REVIEW / SYNTHÈSE

The nervous system in adult tunicates: current research directions[1]

G.O. Mackie and P. Burighel

Abstract: This review covers 25 years of progress on structural, functional, and developmental neurobiology of adult tunicates. The focus is on ascidians rather than pelagic species. The ascidian brain and peripheral nervous system are considered from the point of view of ultrastructure, neurotransmitters, regulatory peptides, and electrical activity. Sensory reception and effector control are stressed. Discussion of the dorsal strand plexus centres on its relationship with photoreceptors, the presence in it of gonadotropin-releasing hormone and its role in reproductive control. In addition to hydrodynamic sense organs based on primary sensory neurons (cupular organs), ascidians are now known to have coronal sense organs based on axonless hair cells resembling those of the vertebrate acustico-lateralis system. The peripheral nervous system is remarkable in that the motor neuron terminals are apparently interconnected synaptically, providing the equivalent of a nerve net. Development of the neural complex in ascidians is reviewed, highlighting recent embryological and molecular evidence for stomodeal, neurohypophyseal, and atrial placodes. The nervous system forms similarly during embryogenesis in the oozooid and blastogenesis in colonial forms. The regeneration of the brain in *Ciona intestinalis* (L., 1767) is discussed in relation to normal neurogenesis. Finally, the viviparous development of salps is considered, where recent work traces the early development of the brain, outgrowth of nerve roots, and the targetting of motor nerves to the appropriate muscles.

Résumé : Notre synthèse couvre 25 années de découvertes sur la structure, la fonction et le développement neurobiologiques chez les tuniciers adultes. Elle porte principalement sur les ascidiens plutôt que sur les espèces pélagiques. Le cerveau des ascidiens et leur système nerveux périphérique y sont étudiés en regard de leur ultrastructure, de leur neurotransmetteurs, de leurs peptides régulateurs et de leur activité électrique. L'emphase porte sur la réception sensorielle et le contrôle des effecteurs. La discussion sur le plexus du cordon dorsal se concentre sur ses relations avec les photorécepteurs, sur la présence d'hormone de libération de la gonadotropine et sur son rôle dans le contrôle de la reproduction. En plus de posséder des organes des sens hydrodynamiques basés sur des neurones sensoriels primaires (organes cupuliformes), on sait maintenant que les ascidiens ont des organes sensoriels coronaires formés de cellules ciliées dépourvues d'axone qui ressemblent beaucoup à celles du système acoustico-latéral des vertébrés et qui ont la même origine embryonnaire à partir de placodes. Le système nerveux périphérique est particulier en ce que les terminaisons des neurones moteurs semblent être interconnectés au niveau des synapses, ce qui produit l'équivalent d'un réseau neural. Nous passons en revue le développement du complexe neural des ascidiens, en particulier les données embryonnaires et moléculaires récentes qui appuient l'existence de placodes stomodéales, neurohypophysaires et atriales. Le système nerveux se forme de façon similaire durant l'embryogenèse du stade oozoïde et la blastogenèse des formes coloniales. Nous présentons la régénération du cerveau de *Ciona intestinalis* (L., 1767) dans le cadre de la neurogenèse normale. Enfin, nous considérons le développement vivipare des salpes, chez lesquelles des travaux récents décrivent le premier développement du cerveau, la croissance vers l'extérieur des racines nerveuses et le ciblage des muscles appropriés par les nerfs moteurs.

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Introduction

The central nervous system of ascidian tadpoles is clearly that of a chordate, albeit on a miniature scale, and is currently the focus of research on patterning genes homologous with those expressed during the early development of the central nervous system of higher chordates (Wada et al. 1998; Holland and Holland 1999; Meinertzhagen and Okamura 2001; Lemaire et al. 2002). The adult ascidian brain, by contrast, tends to be seen as a relatively uninteresting structure. Along with the neural gland, it develops from a segregated portion of neural ectoderm set aside (literally) during the larval stage, and expanding only after metamorphosis (Cole and Meinertzhagen 2001). The segregated portion, which consists of about 40 cells in *Ciona intestinalis* (L., 1767) (hereafter simply *Ciona*), is often termed a rudiment or remnant, a term that somehow reinforces the notion of the adult brain as relic of minor importance, if not actually degenerate. Thus, according to Llinás (2001), "the tadpole-like larva literally digests most of its own brain and reverts to the more primitive, sessile adult form of the species. The take-home lesson…is that a brain is necessary only for actively moving creatures." In fact, adult ascidians have perfectly good brains, an order of magnitude larger than those of their larvae, and their behaviour is as finely adapted to sessility as that of the larvae to motility. Both have evolved sense organs and reflexes appropriate to their lives in competitive environments.

It is certainly true that the larval brain is more instructive than that of the adult for purposes of establishing phylogenetic relationships, but adult neural development and behavioural physiology also provide lessons of evolutionary interest. Tunicates, after all, are a highly successful and diverse group with ancestors going back to the Early Cambrian (Shu et al. 2001). We will consider the major components of the nervous system along with aspects of neural development, emphasizing findings that have accrued since the last major review of the field (Bone and Mackie 1982) and stressing ascidians rather than pelagic tunicates, which are well covered in recent reviews (Anderson 1985; Bone 1985, 1998*a*, 1998*b*; Madin 1995). Ascidian fine structure is treated in detail by Burighel and Cloney (1997).

Central nervous system

Tunicate brains show no obvious longitudinal subdivisions but are compact, ovoid, or elongated structures (Fig. 1). Embryologically, the adult brain arises at a level corresponding to the midbrain and posterior forebrain of higher chordates (Lacalli and Holland 1998). An electron microscope study of the brain of a small colonial ascidian, *Polyandrocarpa misakiensis* Watanabe and Tokioka, 1972 (hereafter simply *Polyandrocarpa*; Fig. 1D), confirms and extends the general picture established by earlier workers that shows a cortical region, containing the majority of the neuronal cell bodies and with the larger (10 μm) ones lying closest to the surface, surrounding a mainly fibrous medulla or neuropil (Koyama and Kusinoki 1993). Although non-nervous, ependymal cells outnumber neurons in the larval nervous system (Nicol and Meinertzhagen 1991), glial cells appear to be absent in the adult ascidian brain (Lane 1972; Koyama and Kusinoki 1993) and the axons are unsheathed. Reissner's fibre, an ependymal secretion product similar to that found in higher chordates, is present in *Oikopleura dioica* Fol, 1872 (hereafter simply *Oikopleura*), as in ascidian tadpole larvae (Holmberg and Olsson 1984). Interneural junctions showing the structural features of chemical synapses occur within both the cortex and the medulla of *Polyandrocarpa*, but gap junctions have not been observed in the brain. Synaptic vesicles range in diameter from 40 to 80 nm and in some cases show electron-dense contents. Most but by no means all of the synapses in *Polyandrocarpa* are axo-axonic. Fritsch et al. (1982) recognize five categories of neurons in *Ciona*, based on location and organellar content. In addition to dense-cored synaptic vesicles, larger (<200 nm) "neurosecretory" granules may also be present (Lane 1972). Tunicate motor neurons injected or backfilled with tracers (Figs. 1B, 1C) are seen as monopolar units with few dendrites (Arkett 1987). They lie near the surface of the cortex and their axons exit via substantial anterior and posterior nerve trunks.

In *Polyandrocarpa*, there are two anterior and two posterior trunks and an unpaired visceral nerve, but in the larger ascidian *Chelyosoma productum* Stimpson, 1864 (hereafter simply *Chelyosoma*), there are four anterior and five posterior trunks (Figs. 1B, 1C). This arrangement is found consistently in all individuals (Arkett 1987). Cell bodies have occasionally been seen in nerve trunks close to the brain (Arkett et al. 1989; Koyama and Kusinoki 1993), but tunicate peripheral nerves are generally composed entirely of axons that are grouped into fascicles separated by layers of fibrous material. The cell bodies of the motor neurons all lie within the brain, along with interneurons and the cell bodies of secondary sensory neurons. Primary sensory neurons in the periphery send their axons to the brain, where they mingle with motor axons. All the major nerves are mixed nerves.

The brain and larger nerve bundles in tunicates are ensheathed in a layer of collagen-like fibrous material and lie within blood spaces with no intervening cellular barrier. Mobile blood cells can apparently enter the brain (Koyama and Kusinoki 1993), but there is no capillary network and it is doubtful if there is anything equivalent to a blood–brain barrier (Lane 1972).

The miniature brains of larvaceans such as *Oikopleura* contain only 50–70 neurons but show functional subdivisions recalling the brains of higher chordates (Fig. 2) (Olsson 1986). Two anterior branches end in a ventral sense organ whose cells resemble chemoreceptors (Bollner et al. 1986). A second pair of nerves originate in presumed tactile sensors in the skin. Their axons contact ciliary control motor neurons in the hindbrain whose processes exit posteriorly via paired, branchial nerves. The sensory vesicle contains a mineralized statolith, sensory neurons, and coronet cells that are considered to be homologues of the coronet cells in the saccus vasculosus of fishes. The ciliated funnel (Fig. 2), like that of ascidians, may be an anterior pituitary homologue. Olsson (1986) notes that all these proposed homologies require further study. Thanks to the small size of the brain, it has been possible to work out some of the internal circuitry from serial ultrathin sections (Olsson et al. 1990). For more details on larvacean central circuitry, see the review by Bone (1998*b*).

Fig. 1. Examples of tunicate brains. A. Developing brain of *Thalia democratica* at a stage where the adult pattern is well established. Bundles of axons (1–7) emerge from clusters of neurons with large cell bodies (C1–C3) and innervate the body-wall muscles. The eye (not shown) lies dorsal to the brain (modified from Lacalli and Holland 1998). B. Dorsal view of the brain of *Chelyosoma productum* showing ciliary arrest motor neurons (CA) located by electrophysiology and filled with Lucifer Yellow. The axons of all except CA_2 exit through root AM_3, which leads to the visceral nerves supplying the branchial sac. Roots are labelled according to their orientation with respect to branchial (B) and atrial (A) siphons and lateral (L) or medial (M) location (from Arkett 1987, reproduced with permission of J. Comp. Physiol. A, Vol. 161, © 1987 Springer-Verlag). C. Camera lucida tracing of cell bodies in *Chelyosoma* backfilled with $NiCl_2$ via root AM_3. Roots are labelled as in Fig. 1B. Solid cell bodies are on the dorsal surface and open cell bodies are on the ventral surface. The mean number of filled cells was 83. The main dorsal clusters were consistently located near the bases of the BM roots and along the left side and near AM3 (from Arkett et al. 1987). D. Schematic representation of the brain of *Polyandrocarpa misakiensis*. N1, large neuron; N2, small neuron; N3, neuron containing dense granules; NF1, axon associated with fibrous sheath; NF2, axon entering blood sinus; NN, non-nervous cell; S1–S6, synaptic sites (modified from Koyama and Kusinoki 1993).

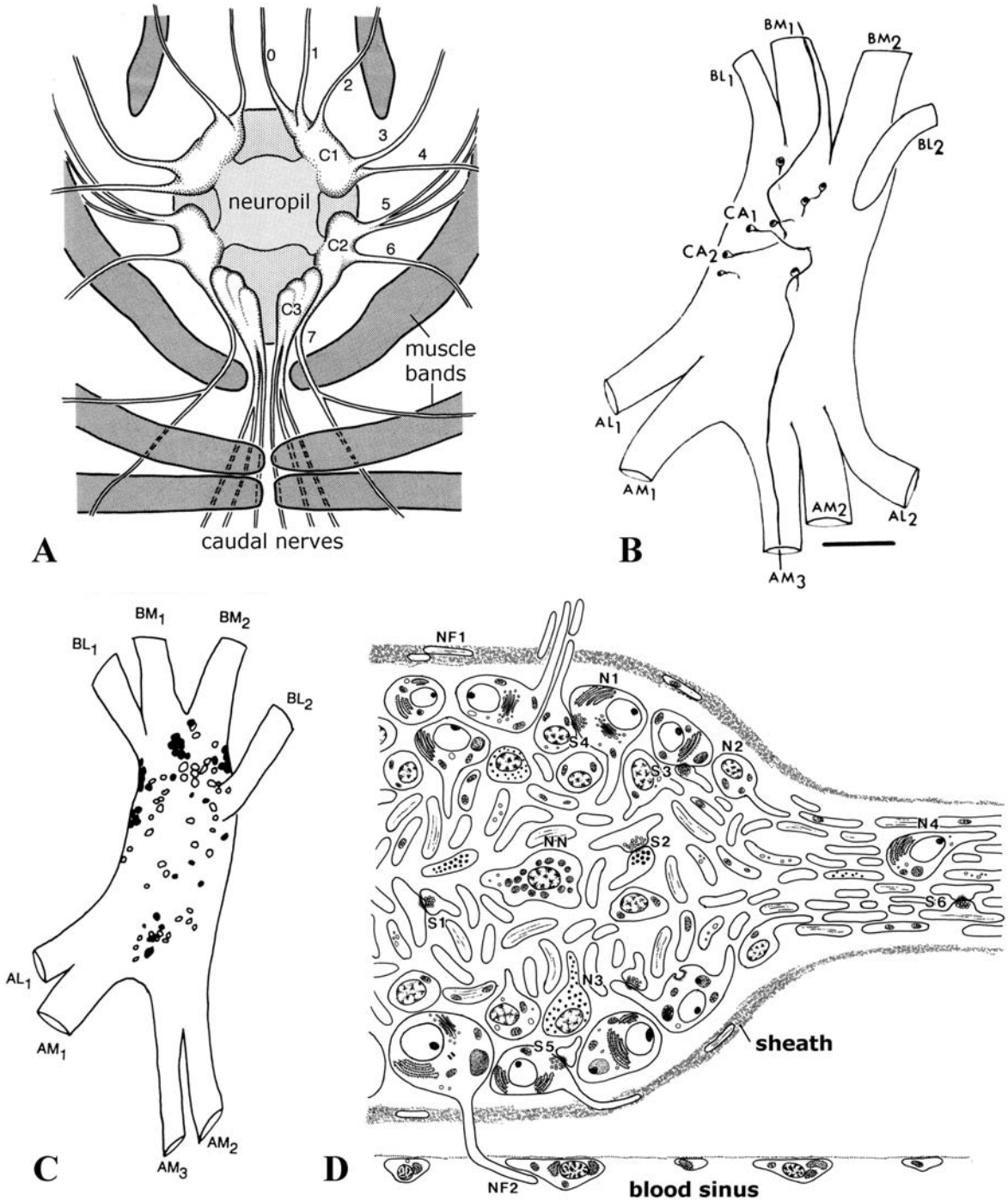
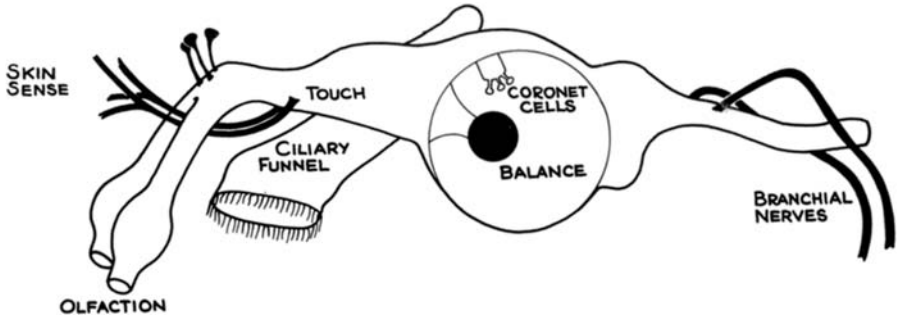


Fig. 2. The brain of *Oikopleura dioica* showing sensory inputs and features reminiscent of brains in higher chordates (from Olsson 1986, reproduced with permission of the Ichthyological Society of Japan © 1986).



