any, can we attach to the smallness of the number of cells in the ascidian larval brain? The neural plates in different embryos give rise to a series of animal brains of widely differing complexities, comprising what must be the widest range of cell numbers, from 10^2 to 10^{14}, in any single series of homologous animal brains. At the low end, network complexity, and with it behavioural diversity, is limited; at the upper end, genetic, developmental, and metabolic costs are very high. We have, of course, no direct ways of computing these limits and their underlying costs, but it does appear that the ascidian larval CNS is close to a lower limit. It is also associated with a genome that lacks many of the genes for rapid conduction and long-range interaction, and thus seems suited to the life of a small, slow animal (Okamura et al. 2004).[2] Remarkably, larvaceans such as *Oikopleura* and the genus *Fritillaria* Lohman, 1896, have even fewer CNS cells. For example, despite its behavioural complexity (Bone 1985), the CNS in an adult larvacean has only about a quarter the number of cells (Martini 1909*a*, 1909*b*; I.A. Meinertzhagen, unpublished observations) found in a *Ciona* larva. Together with the rapid embryonic development, this number suggests that the larvacean brain contains cells of, on average, two fewer generations. For both cases, ascidian and larvacean, and despite the minor variations in cell number recorded in previous reports, it seems that it should still be possible to characterize the nervous system, cell by cell, as in forms such as *C. elegans*. That opportunity rests on a cell constancy that is in turn based on the fixed generations of cells in an embryo with determinate cleavage.

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