The small colonial styelid ascidian *Botryllus schlosseri* (Pallas, 1766) (hereafter simply *Botryllus*) is estimated to have about 1000 neurons in its brain, including not only motor and inter-neurons but secondary sensory neurons supplying the coronal organ (Burighel et al. 2003). As there are some 2000 hair cells in the organ, each sensory neuron in the brain must receive convergent input from many hair cells. Some ascidian and salp brains are much larger. Electron micrographs of the visceral nerve in *Chelyosoma* show the profiles of about 4500 axons in cross section, about a quarter of which are estimated to derive from primary sensory neurons lying in the branchial sac. Assuming that the other major nerves have a similar composition, it is estimated that more than 35 000 axons enter or leave the brain (Mackie and Singla 2003). In the developing brain of the salp *Thalia democratica* Forskål, 1775 (Fig. 1A) by the end of the proliferative stage, about 6400 cell bodies are present (Lacalli and Holland 1998). Here, primary motor neurons form in clusters around the equatorial margin on the ventral side and are greatly outnumbered by other neurons, presumably interneurons or secondary sensory neurons. The mature salp brain certainly also contains many axons deriving from sensory neurons whose cell bodies lie in the periphery. Though the ascidian brain shows no obvious subdivisions, there is evidence of regional specialization within it. In *Chelyosoma*, nickel backfills of the visceral nerve consistently showed cell bodies clustered in specific areas on the dorsal side (Fig. 1C) (Arkett et al. 1989).

The presence of a large number of regulatory peptides in the ascidian brain is evident from immunocytochemical investigations, although interpretation of these findings is often tentative, owing to the possibility of cross-reactivity. Some 20 neuropeptides are listed in a recent review by Pestarino (1991). In some cases more than one peptide may be located in the same cell (Pestarino et al. 1993). Genes for insulin and insulin-like growth factor are expressed in the nervous system of *Chelyosoma* (McRory and Sherwood 1997). Cells in the brain of *Styela plicata* (Lesueur, 1823) express the mRNA for a proopiomelanocortin-like (POMC) molecule (Masini et al. 1998) and prohormone-converting enzymes associated with production of POMC derivatives such as adrenocorticotropin are reported in the brain and along the dorsal strand in *Halocynthia roretzi* (von Drasche, 1884) (hereafter simply *Halocynthia*; Kawahara et al. 2003*a*). Cell bodies immunoreactive for gonadotropin-releasing hormone (GnRH) are reported to occur in regenerating brain of *Ciona* (Bollner et al. 1997). Substance P- and cholecystokinin-like immunoreactivity has been detected in specific locations of the developing and regenerating brain of adult *Ciona* (Bollner et al. 1992, 1993*a*). Cionin, a peptide showing similarities with cholecystokinin and gastrin, is expressed in the gut and brain of *Ciona* and a putative cionin receptor has been cloned (Nilsson et al. 2003). GABA-like immunoreactivity is present in both *Ciona* (Bollner et al. 1993*b*) and *Oikopleura* (Bollner et al. 1991). Hopefully, these studies will stimulate physiological investigation. Similarly, evidence for biogenic amines and their receptors (Takeda 1992; Kuznetsova et al. 1995), and endogenous opioids or their precursors (Piccioli et al. 1985; Masini et al. 1998), requires physiological backup before its significance can be evaluated. No histochemical evidence has yet been found for neurons producing nitric oxide in tunicates (Elofsson et al. 1993). Finally, Pestario et al. (1997) found that neurons in the brain of *Styela plicata* express mRNAs for an interleukin-1β-like peptide. In vertebrates, this cytokine acts as a circulating hormone, autocrine hormone, and modulator of many biological functions, including the body's response to infection and inflammation; thus, its presence in a tunicate suggests that a neuroimmune axis appeared early during chordate evolution.

We can be reasonably sure that the motor innervation is cholinergic, as d-tubocurarine (curare) blocks muscle contractions in *Ciona*, while direct application of acetylcholine to the muscles causes them to contract (Florey 1967). Similarly in salps, doliolids, and larvaceans, acetylcholine iontophoresis evokes muscle depolarizations (see Bone 1998*a*). Extracts of the *Ciona* brain bioassayed on clam hearts showed an acetylcholine content of 20–120 μg/g wet mass (Florey 1963). Curare also blocks the cilio-motor innervation of the branchial sac of ascidians (Mackie et al. 1974; Arkett 1987) and *Fritillaria pellucida* Busch, 1851 (hereafter simply *Fritillaria*; Bone et al. 1979), as well as the body-wall muscles in *Ciona* (Nevitt and Gilly 1986). The electrical correlates of ciliary reversal in the branchial sac are potentiated by neostigmine, an anticholinesterase (Arkett et al. 1989). The motor innervation of ascidians is readily demonstrable by cholinesterase histochemistry (Arkett et al. 1989; Mackie and Wyeth 2000; Burighel et al. 2001; Zaniolo et al. 2002), although such reactivity is not in itself diagnostic of a cholinergic mode of transmission. In *Oikopleura*, the reaction product is concentrated in the synaptic cleft at neuromuscular junctions, as seen in electron micrographs (Flood 1973). A choline acetyltransferase gene, as well as a vesicular acetylcholine transporter gene, has been isolated

from *Ciona*, both being expressed first during early larval development (Takamura et al. 2002). Not all neurons in tunicates show cholinesterase reactivity. There appear to be no reports of reactivity in sensory neurons, and neurons of the dorsal strand plexus in corellids do not react (Mackie and Wyeth 2000). Anti-tubulin immunolabelling provides a simple way of showing most components of the peripheral innervation in both larval and adult ascidians (Crowther and Whittaker 1992; Mackie and Wyeth 2000).

Ascidian tadpole larvae probably employ many of the same neurotransmitters and neuromodulators as adults, and work on larvae may therefore provide pointers for studies dealing with adult nervous systems. For example, evidence for the involvement of both glutamate and GABA in swimming in *Ciona* tadpole larvae (Brown et al. 2003) suggests that the action of these amino-acid transmitters should be investigated in adults, where analysis of the circuitry underlying reflex activities such as squirting and ciliary arrests is still in its infancy.

Electrophysiological investigation of brain functions has not been pursued very far, and merits further attention as it is relatively easy to record extracellularly from the larger nerve trunks and intracellularly from cell somata within the brain. The first intracellular recordings were made from salps (Mackie and Bone 1977; Anderson et al. 1979), where regular, synaptically driven bursts of impulses were recorded from motor neurons during swimming. The firing rate of certain neurons in the salp brain is affected by input from excitable epithelia. As noted below, salps can switch from forward to reverse swimming depending on the directionality of sensory input, but the details of the central switching mechanism are still largely unknown (see Anderson 1985).

Arkett (1987) was the first to record intracellularly from brain neurons in ascidians. He showed conclusively that pacemaker neurons in the brain drove ciliary arrests in the branchial sac (see further below under section 'Motor innervation and the "peripheral nerve net"'). The ciliary arrest motor neurons were injected iontophoretically with Lucifer Yellow (Fig. 1B). This was followed up with nickel chloride backfills of the visceral nerve, which showed distinct populations of central cells (Fig. 1C); some of which were presumably the cilio-motor neurons (Arkett et al. 1989). Extracellular recordings of motor output from the brain are perfectly feasible in larger ascidians (Mackie and Wyeth 2000), but sensory input has proven harder to record. Compound action potentials have, however, been recorded from the visceral nerve in *Chelyosoma* following vibratory stimuli. The impulses presumably originate in the capsular sense organs located in the branchial sac (Mackie and Singla 2003).

No one appears to have carried out patch or voltage clamp analysis on adult tunicate neurons and we know nothing about their membrane channels or ionic currents, but the ability to grow brain tissue from *Ciona* in culture (Moss et al. 1998) means that a way may now be open for electrophysiological study of central neurons in vitro.

Dorsal strand plexus

The dorsal strand, or dorsal cord, in solitary phlebobranch ascidians such as *Ciona* is a tubular epithelial structure originating from the posterior end of the neural gland and running back within the dorsal blood sinus (Fig. 3). In some species the strand extends as far as the gonads, which appear to form in association with it during development (Huus 1924). In *Polyandrocarpa*, it terminates abruptly just behind the brain (Koyama 2002), whereas in *Halocynthia*, it takes the form of extensively branched tubules arising from the dorsal side of the neural gland (Terakado et al. 1997). The colonial stolidobranch *Botryllus* has a "dorsal organ", unconnected to the neural gland in the adult state (Burighel et al. 1998). Some ascidians (e.g., *Ascidia interrupta* Heller, 1878) apparently lack the structure altogether (Ruppert 1990). Neither the dorsal strand nor the neural gland are nervous structures, although they and the brain share a common embryonic origin from neural ectoderm (Manni et al. 1999).

Regardless of the form the dorsal strand takes, it is typically associated with a plexus of neurons termed the dorsal strand plexus (Figs. 3B, 4A). This is a classical nerve net, composed of bi- and multi-polar neurons with their cell bodies in the periphery (Mackie 1997). The cell bodies are concentrated around the dorsal strand and brain, but extend into other regions as shown in several phlebobranch ascidians (Markman 1958; Mackie 1995*a*; Powell et al. 1996). In addition to mature nerve cells, the plexus sometimes contains rounded, axonless cells ("neuroblasts", Fig. 4B) and stages apparently intermediate between these and formed nerve net elements (Mackie 1995*a*). It has long been thought (Brien 1933; Lender and Bouchard-Madrelle 1964; Bollner et al. 1997) that the dorsal strand acts as a source of nerve cells during brain regeneration (see section "Formation of the adult ascidian innervation"). Fedele (1938) believed that the nerve cells were produced by delamination from the dorsal strand epithelium. This is supported by unpublished electron microscope observations on *Halocynthia* (K. Terakado, personal communication). If this is correct, bromodeoxyuridine (BrdU) labelling should elucidate the processes of neurogenesis and migration of neuroblasts to their final destinations, but this awaits demonstration.

The dorsal strand plexus has attracted recent attention chiefly because the neurons composing it are immunoreactive for various neuropeptides. The presence of GnRH in the dorsal strand plexus is now well established. Two forms of the molecule (tGnRH-I, tGnRH-II) are present in *Chelyosoma* and tGnRH-I immunoreactivity has been demonstrated in the dorsal strand plexus of this species, localized to dense-cored vesicles in axons of the plexus (Powell et al. 1996; Craig et al. 1997).

A GnRH-immunoreactive (GnRH-ir) plexus is also well developed in *Ciona* and six forms of GnRH are encoded in the genome of this species (Adams et al. 2003). Posteriorly, elements of the plexus lie in close proximity to the gonads and gonoducts (Mackie 1995*a*; Powell et al. 1996; Tsutsui et al. 1998; Terakado 2001). Anteriorly, it extends into the region around the brain where a dense population of GnRH-ir cell bodies and processes is found, particularly on the right side (Tsutsui et al. 1998). GnRH-ir cells have been observed close to the brain even in *Botryllus*, which lacks a dorsal strand plexus (Burighel et al. 2001). In some ascidians like *Halocynthia*, many GnRH-ir cells are present in the brain, both within the cortex and the medulla, and GnRH-ir nerve fibres originating in the brain run out to the viscera (Terakado 1997). However, in *Ciona* the GnRH-ir cells ob-

Fig. 3. (A) Diagram of an ascidian such as *Ciona* showing location of brain, neural gland, and dorsal strand. The visceral nerves run back from the brain along the dorsal blood sinus. (B) Ventral view of the neural complex showing the relationships of the dorsal strand, dorsal strand nerve plexus, and visceral nerve (modified from Mackie 1995).

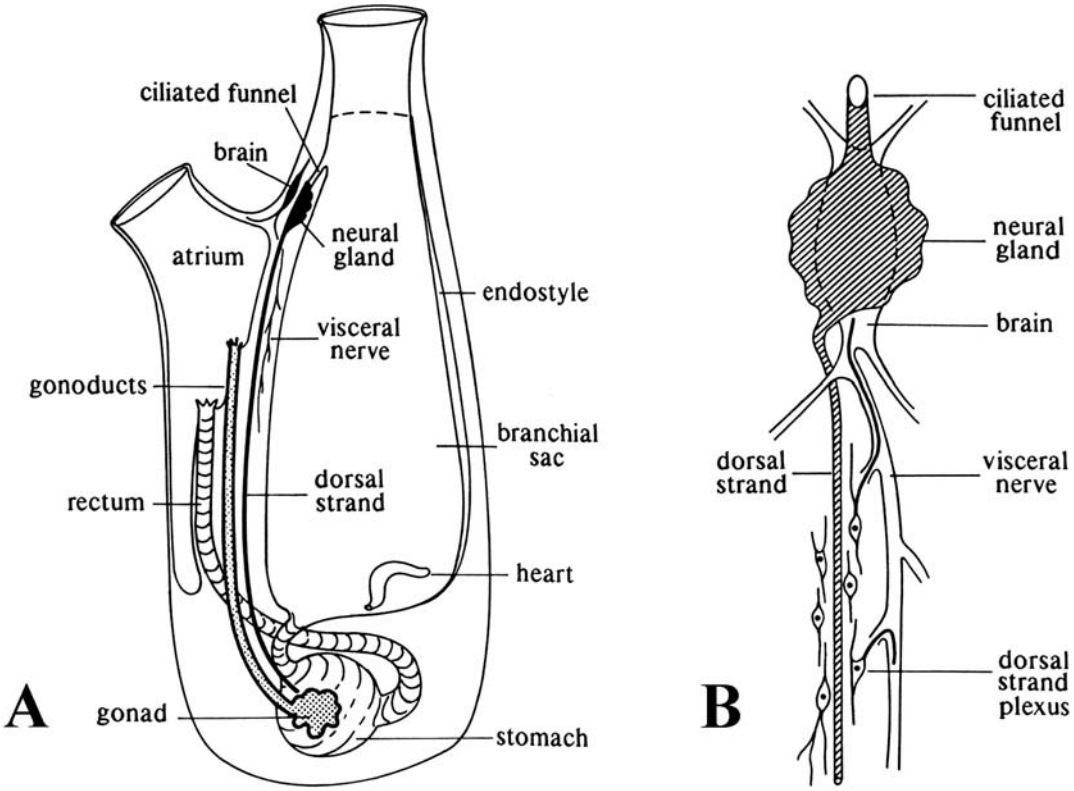
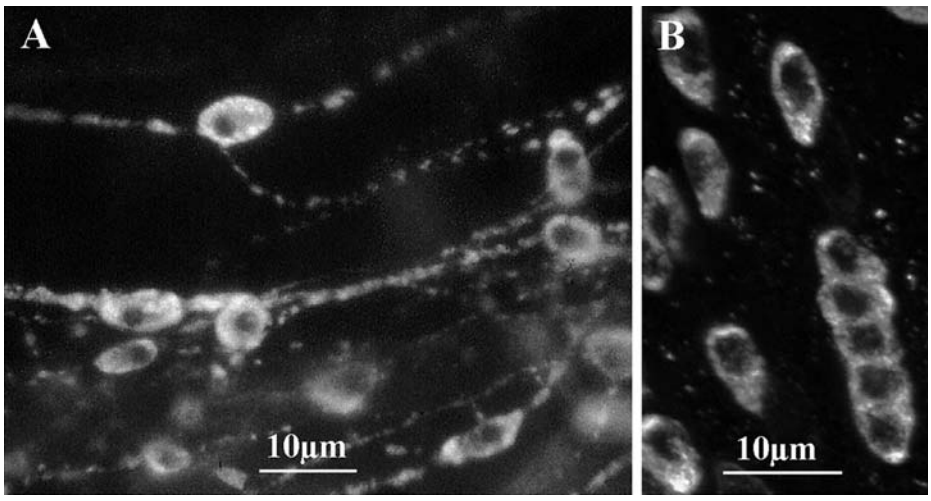


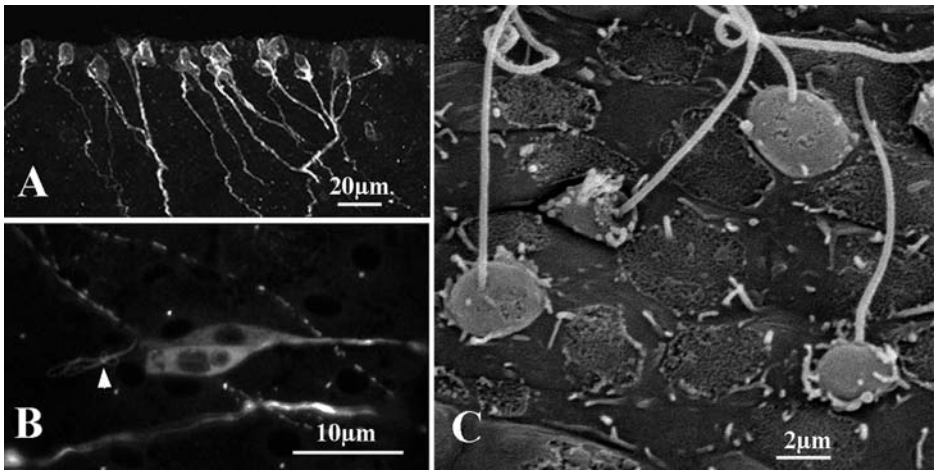
Fig. 4. GnRH-immunoreactive cells associated with the dorsal strand in *Corella inflata*. (A) Dorsal strand nerve plexus. (B) Cells lacking neurites ("neuroblasts").



served in the vicinity of the brain actually lie outside it on the surface (Mackie 1995*a*; Terakado 2001) and appear to be part of the dorsal strand plexus. Evidence from a variety of sources that GnRH secreted by the dorsal strand plexus regulates reproduction in ascidians (Sherwood et al. 2005) is strengthened by the recent finding of genes for GnRH receptors which have been identified by cDNA cloning from the neural complex in *Ciona*. Injected into *Xenopus* eggs, the mRNA for one of these genes produced membrane channels that developed inward current in response to ascidian GnRH. The receptors are expressed in the gonoducts and other visceral organs (Kusakabe et al. 2003).

Prolactin-like immunoreactivity (PRL-ir) has been demonstrated in dense-cored vesicles within cells scattered along the dorsal strand in *Halocynthia* (Terakado et al. 1997). The cells are spherical or ovoid and lack neurite-like extensions, although they lie in close proximity to cells bearing such extensions, both sorts of cells being strung along close to the dorsal strand as in the dorsal strand plexus of *Ciona* (Mackie 1995*a*). GnRH-ir neurons appear to be present in the dorsal

Fig. 5. Primary sensory neurons in *Corella inflata*. (A) Sensory cells clustered at the rim of the atrial siphon and labelled with anti-tGnRH-I antibody. Their cilia (not stained) project into the incurrent water path. (B) A pair of sensory cells in the outer mantle epithelium labelled with anti-tubulin. Their cilia (arrowhead) contact or enter the covering tunic. (C) Tips of cupular sensory cells shown by scanning electron microscopy after removal of the cupula by pronase treatment.



strand of *Halocynthia* (Terakado 2001), but the exact relationship between them and the PRL-ir cells remains unclear.

Adrenocorticotropin-like immunoreactivity has been detected in cells scattered along the dorsal strand in *Halocynthia* (Kawahara et al. 2003*b*). The peptide lies within secretory granules with diameters of 300–500 nm. These cells evidently represent a different population from those showing PRL-ir, where the granules are smaller (100–250 nm; Kawahara et al. 2002). This work was done by immunoelectron microscopy, with appropriate preabsorption controls.

The findings reviewed above suggest that the dorsal strand plexus, broadly defined, is a composite structure including cells both with and without neurites, extending to the area around the brain as well as posteriorly in the dorsal blood sinus, and containing several different neuropeptides. It may recruit new cells by delamination from the dorsal strand, but this awaits proof, as does the ability of GnRH-ir cells to migrate to other regions.

Sensory receptors and sense organs

Sensory cells, presumed to be mechanoreceptors, occur singly, in pairs, or in small clusters in the inner and outer mantle epithelia of ascidians (Fig. 5B). They are primary sensory neurons, with a single axon going to the brain. A single cilium projects to the exterior or, in the case of the outer mantle epithelium, into the tunic. A slim (ca. 0.3 µm) axon joins with other nerves running to the brain. Such cells are concentrated in a row around the edge of each siphon (Fig. 5A) and are generally most abundant in the area of the siphons (Millar 1953). They show well in preparations labelled with anti-tubulin (Mackie and Wyeth 2000). Similar cells are found in pelagic tunicates; for instance, around the lips in doliolids and salps and at the entrance to the branchial siphon in pyrosomes. These and many other varieties of presumed receptor cells in pelagic tunicates are reviewed by Bone (1998*b*), but two remarkable cases deserve special mention here.

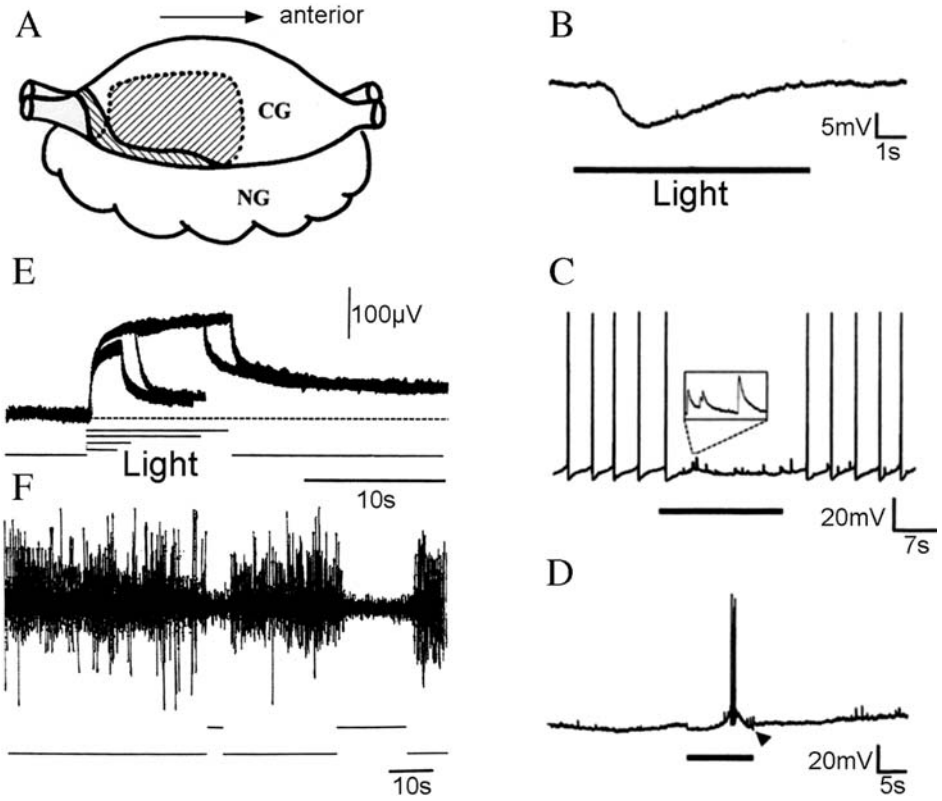
A unique type of sensory cell is seen in salps at the plaques where adjacent blastozooids adhere to one another. The tip of its cilium is branched, and the branches terminate in small expansions that abut upon and form specialized junctions with excitable epithelial cells of the adjacent zooid (Bone et al. 1980). These junctions are polarized in the direction of the sensory cilium and evidently mediate communication between zooids, allowing epithelial impulses to "jump" across the adhesion plaque and enter the nervous system of the next zooid.

Another highly peculiar sensory cell is the Langerhans receptor of *Oikopleura*. It is a secondary sensory cell that lies in the caudal epithelium and is coupled to adjacent epithelial cells by gap junctions. Thus, it can transmit excitation from the epithelium, which is excitable, to an afferent neurite that enters the caudal ganglion, as well as responding directly to the movement of the bristle-like ciliary process (Bone and Mackie 1975; Bone 1985). Excitation of the Langerhans pathway evokes escape swimming. The junction between the receptor cell and the afferent neurite is a gap junction, not a synapse, so the cell can hardly be a genetic forebear of the secondary sensory neurons (hair cells) characteristic of coronal organs. Another larvacean, *Fritillaria,* lacks or loses its epidermal epithelium, lacks the skin conduction system and Langerhans receptors, and shows no escape behaviour when touched (Bone et al. 1977). *Fritillaria* may, however, have secondary sensory neurons in the lower lip (Bone et al. 1979).

Photoreception

Well-defined photoreceptor organs (ocelli) are known in salps and pyrosomes, and probably play a part in sensing the light-intensity changes that along with other factors regulate diurnal vertical migrations. Their structure, physiology, and behavioural importance have been reviewed recently by Madin (1995) and Bone (1998*b*) and will not be covered here. Surprisingly, complex ocelli are also present in some ascidian tadpole larvae (see Bone and Mackie 1982). Intracellular recordings from tunicate photoreceptor cells show graded hyperpolarizations during illumination that is associated with increased membrane conductance, as in vertebrate cones (Gorman et al. 1971; McReynolds and Gorman 1975).

Fig. 6. Responses to illumination in *Ciona* (A–D) and *Halocythia* (E, F). (A) Neural complex showing the brain (CG) and neural gland (NG). The shaded areas show populations of GnRH-immunoreactive neurons. Intracellular recordings in Figs. 6B–6D were made from photoreceptor neurons in close proximity to GnRH-immunoreactive cells in the ventral zone. (B) Transient hyperpolarization in response to light. (C) Suppression of spontaneous discharge pattern by illumination. Small depolarizing events (enlarged in inset) are presumably excitatory synaptic potentials, which may also be light-induced. (D) Transient depolarizations leading to spikes. (E) Light-evoked slow potentials recorded extracellularly, representing summed hyperpolarizations of numerous cells in areas where retinal-containing cells are concentrated. (F) High-frequency spontaneous discharges of numerous neurons blocked by illumination. Figure 6A was modified from Tsutsui et al. (1998); Figs. 6B–6D were modified from Tsutsui and Oka (2000); Figs. 6E and 6F were modified from Ohkuma et al. (2000).



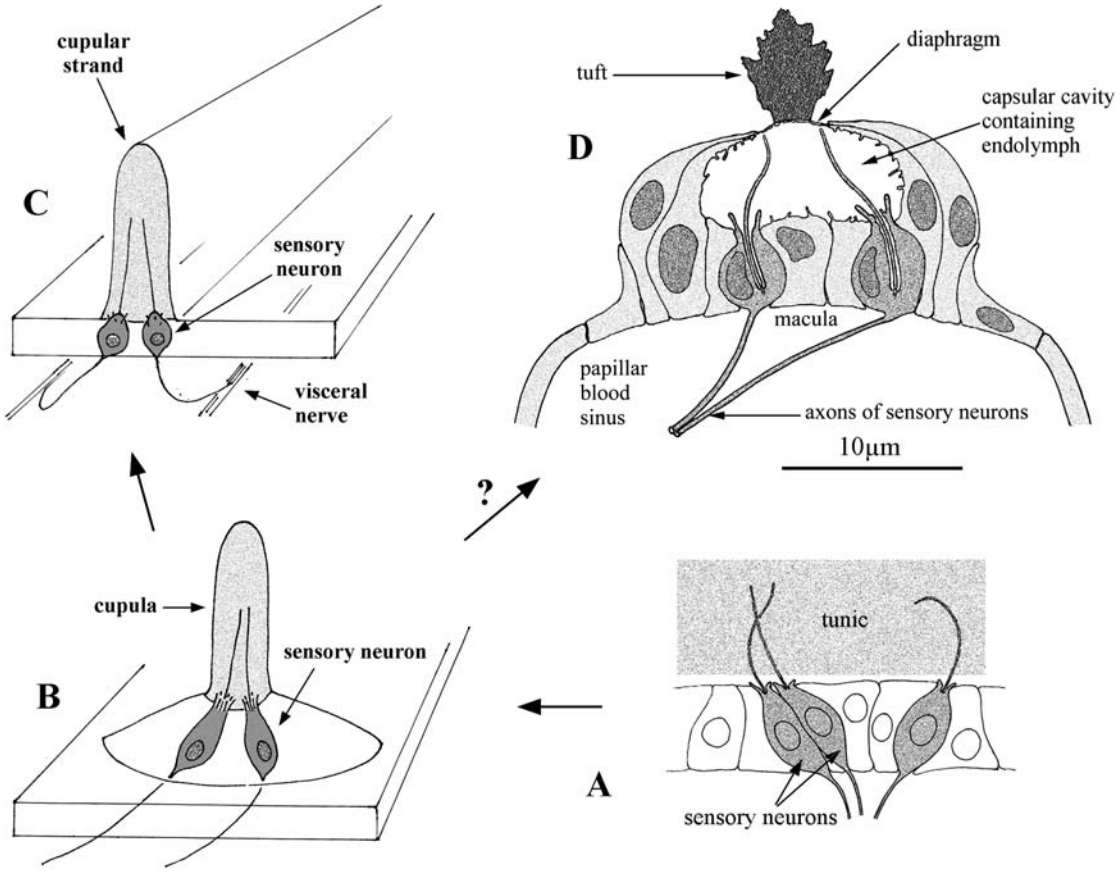
Adult ascidians lack ocelli, but some are clearly sensitive to light. Hecht (1918*a*, 1918*b*) showed that illumination of the oral siphon of *Ciona* evoked the crossed reflex (see section "Control of body-wall muscles", p. 163). This and other older work is reviewed by Goodbody (1974). Gamete release is triggered by light in many ascidians (Lambert and Brandt 1967; Numakunai and Hoshino 1973, 1980; Svane and Young 1989; West and Lambert 1976). It is only recently that electrophysiological evidence regarding the nature and location of the photoreceptors has begun to emerge.

Retinal isomers that undergo interconversion between all-*trans* and *11-cis* forms when illuminated by blue and orange light, respectively, were extracted from the minced brains of *Halocynthia* (Kajiwara et al. 1990), suggesting that the brain is the site of the photoreceptors which control gamete release, a process that probably involves GnRH. Ohkuma and Tsuda (2000) successfully visualized retinal proteins in the brain of the same species. The cells that contain the retinal proteins (the presumed light sensors) do not show GnRH-like immunoreactivity and they lie within the brain cortex, whereas GnRH-ir cells lie just outside on the surface, and are here considered part of the dorsal strand plexus.

Intracellular recordings from the ventral side of the brain in *Ciona savignyi* Herdman, 1882 (Figs. 6A–6D) showed light-sensitive voltage responses (Tsutsui and Oka 2000). About half the neurons penetrated showed regular trains of action potentials, and about a quarter showed transmembrane voltage changes in response to light, of which almost all were to light "on". The commonest response was a transient hyperpolarization (Fig. 6B). In cells showing a spontaneous firing pattern, action potentials were suppressed during illumination (Fig. 6C). Transient depolarizations with spike generation were observed in a few cells (Fig. 6D). The proximity of these neurons to GnRH-ir cells of the dorsal strand plexus suggests a functional link with gamete release that is mediated by GnRH.

Electrical recordings of responses to light were also obtained from *Halocynthia*, using extracellular recordings from the surface of isolated brains (Ohkuma et al. 2000) in areas where retinal-containing neurons are known to occur (Ohkuma and Tsuda 2000). Illumination evoked positive slow waves whose duration corresponded to the duration of illumination (Fig. 6E). These recordings may be considered equivalent to electroretinograms. As with *Ciona*, recording from within the brain with low-resistance glass microelectrodes showed blockage of spontaneous discharge activity during illumination (Fig. 6F). Finally, the distribution of photoreceptors and GnRH cells was checked in paraffin sec-

Fig. 7. Hydrodynamic sensors in phlebobranch ascidians. (A) Presumed evolutionary starting point where the cilia of epithelially located, primary sensory neurons project into the tunic. This condition is seen in the outer mantle epithelium. (B) Simple cupular organ as seen in *Ciona intestinalis* and *Corella eumyota*. (C) Cupular strand of *Corella inflata*. (D) Capsular organ of *Chelyosoma productum*. It is suggested that the cupular strand evolved from the simple type of cupular organ and that the capsular organ may also have done so. Figures 7A and 7D were modified from Mackie and Singla (2003); Figs. 7B and 7C were modified from Mackie and Singla (2004).



tions by immunocytochemistry. Doubly labelled preparations confirmed that the photoreceptor neurons are a distinct population from the GnRH cells but lie very close to them.

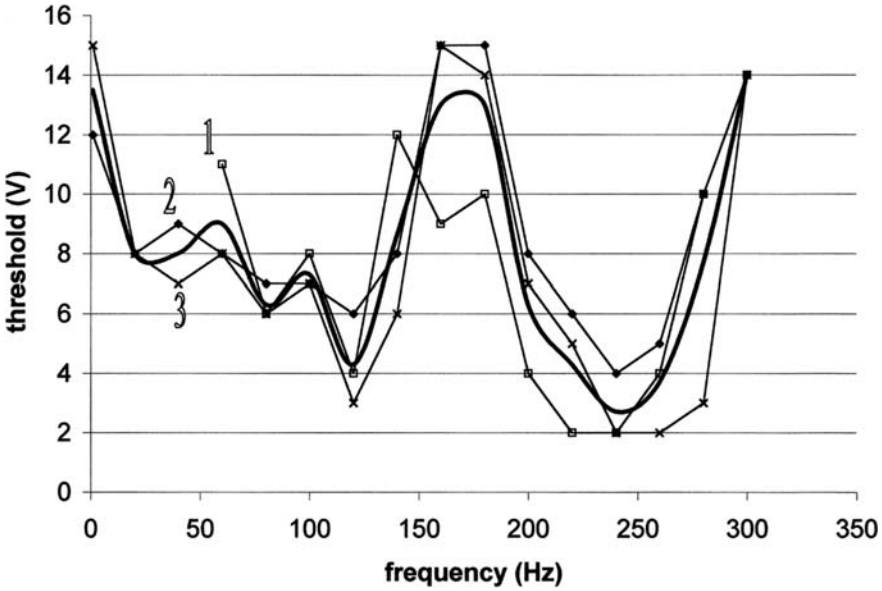
The population of GnRH-ir neurons located at the surface of the brain may not be the only such cells linked to photoreceptors. Debrained *Chelyosoma* not only survive for long periods but continue to show seasonal reproductive cycles (Hisaw et al. 1966). Photic induction of gamete release was demonstrated in isolated sperm ducts of *Ciona* by Woollacott and Porter (1977), who attributed it to the direct action of light on microfilaments in the gonoduct epithelial cells. Mackie (1995*a*), however, found a rich innervation of the gonoducts by neurons of the GnRH-ir dorsal strand plexus (confirmed by Powell et al. 1996) and suggested that GnRH was responsible for the sperm release observed in isolated gonoducts. Rather than the gonoduct epithelium being photosensitive, GnRH cells in the gonoduct region might themselves be photoreceptors, or be associated with photosensitive cells as in the region around the brain.

Hydrodynamic sensors

Until recently, the only known ascidian organs of this type were the cupular sense organs (Fig. 7B) found in the lining of the atrial siphon in *Ciona* (Fedele 1923; Bone and Ryan 1978; Corette et al. 1985). Similar structures have also been described in salps (see Bone 1998*b*). Cupular organs much like those in *Ciona* have since been reported in *Corella eumyota* Traustedt, 1882, where they are located not in the siphons but on the atrial surface of the branchial sac. These organs are termed simple cupular organs to distinguish them from the cupular strand (Fig. 7C), which is a much larger, elongated organ found in *Corella inflata* Huntsman, 1912 (hereafter simply *Corella;* Mackie and Singla 2004).

Simple cupular organs (Fig. 7B) consist of a pad of tissue (macula) containing supporting cells and sensory cells (primary sensory neurons) whose cilia project into a finger-like process composed of tunic-like material, the cupula. About 75–100 of these organs are found in *Ciona*, each containing 15–20 sensory cells. Their counterparts in *Corella eumyota* are fewer but larger, with 20–30 sensory cells. Direct evidence of function is lacking. *Ciona* appears insensitive to vibrations issuing from sources more than a few millimetres away (Bone and Ryan 1978; Corette et al. 1985) and similar levels of vibration sensitivity are observed in species lacking cupular organs. It seems unlikely therefore that they are specialized for detection of near-field vibrations. Their location close to the atrial siphon suggests that they may monitor changes in local water flow, like neuromasts of the lateral

Fig. 8. Sensitivity to vibration in *Chelyosoma*. Values on the *y* axis represent the lowest voltages used to drive a vibrating probe over a range of frequencies shown on the *x* axis that were effective in evoking ciliary arrests in the branchial sac (from Mackie and Singla 2003, reproduced with permission of Brain Behav. Evol., Vol. 61, © 2003 S. Karger AG).



line of fish. The generalized vibration sensitivity shown by the great majority of ascidians is more likely due to the scattered receptors in the outer mantle epithelium (Fig. 7A).

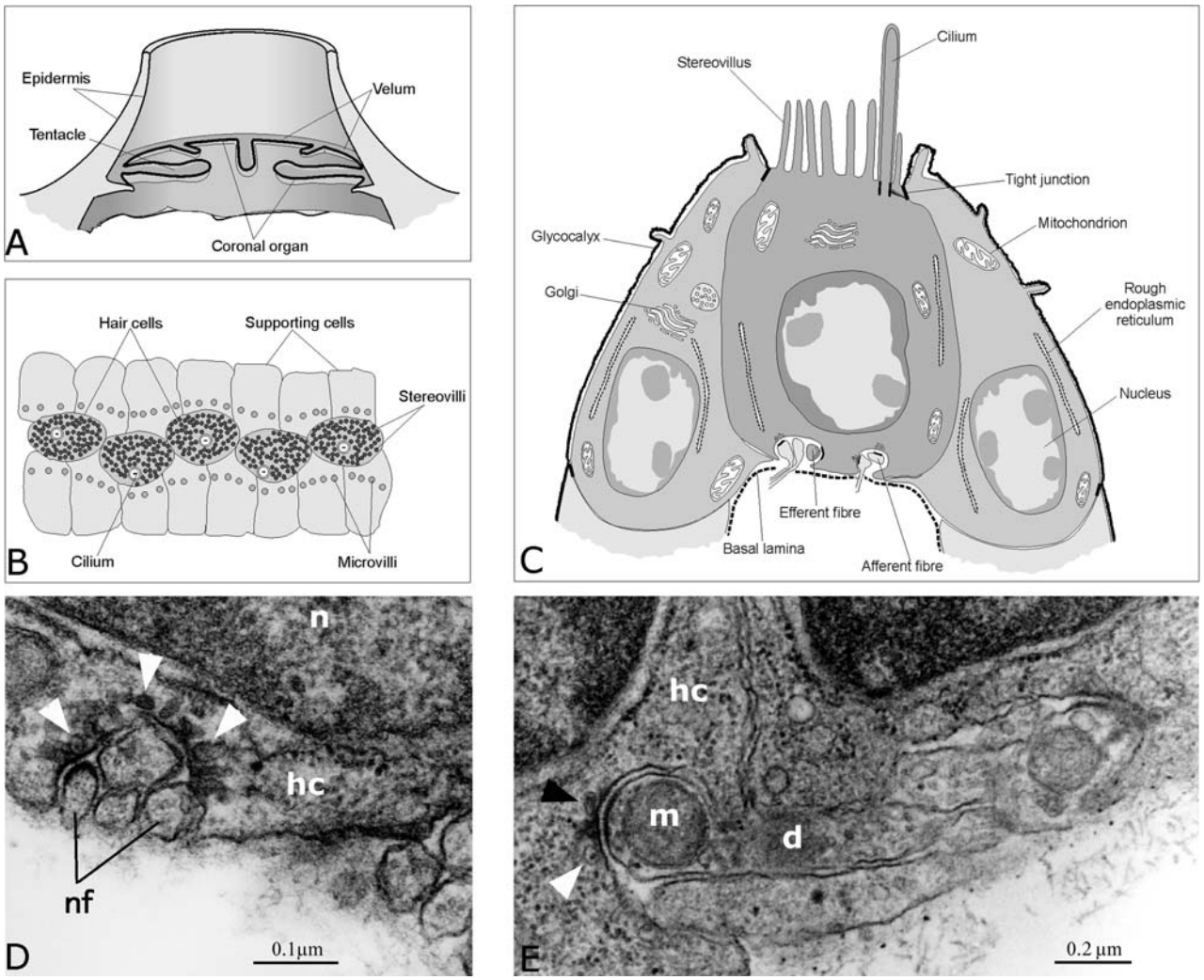
In the cupular strand of *Corella* (Fig. 7C), the cupula is elongated in the animal's longitudinal axis, running along the dorsal midline of the branchial sac. There is only one such structure in the animal, but it is 7–8 mm long in a typical animal and has about 1500 sensory neurons (Fig. 5C). Curiously, these sensory neurons show GnRH-like immunoreactivity, suggesting that GnRH may function here as a neuromodulator. The form of GnRH present has not been identified, but its reactivity with antisera raised against various known forms of GnRH suggests that it is a distinct form of the molecule, immunologically different from the form or forms present in the dorsal strand plexus which control reproduction (Mackie and Singla 2004).

In another corellid, *Chelyosoma*, sense organs of a unique type termed capsular organs (Fig. 7D) have recently been described (Mackie and Singla 2003). There are about 200 of these structures in the atrial epithelium of the branchial sac. The macula contains 5–6 primary sensory neurons whose cilia project, not into a cupula, but into a fluid-filled capsule. The apex of the dome-shaped capsule contains an oculus that is covered with a thin acellular diaphragm, which is surmounted by a tuft of tunic-like material. Although this organ can be seen as a possible derivative of the simple cupular type of organ, the location of the sensory cilia within the capsular fluid (endolymph) with their tips just below the diaphragm, strongly suggests a structure specialized for detection of water-borne vibrations. Monitoring the animal's responses with a thermistor flow meter during artificially generated vibrations showed that *Chelyosoma* could detect 104 dB (re 1 μPa) from a source 80 cm away, with peak frequency in the 240–260 Hz range (Fig. 8). This is a level of sensitivity equivalent to that shown by fishes such as salmon. By contrast, neither *Corella* nor *Ciona* appear sensitive to vibrations from sources farther than 4 cm away.

Botryllus and *Botrylloides violaceus* Oka, 1927 have a novel hydrodynamic sensor, the coronal organ, composed of a continuous row of some 2000 sensory cells and their supporting cells that run around the velum and tentacles at the base of the oral siphon (Figs. 9A, 9B). Contrary to the situation in cupular and capsular organs, the sensory cells here are axonless hair cells that synapse locally on the dendrites of secondary sensory neurons whose cell bodies lie in the brain (Figs. 9C–9E); lack of an axon has been confirmed by DiI staining (Burighel et al. 2003). The obvious resemblance to sensory structures in the vertebrate acusticolateralis system is enhanced by the presence of efferent synapses, both on the hair cells and on the afferent neurons they synapse with. The hair cells have a single, nonmotile cilium and numerous stereovilli. The cilium may be located centrally or to one side. Unlike other epithelial cells, the hair cells are not coupled to adjacent cells by gap junctions. It seems likely that the coronal organ detects small particles in the incoming water.

A new survey shows that coronal organs are present in representatives of all three orders of Ascidiacea and that there is considerable variability in the apical structures. In *Styela plicata*, there are two sorts of hair cells in the coronal organ. One sort has two cilia lying within a crescent-shaped bundle of stereovilli that are graded in length, short on one side and long on the other, as in typical vertebrate hair cells (Manni et al. 2004*a*). As long as the only known hydrodynamic sense organs in tunicates were the cupular and capsular organs described above, both of which are based on primary sensory neurons, it was difficult to accept a tunicate evolutionary starting point for the vertebrate acusticolateralis system, and it appeared more likely that the resemblances were due to convergence (Mackie and Singla 2003). The finding of the coronal organ, a tunicate sense organ in which the receptors are axonless hair cells synapsing on afferent neurons, makes it reasonable to consider a possible homology with fish neuromasts and related vertebrate or-

Fig. 9. Coronal organ of *Botryllus schlosseri*. (A) Location of the organ in the oral siphon. (B) Zig-zag arrangement of hair cells in surface view. (C) Summary diagram showing the relationship between hair cells, nerve endings, and supporting cells. (D) Four nerve fibres (nf) are seen enclosed in a groove in the base of a hair cell (hc). n, hair-cell nucleus. Numerous vesicles (white arrowheads) encircle the synaptic zone. (E) Vesicles with dense contents (black arrowhead) and clear vesicles (white arrowhead) are both seen on the hair-cell (hc) side of the junction. The dendrite (d) of the sensory neuron receiving the synapse contains a prominent mitochondrion (m). Modified from Burighel et al. (2003).



gans. Embryological and gene-expression studies suggest that the atrial primordia of ascidians, where the hydrodynamic sensors later differentiate, are homologous to the otic placodes of vertebrates (Katz 1983; Wada et al. 1998). This is discussed further in the second paragraph of section "Stomodeal placode" (p. 172).

Other sensors

Although it seems likely that some tunicates are chemosensitive, clear evidence is lacking. Possible examples of olfactory sensors are cells located in the region around the lips in doliolids and salps (see Madin 1995), and in the ventral organ a group of 30 cells lying under the lower lip in *Oikopleura* (Fig. 2). The cells of the ventral organ are primary sensory neurons that send their axons into the paired frontal lobes of the brain, recalling the situation in higher chordates (Bollner et al. 1986; Olsson 1986). The coronet cells in the sensory vesicle of *Oikopleura* (Fig. 2) may be homologues of cells in the saccus vasculosus of fish, which are thought to be chemoreceptors monitoring the composition of the central fluid.

The tunicate neural gland is connected to the exterior by a duct leading to the ciliated funnel (Fig. 3). The cilia beat inwards, allowing particulates and tracer substances to enter the gland (Godeaux and Beros-Dubroux 1979; Ruppert 1990). Barrington (1975) suggested that the gland was a chemoreceptor, which was sensitive to secretions of other individuals taken in through the duct, and that these might regulate reproductive activities; a proposal echoed by Gorbman (1995) and Gorbman et al. (1999) in the context of GnRH. Gorbman et al. (2003) have since reported that GnRH acts as a pheromone in a chiton and an enteropneust, causing synchronous spawning. Given the evidence for an endocrine role for GnRH in tunicates, it would therefore not be surprising if GnRH were secreted to the exterior and were taken in by conspecifics via the ciliated funnel, acting as a pheromone to coordinate spawning. However, preliminary tests on *Ciona* proved inconclusive (A. Whiteley, personal communi-

cation) and there is still no solid evidence for either sensory cells or chemoreception in the neural gland.

Hecht (1918*b*) explored sensitivity to temperature, to osmotic pressure, and to various chemicals in *Ascidia atra* Lesueur, 1823 (a synonym of *Ascidia nigra* Savigny, 1816). He found this ascidian to be thermosensitive, responding to temperatures above 32 °C and below 20 °C. He also found that the most chemosensitive region was the inside of the oral siphon. Removal of the oral tentacles, deemed to be chemoreceptive sites, did not qualitatively affect the responses observed. Day (1919) found that the oral tentacles were actually less sensitive to quinine than the lobes of the siphons. As noted earlier in the fifth paragraph in section "Hydrodynamic sensors" (p. 160), nerves and sensory cells are present in oral tentacles of *Botryllus* (Burighel and Cloney 1997; Burighel et al. 2003), but their function is almost certainly mechanoreception. *Clavelina huntsmani* Van Name, 1931 is reported to respond by squirting to fluids extracted from the tissues of conspecifics (Pelletier 2004). There was no response to injury fluids from *Corella willmeriana* Herdman, 1898. This appears to be the first evidence of alarm signaling in tunicates and may indeed be the first indication of any sort of chemosensitivity as currently understood, because the tests by Hecht and others used substances that would have had very general effects (acids, bases, salts) or were neuroactive (strychnine, morphine).

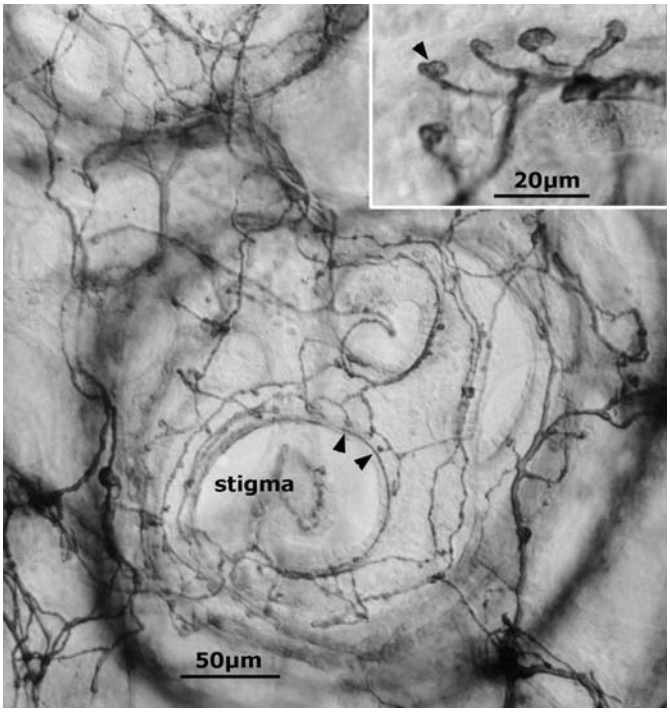
Gravity receptors have never been described in adult ascidians, but larvaceans and ascidian tadpoles have sensory vesicles containing structures termed statocysts or otocysts, as well as other sensory structures (Burighel and Cloney 1997; Sorrentino et al. 2000). They might detect gravity, acceleration, or vibration, but physiological evidence is lacking. Bone (1998*b*) suggests that the otocyst of doliolid oozooids is more likely to be a vibration receptor than a gravity receptor. Proprioceptors are thought to be present in the tail of *Dendrodoa* tadpoles (Mackie and Bone 1976), but no such structures have been found in adult tunicates.

Motor innervation and the "peripheral nerve net"

Like sensory nerves, tunicate motor fibres are hard to visualize with traditional methods, although some workers (Hunter 1898; Markman 1958) have had success with oxidized or reduced methylene blue, and Bone (1959) with silver impregnations. Phase-contrast microscopy shows the thicker nerve bundles and some of the finer terminations, but the picture of the branchial motor innervation obtained by Mackie et al. (1974) by this method gave no idea of the richness of the innervation revealed in a later study using cholinesterase histochemistry (Arkett et al. 1989). Immune labelling with anti-tubulin is currently a simple and effective option, showing both motor and sensory nerves, but it fails to reveal the finest details such as the synaptic boutons in the branchial sac of *Corella*, which are very clear in cholinesterase preparations (Fig. 10).

The general layout of the motor innervation has been described most completely in *Botryllus* (Burighel et al. 1998, 2001; Zaniolo et al. 2002), where all organs, except for the neural gland, dorsal organ, and gonads, show an extensive cholinesterase-reactive innervation. Both in developing

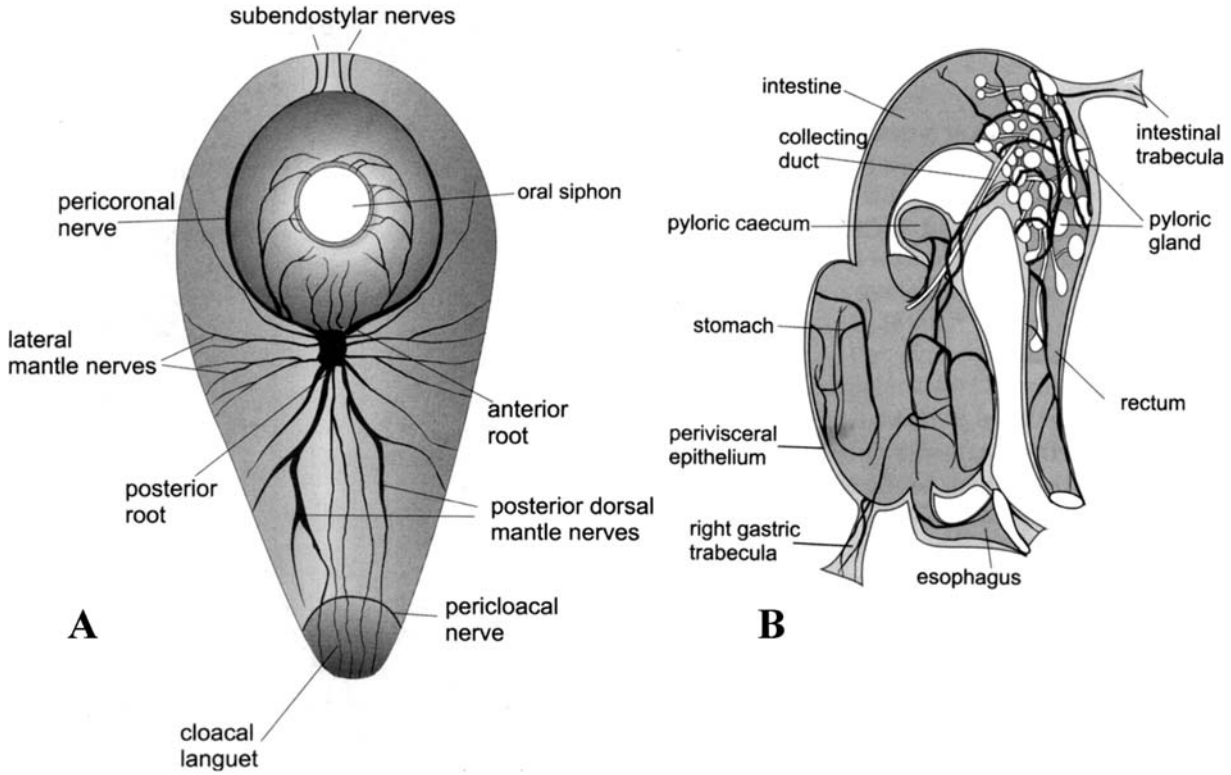
Fig. 10. The rich branchial innervation of *Corella eumyota* shown by cholinesterase histochemistry. Numerous fine nerve endings run to the bases of the ciliated cells lining the stigmata. Synaptic boutons (arrowheads) are shown enlarged in the inset.



oozooids and asexual buds, motor axons run out from cell bodies in the brain to the main effectors (Fig. 11A); i.e., the muscles of the body wall and siphons and the ciliated cells lining the branchial stigmata. They also extend to organs such as the gut, endostyle, and heart, where their functions are still poorly understood.

Feeding in doliolids involves coordinated activity of the cilia of the branchial sac, endostyle, peripharyngeal bands, and oesophagus, and is evidently controlled by the largely autonomous visceral innervation, as it continues after the brain has been removed (Bone et al. 1997). Though no visceral ganglion has been described, "a small group of two or three neuron somata" was seen by these workers using methylene blue staining. They lie in the dorsal epithelial fold from which the branchial sac is suspended. Multipolar neurons have also been described in the gut and endostyle of salps (Fedele 1933; Bone 1959). Fedele (1938 and earlier papers) described a visceral innervation in *Ciona*, but this now appears to be a complex of motor axons whose cell bodies lie in the brain, dorsal strand plexus elements, and possibly sensory elements. In *Botryllus*, AChE histochemistry showed a remarkably rich innervation of the whole gut (Fig. 11B), but there were no local cell bodies corresponding to those seen in salps and doliolids. The coordination of the various action systems involved in feeding in ascidians has not been comprehensively addressed from the neurophysiological standpoint. Almost all neurological studies have focussed on the control of the cilia in the branchial stigmata without regard to events in the endostyle or other visceral organs. It may be that the production, transport, and ingestion of the mucous filter are all coordinated locally by elements of a visceral nerve complex, as in doliolids. A visceral nerve

Fig. 11. (A) Principle nerves of the dorsal mantle of *Botryllus* as shown by cholinesterase histochemistry. (B) Innervation of the gut in dorsal view. Modified from Burighel et al. (2001).



plexus with local neuronal somata cannot be completely excluded.

In both ascidians and doliolids, the major conduit for nerves running between the brain and viscera is the visceral nerve; however, in ascidians the other major nerves have branches going to some of the same regions, suggesting the possibility of dual innervation (Burighel et al. 2001). A dual motor innervation (cholinergic and GABAergic) is also suspected in the tail muscles of *Oikopleura* (see Bone 1998*a*).

A peculiar feature of colonial tunicates is that nerves are apparently unable to pass from one zooid directly to another. This may mean that the tunic is an inhospitable environment for nerve growth (Mackie 1995*b*). Accordingly, colonial forms have evolved other, more exotic, means of communication (section "Coordination of tunicate colonies").

Control of the heart

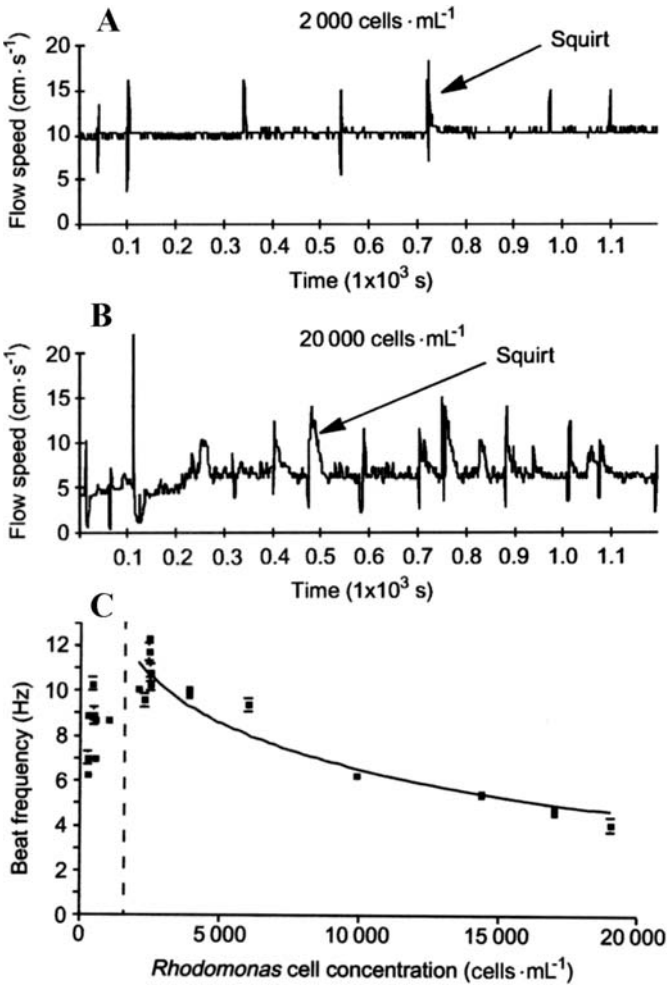
Although the basic cardiac rhythm is undoubtedly myogenic (see Goodbody 1974; Bone and Mackie 1982), heart rate may be under some degree of nervous control. Florey (1951) found that strong contractions of the body wall were accompanied by alterations of the cardiac rhythm and often preceded changes in the direction in which the blood was pumped. He also found that strychnine and picrotoxin caused dramatic alterations in both the rhythm and the direction of beating but only if the brain was present, which implied that these excitatory nerve drugs were acting through the nervous system. Arai et al. (1999*b*) observed an increase in heart rate (monitored electrically as an electrocardiogram) along with squirting, following stimulation of the atrial siphon. The increase was not seen after brain extirpation. Several workers have observed nerves in the pericardium, most recently Burighel et al. (2001) using cholinesterase histochemistry, but nerves do not enter the myocardium. Any nervous effect on the heart rate is therefore likely to be indirect, via blood-borne neurochemicals.

Control of body-wall muscles

Ascidian squirting behaviour and related muscular activities were much studied in the first half of the last century, with research by distinguished physiologists such as Loeb, Jordan, Bacq, and Hecht (see ten Cate 1931). Following the work of Florey (1951) and Hoyle (1952, 1953), the topic entered a dormant phase and attention switched more to pelagic tunicates. The simple and regular layout of muscle bands in salps and doliolids and the caudal musculature of larvaceans make these more suitable objects for neuromuscular physiology than the rather confusing array of body-wall muscles in ascidians, where the responses are also less clearly defined. Pelagic tunicates have attracted a series of groundbreaking studies mostly by Q. Bone and coworkers. Because these are covered in recent accounts (Bone 1998*a*, 1998*b*; Bone et al. 1998), we will focus here on the less understood ascidians.

Squirting behaviour in ascidians involves contraction of smooth or obliquely striated muscle bands in the mantle, siphons, and branchial sac. The resulting compression of the body wall causes ejection of water from one or both siphons. Squirting can be monitored using flow meters in the siphons (Petersen et al. 1999; Mackie and Singla 2003) or pressure tranducers inserted into the atrial chamber, a method favoured by Japanese aquaculturists working on *Halocynthia* (Arai et al. 1998, 1999*a*, 1999*b*; Katayama et al. 1999; Hoshiai et al. 2001). Squirting is typically accompanied by

Fig. 12. Effect of the concentration of *Rhodomonas* cells on squirting (A, B) and ciliary beating (C) in *Ciona*. In Figs. 12A and 12B, flow was monitored at the atrial siphon using a thermistor flow meter. Squirting increased in frequency with algal concentration. In Fig. 12C, ciliary beat frequency declined with algal concentration. (From Petersen et al. 1999, reproduced with permission of Mar. Biol. (Berl.), Vol. 133, © 1999 Springer-Verlag.)



ciliary arrests in the branchial sac (see below). It varies in duration and intensity, depending on the strength and type of stimulation. It often seems to happen spontaneously, but it is difficult to be certain that no external stimulus was involved. For one thing, squirting frequency can vary according to the amount of food in the water (Figs. 12A, 12B) (Petersen et al. 1999). Tunicates like other filter feeders are acutely "aware" of particulates in the water they process. This is evident from recent work on *Oikopleura*, where tail beat frequency and particle rejection at the mouth both vary according to food concentration (Selander and Tilesius 2003). In *Fritillaria*, periodic muscular contortions interrupt the regular pumping process, serving to backwash coarse particles and prevent filter clogging (Flood 2003).

Arai et al. (1998, 1999*a*) distinguish tonic from phasic squirting. Tonic squirting is characterized by lower intrabranchial pressure, longer duration, and longer inter-squirt interval than phasic squirting. Unlike phasic squirting, tonic squirting does not appear to be evoked on a neural reflex basis. Long-term recordings from *Halocynthia* with pressure transducers (Katayama et al. 1999) showed no rhythmic squirting in the dark, but periodic, tonic squirting was seen during periods of illumination. These workers do not appear to have considered variations in ciliary beat frequency as a possible basis of tonic squirting.

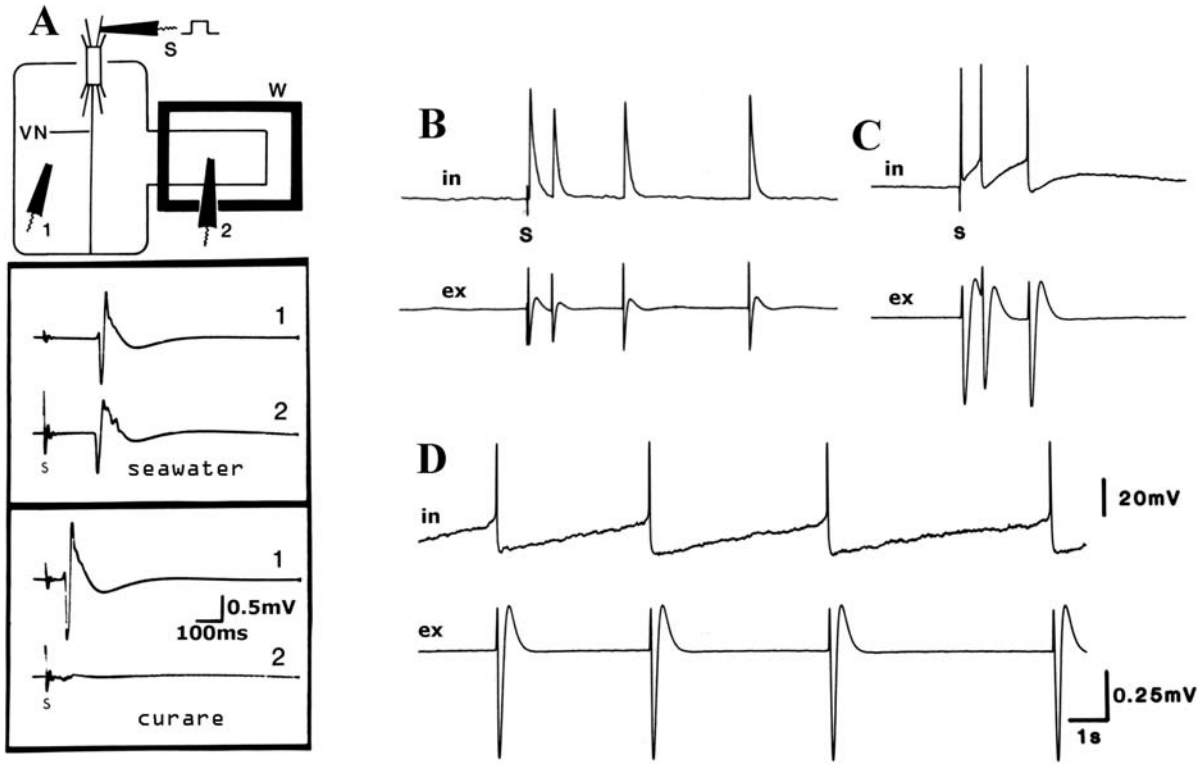
In mechanically induced squirting, tactile stimulation evokes afferent nerve impulses that travel to the brain, which result in motor discharges that can be recorded extracellularly from the larger nerve roots (Mackie and Wyeth 2000). The number and time relationships of afferent signals evidently determine how large and widespread the response will be. Full-scale squirting behaviour grades down into local twitches of stimulated siphons in which the brain may not be involved at all. The brain is more than just part of a fast pathway mediating the spread of responses between the two sides. For instance, it is possible by stimulating the interior of the oral siphon to get the atrial siphon to close while the oral one stays open (the "crossed reflex" of Hecht 1918*b*). The result is a brief interruption in the incurrent flow of water through the oral siphon, which may serve to dislodge material lodged in the oral tentacles, but does not produce a full-scale squirt and is not accompanied by ciliary arrest (G.O. Mackie, unpublished data). The crossed reflex is lost after removal of the brain even though slow conduction of contractions still takes place via the peripheral pathways that survive debraining (see below).

Motor neurons driving the body-wall muscles have not been identified in ascidian brains or recorded intracellularly, but the picture will probably turn out to be similar to that described for ciliary arrests. The only detailed electrophysiological work on adult ascidian muscles is that of Nevitt and Gilly (1986) who recorded from body-wall muscles of *Ciona*. These workers have found that the muscles resemble vertebrate skeletal muscle more than smooth muscle in that they are composed of discrete bundles of small-diameter muscle fibres arranged in parallel and innervated by neuromuscular junctions resembling motor end plates. There is no evidence of electrical coupling between fibres, and the action potential is a fast-rising, all-or-none propagating calcium spike. In doliolids, Inoue et al. (2002) have recently shown that muscle contraction depends on Ca^{2+} influx from the exterior through L-type calcium channels. There is no sarcoplasmic reticulum or transverse tubular system. Work on ascidian muscle proteins is covered by Burighel and Cloney (1997); actin genes and their expression in *Oikopleura* are treated by Nishino et al. (2000).

Control of branchial cilia

Fedele (1923) noted that the cilia lining the branchial stigmata in *Doliolum* underwent arrest when the animal was subjected to tactile stimulation but beat continously after anaesthesia with chloral hydrate. MacGinitie (1939) described ascidian ciliary arrests as occurring in synchrony with squirting. Further work by various authors (Takahashi et al. 1973; Mackie et al. 1974; Arkett 1987; Arkett et al. 1989) has confirmed these observations and provided satisfactory, detailed evidence of nervous control. Curare blocks the spread of arrests in the branchial sac (Fig. 13A). Arrests similar to those seen in ascidians are described in doliolids

Fig. 13. Ciliary control in *Chelyosoma*. (A) Effect of curare on transmission of ciliary arrest potentials (CAPs) recorded extracellularly from the branchial sac with suction electrodes (1, 2). A tongue of the branchial sac was placed in a vaseline well (w), which allowed it to be immersed in a test solution while the rest remained in seawater. Stimuli (s) applied to an anterior brain root caused CAPs in the branchial sac, which were conducted via the visceral nerve (vn) and its lateral branches. When both parts were in seawater, CAPs spread into the well. When the well contained 10 $\mu g \cdot mL^{-1}$ of d-tubocurarine, CAPs were no longer recorded. (B) Following stimulation of an anterior brain root (s), CAPs were simultaneously recorded intracellularly (in) and extracellularly (ex) from stigmatal ciliated cells. (C, D) Intracellular recordings from ciliary arrest motor neurons in the brain and the resulting CAPs recorded extracellularly in the branchial sac. In Fig. 13C, a short burst of spikes was evoked by stimulation of an anterior brain root. In Fig. 13D, a ciliary arrest motor neuron was firing spontaneously, producing a long-lasting spike train. Each spike evoked a CAP. The interval between motor neuron spikes reflects the recovery rate from the hyperpolarizing after potential. (From Arkett 1987, reproduced with permission of J. Comp. Physiol. A, Vol. 161, © 1987 Springer-Verlag).



(Bone and Mackie 1977) and pyrosomes (Mackie and Bone 1978). In *Oikopleura*, the cilia do not arrest but simply reverse the direction of beating (Galt and Mackie 1971).

Flow-meter recordings from the siphon openings in ascidians provide an easy and sensitive way of detecting ciliary arrests (Mackie and Singla 2003), but direct observation of ciliary activity through the transparent body wall (Petersen et al. 1999) is feasible given suitable optics and is equally nonintrusive. The electrical correlates of ciliary arrests are often large enough for arrests to be monitored relatively noninvasively with a suction electrode attached to the outside of the tunic (Mackie 1974; Mackie and Bone 1978; Pelletier 2004) or attached to the branchial sac through a window cut in the mantle (Arkett 1987).

Ciliary arrests are typically evoked by external stimuli, including water- and substrate-borne vibrations and external contact with foreign objects. Any type of stimulation that evokes contraction of the body wall or siphon muscles is likely also to cause one or more ciliary arrests, but the coupling between the two effector systems is not necessarily one-to-one. Ciliary arrests do not accompany closure of the atrial siphon during the crossed reflex. Tactile stimuli evoking arrests are presumably detected by mechanoreceptors and relayed via afferent pathways to the brain. With strong stimulation, prolonged arrests are seen.

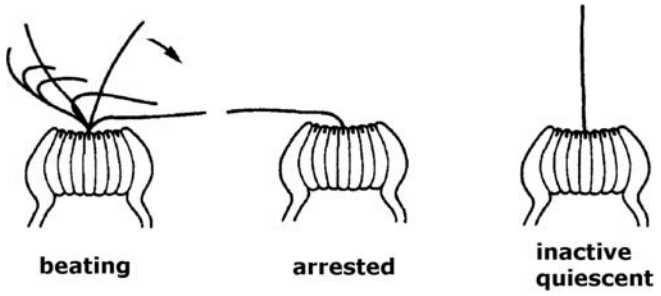
Intracellular recordings from motor neurons in the brain reveal a population of ciliary arrest motor neurons (Arkett 1987). Excitatory postsynaptic potentials (epsps) are recorded in the motor neurons when single stimuli are applied to an anterior brain root. With successive shocks, the epsps sum and facilitate to reach spike threshold. When excited, the motor neurons tend to fire in bursts (Fig. 13C), producing efferent discharges that propagate primarily (but not exclusively) via the visceral nerve to the branchial sac, causing ciliary arrests. Arrests occur one for one with action potentials recorded from the ciliary arrest motor neurons after a delay that varies according to the length of the conduction pathway. The frequency of potentials within bursts is evidently determined by the rate at which the cell recovers from the hyperpolarized state that follows each spike. This can take several seconds (Fig. 13D). Injection of depolarizing current that would normally spike the cell fails to do so during the hyperpolarization phase. Although Arkett's recordings make it clear that the ciliary arrest neurons are "pacemakers" in the sense that they produce bursts of spikes with regular inter-spike intervals, they probably normally re-

quire excitatory input to initiate such bursts. The trains of ciliary arrests observed in intact animals probably reflect summed afferent input from various sensors rather than being the simple product of a central pattern generator.

The ciliated cells lining the stigmata are individually innervated, with clearly visible synaptic boutons (Fig. 10). The ciliated cells are arranged in groups joined by gap junctions, as shown in electron microscope thin sections (Mackie et al. 1974) and freeze-fracture replicas (Martinucci et al. 1992; Burighel et al. 1992; Lane et al. 1995). It was originally thought that local electrical currents or propagating action potentials spread through gap junctions from scattered neurociliary synapses, triggering arrests. The discovery of the richness of the motor innervation (Arkett et al. 1989) made this hypothesis unnecessary, and (more to the point) Arkett (1987) injected depolarizing current into ciliated cells and found that arrests failed to spread to more than a few ciliated cells on either side. Nevertheless, some of Arkett's results with octanol, a gap junction blocker, seem to suggest that propagation of arrests in the branchial sac somehow involves gap junctional transmission either through nerves or epithelia.

The arrest event recorded intracellularly from ciliated cells is an overshooting, 45–55 mV spike (Fig. 13B) (Mackie et al. 1974; Arkett 1987). Full arrests, where the cilia lie flat against the stigmatal wall, and the inactive state, where the cilia are upright but not beating (Fig. 14), are calcium-dependent states, but the inactive state has a lower threshold than the fully arrested state. Experiments with membrane-permeant cAMP analogues, complemented by tests on saponin-extracted models, show that cAMP acts antagonistically to calcium, activating ciliary beating as in the lateral gill cilia of *Mytilus* L., 1758 and other preparations (Bergles and Tamm 1992). In the *Mytilus* preparation, elevation of cAMP levels and beating are caused by neuronal release of 5-hydroxytryptamine, but no such second nervous pathway has come to light in tunicates. It is beginning to appear, however, that the stigmatal cilia not only undergo arrests but also vary their rate of beating. Direct observation of ciliary metachronal waves allowed Petersen et al. (1999) to show that the rate of beating varies with particle concentration. The cilia beat at higher frequencies when particle concentration is low than when high (Fig. 12C), and the flow rate recorded at the siphons varies accordingly. These changes are evidently not attributable to variations in water resistance or other mechanical factors. They occur very rapidly and are related to the state of fullness of the gut, so they may well be nervously mediated. Terakado (2001) and Adams et al. (2003) independently report that injection of GnRH into the body cavities of *Ciona* results in increased water flow. Although it is not certain that this is due to accelerated ciliary beating rather than to muscular constriction of the siphons, the fact that GnRH is present in neurons of the dorsal strand plexus located in branchial blood sinuses suggests an effect upon cilia beat frequency. This should be investigated further. It may yet transpire that the branchial cilia of tunicates, like the lateral gill cilia in bivalve molluscs (Jørgensen 1990), are under dual nervous or neurohormonal control.

Fig. 14. Three different states of *Ciona* branchial cilia as seen in a cross section through the edge of a stigma, showing ciliary profiles. For simplicity, only one cilium is shown. Modified from Bergles and Tamm (1992).



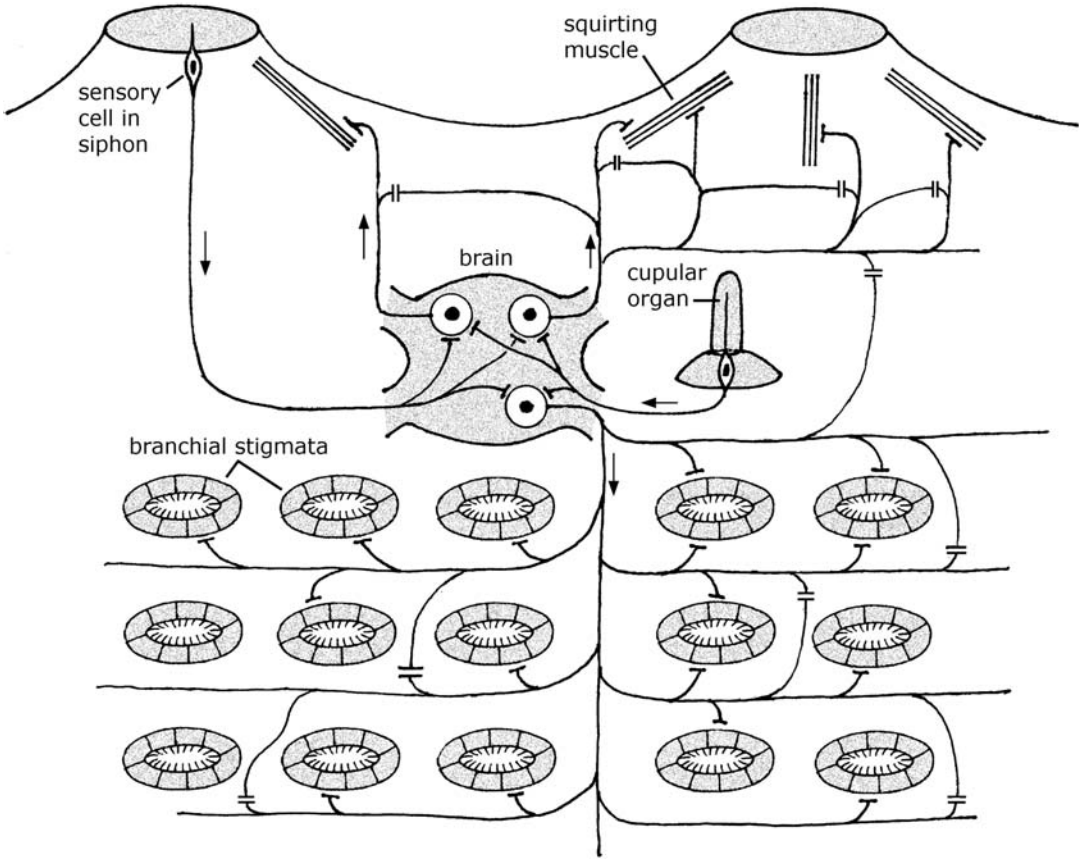
Local activity and the "peripheral nerve net"

It has long been known that ascidians resemble sea anemones and other cnidarians in showing autonomous activity of isolated portions of the body. Many activities continue after brain removal. This has prompted suggestions that in addition to the motor innervation from central neurons the animals possess a peripheral nerve net (see, for instance, Florey 1951). This applies not only to visceral functions but also to body-wall contractions and branchial ciliary arrests. Histological study of these effector regions, however, has consistently failed to show anything resembling a nerve net, in the sense of a system of interconnected neurons with their cell bodies in the periphery. (The only true nerve net in the animal is the dorsal strand plexus, which is confined to a specific location (the dorsal blood sinus) and does not extend into the body wall or into the greater part of the branchial sac.)

In a recent electrophysiological study of *Chelyosoma*, Mackie and Wyeth (2000) summarize the long history of the nerve-net debate and confirm that body-wall preparations of debrained animals continue to respond to stimulation with local and spreading contractions. The ability is retained for many months and there is no brain regeneration in this ascidian. Spread is decremental and facilitative and much slower than in intact animals. Recordings from peripheral nerve bundles in debrained animals show bursts of spikes preceding contractions, as in the intact animal, and curare abolishes all responses. There can be no doubt therefore that spread is nervous. The siphons of debrained animals sometimes show coordinated contractions, accompanied by bursts of regularly spaced potentials, showing that the peripheral nervous system retains pacemaker capability as well as the ability to conduct. The conclusion from this work was that the "residual" innervation, which survives debraining, is composed either of interconnected motor nerve terminals (the most likely explanation) or of interconnected sensory neurites or of some combination of the two.

Similar findings were earlier made in the branchial sac. Mackie et al. (1974) found that isolated branchial sacs of *Corella* continued to show propagated bursts of ciliary arrests when stimulated, and sometimes showed such bursts spontaneously. Arrests, and the system coordinating them, were both blocked by curare. Trains of arrests were through-conducted across the whole, isolated branchial sac, but travelled fastest between points connected by nerves running in straight lines and slowest when measured along zigzag, indirect routes. As with the innervation of the muscles of the body wall, histological study revealed a rich motor inner-

Fig. 15. Reflex pathways through the brain in corellids and conduction pathways in the periphery. Sensory input from primary sensory neurons located around the siphon rim and in cupular organs triggers motor output to the squirting muscles and branchial ciliated cells. After removal of the brain, impulses spread through the interconnected motor nerve terminals, providing the functional equivalent of a nerve net. Spread through these peripheral pathways both in the body wall and branchial sac involves axo-axonic transmission through curare-sensitive synapses.



vation deriving from motor neurons whose cell bodies lay in the brain, and the complete absence of a peripheral nerve net. This led to the conclusion that the motor nerves running through the branchial sac must synapse with one another peripherally, as shown schematically by Bone and Mackie (1982) and updated here in Fig. 15.

Not only do the body-wall muscles and the branchial sac both retain the ability to generate and conduct trains of impulses when isolated from the brain, they also remain in communication with one another. Ciliary arrests in the branchial sac occur in synchrony with siphonal twitches. Thus, the coordination of the two sets of effectors mediated by central pathways in the intact animal is retained by virtue of the "residual" peripheral pathways in the debrained condition. Conduction, however, is an order of magnitude slower than when the brain is present (Mackie and Wyeth 2000).

Arkett (1987) repeated the earlier work on the branchial sac and confirmed that curare blocked arrests transmitted through the branchial sac via the motor innervation, but puzzlingly, he also found that it was still possible to obtain waves of arrest in curarized preparations. These spread extremely slowly (0.8–1.0 $mm \cdot s^{-1}$) and were abolished by octanol treatment, pointing to gap junctional coupling in the conduction pathway; however, whether the conducting cells were nerves or epithelial cells was not determined. The conduction velocity in Arkett's mysterious octanol-sensitive pathway sets it sharply apart from even the slowest curare-sensitive peripheral pathway, and its nature is still unresolved.

If a true nerve net was present, or if peripheral sensory neurites were interconnected to form the equivalent of a local net, these might provide an alternative to interconnected motor nerve terminals as the basis for peripheral conduction. In a few regions of the body wall, the sensory innervation is dense enough for this to be imaginable, but sensory neurons are completely absent from many parts of the body wall and branchial sac. Sensory neurons are present in a specific region along the dorsal side of corellid branchial sacs, but they are associated exclusively with cupular or capsular organs (see above) and show no net-like interconnections. Their axons do not extend into the sac as a whole but go to the brain. The ciliary arrest responses evoked by vibration of the capsular organs in *Chelyosoma* cannot be obtained if the brain is removed from the reflex pathway, indicating that there are no local links between sensory and motor neurites in the periphery (Mackie and Singla 2003). This leaves synaptically interconnected motor nerve terminals as the only plausible alternative.

This model, in which interconnected motor nerve terminals provide the basis of the "residual" innervation that survives brain removal, presumably applies throughout the animal, both in the body wall and branchial sac, as shown

here schematically (Fig. 15). To prove conclusively that the model is correct would require ultrastructural demonstration of axo-axonic synapses between peripheral motor neurons. Axo-axonic synapses have been reported between efferent axons in the oral tentacles of botryllids (Burighel et al. 2003), but not yet in the body wall or branchial sac. The argument for the proposed arrangement (one which must be unique in the animal kingdom) rests largely on the lack of any viable alternative. The physiological findings on conduction in the peripheral pathway, notably the graded, decremental type of spread observed in the body wall, are consistent with the model. Here, we assume the axo-axonic junctions do not transmit on a one-for-one basis but require a train of impulses to transmit. Numerous synapses would have to be crossed by impulses travelling for any distance across the "net" and, without sustained stimulation, spread will remain local. Passage across the branchial sac is evidently easier, and here we may assume that some or all of the synapses transmit on a one-for-one basis.

Peripheral interconnections between motor neurons would inevitably result in some loss in the selectivity of the excitation of specific muscles and ciliary fields, but most responses seen in intact ascidians are of a fairly diffuse nature, involving unified muscle contractions or ciliary arrests over wide areas. Little directionality is observed in ascidian behaviour. It can hardly be argued that the peripheral interconnections evolved to enable the animal to function in the debrained condition, as loss of the brain without simultaneous, massive destruction of the rest of the animal would be an improbably rare event. Instead, we may suppose that the interconnections between peripheral motor terminals help in the normal, intact animal to smooth out motor responses by equalizing spread within effector subsets following the arrival of motor excitation from the brain and also to transmit certain local responses that do not involve the brain.

Coordination of tunicate colonies

In contrast to cnidarian and bryozoan colonies, the zooids in tunicate colonies are never directly interlinked by nerves. Possibly the tunic material represents a hostile environment for nerve growth, although several other sorts of cells thrive in it. Alternative modes of communication have evolved in several cases both in ascidians (see Mackie 1986, 1995*b*) and in pelagic tunicates (see Anderson 1985; Bone 1998*b*).

In *Pyrosoma*, tactile stimulation of a zooid leads to ciliary arrest as in ascidians, accompanied by a flash of light from a light organ that lies close to the branchial sac. The flash is evidently detected by the eyes of neighbours, which respond by flashing and arresting their cilia themselves. This "serial photic excitation" probably serves as an early warning device, allowing zooids at some distance from sites of noxious stimulation to close down their feeding currents in advance (Mackie and Bone 1978).

Salp chains can reverse their swimming direction when stimulated at the front while swimming forward. A signal spreads along the chain at 12 $cm \cdot s^{-1}$ in *Salpa fusiformis* Cuvier, 1804. The salp outer epithelium is excitable (Mackie and Bone 1977), responding to touch and conducting action potentials ("skin impulses"), but these do not spread directly to adjacent zooids. Impulses initiated in the skin of the leading blastozooid in a swimming chain enter the zooid's central nervous system and cause it to switch into reverse gear. At the same time the skin impulses propagate to the region where the stimulated zooid attaches to the one behind it. Here at adhesion plaques polarized in the reverse direction, impulses are picked up by sensory neurons and travel via afferent nerves to the brain of the second zooid, again causing a change in swimming direction, and so on down the chain. Chains stimulated at the rear show accelerated forward swimming and this too involves an alternation between epithelial and nervous conduction, with epithelial impulses crossing through adhesion plaques polarized in the forwards direction (Anderson and Bone 1980; Anderson et al. 1979; Bone et al. 1980). As Anderson (1985) notes, interposition of nervous elements in the pathway allows positional information to be relayed by a diffusely conducting system (the excitable epithelium) without sacrificing the economy of means achieved by using epithelia as conduction pathways.

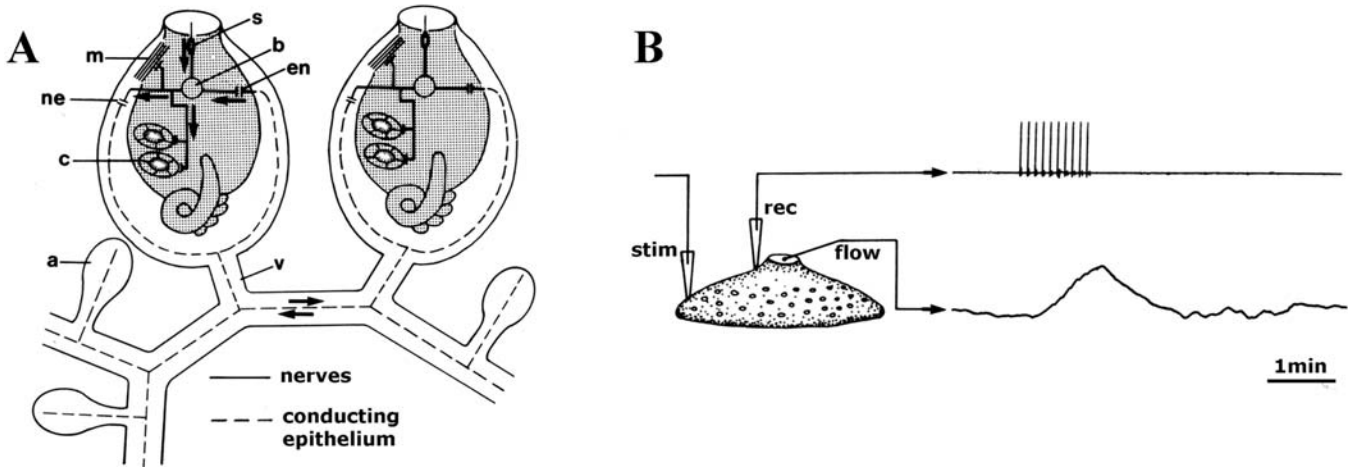
Excitable epithelia also play a role in coordination in *Botryllus* and in other colonial styelid ascidians. The zooids in these colonies are interconnected by blood vessels in which fluids are pumped to and fro by coordinated contractions of the vascular ampullae. Coordination of these contractions is achieved by propagated electric impulses conducted by epithelial cells lining the blood vessels. This signaling pathway also serves to coordinate protective behaviour of the zooids, as the epithelial impulses enter the nervous stem and activate efferent pathways that trigger squirting and ciliary arrests (Fig. 16A) (Mackie and Singla 1983).

Finally, some didemnid ascidians have evolved a novel way of controlling the degree of opening of the cloacal apertures through which the water pumped in by the zooids passes to the exterior, thereby regulating flow through the colony. The zooids themselves do not intercommunicate, but stimulation of the common tunic at any point leads to propagated electrical events that travel to the cloacal sphincter, thus causing it to contract (Fig. 16B). The tunic conducting network also generates spontaneous trains of impulses. In *Diplosoma similis* (Sluiter, 1909), a species having photosynthetic symbionts, the system is light-sensitive and the colony contracts and expands on a diurnal basis. There are no nerves in the tunic and it appears that the impulses are conducted by a network of contractile cells (myocytes) distributed throughout the entire tunic, of which the sphincter is a specialized part (Mackie and Singla 1987). Though the myocytes are not fully fledged muscle cells, the ability of the network to spread its own contractions by means of propagated, all-or-none electrical impulses is an example of myoid conduction, similar in principle to the process seen in vertebrate hearts.

Early development of the ascidian neural complex

The central nervous system of ascidian tadpole larvae shows the characteristic regionalization and expression of key developmental genes seen in the vertebrate central ner-

Fig. 16. Coordinated activities in ascidian colonies. (A) In *Botryllus*, electrical impulses propagate throughout the vascular network in the cells of the epithelial lining and coordinate contractions of the ampullae. The system also coordinates defensive responses in the zooids when they or the canals are stimulated. Impulses enter the zooid nervous systems and trigger contractions and ciliary arrests. a, ampulla; b, brain; c, ciliated cells of branchial stigmata; en, epithelio-neural transmission step; m, body-wall muscle; ne, neuro-epithelial transmission step (modified from Mackie and Singla 1983). (B) In *Diplosoma*, stimulation of the tunic with a suction electrode (stim) causes contraction of myocytes that form a network throughout the tunic and are concentrated as a sphincter around the cloacal aperture. The sphincter closes progressively, impeding water flow through the colony. The zooids continue pumping normally, so flow velocity out of the narrowed aperture increases, as shown with a flow meter (lower trace). Passage of excitation in the myocyte network was accompanied by electrical potentials that were recorded through a second suction electrode (rec) in the upper trace. The activities of the zooids are not affected by events conducted in the tunic (modified from Mackie and Singla 1987).



vous system (Corbo et al. 1997; Wada et al. 1998; Lemaire et al. 2002). The larval central nervous system consists of a hollow anterior sensory vesicle (or brain), the neck region, the visceral ganglion, and the caudal nerve cord. It gives rise to an ectodermal neurohypophyseal duct, sometimes called the hypophysis (Elwyn 1937; Satoh 1994), located anteriorly and on the left side of the sensory vesicle. Both the sensory vesicle and the neurohypophyseal duct derive from elements of the neural plate, which rolls up to form the central nervous system (Lemaire et al. 2002). The neurohypophyseal duct represents the first rudiment of the neural complex (neural gland, cerebral ganglion, and dorsal strand) of the adult ascidian. In larvae of different species it shows some variations in size and form, depending on the duration of embryonic development and of larval life. As a rule, the state of its differentiation is much less advanced in larvae of solitary forms than in colonial forms (Torrence 1983). For example, in the larva of the solitary *Ciona*, the neurohypophyseal duct is represented by a short, thin canal of about 40 cells that is embedded in the thickness of the wall of the sensory vesicle (Willey 1893; Meinertzhagen et al. 2000; Lemaire et al. 2002), whereas in the larva of the colonial *Botryllus*, it is already a conspicuous tubular structure proliferating elements for the formation of the cerebral ganglion (Fig. 17) (Manni et al. 1999).

At metamorphosis, complex transformations occur in all parts of the larval body (Cloney 1978), and these were recently described by Chiba et al. (2004) in *Ciona* as a series of nine stages starting with swimming larva and proceeding through to juveniles. The larval central nervous system regresses completely except for the neurohypophyseal duct that grows and proliferates to form the adult neural complex. During metamorphosis, the differentiation of the neurohypophyseal duct was followed by light and electron microscopy in larvae of *Botryllus* (Manni et al. 1999) and *Ciona* (Manni et al. 2004*b*). This work showed that almost all the components of the neural complex derive from the neurohypophyseal duct, but there is debate about the extent of the contribution of the stomodeal area to the formation of the aperture of the neural gland (ciliated duct) that opens into the pharynx. In *Ciona* (Fig. 18A) (Katz 1983; Nicol and Meinertzhagen 1991; Lemaire et al. 2002), *Halocynthia* (Satoh 1994), and *Molgula citrina* Alder and Hancock, 1848 (Vorontsova et al. 1997), this stomodeal area (sometimes termed pharynx) can be defined as a dorsal invagination of the ectoderm representing both the prospective mouth and the prospective prebranchial zone where the ciliated duct opens. In *Botryllus*, where such an invagination is absent, the stomodeal region includes the anterior portion of the dorsal groove and the anterior region of the pharynx (the prebranchial zone) where both the neurohypophyseal duct and the mouth open (Manni et al. 1999).

During embryonic development, the neurohypophyseal duct rapidly assumes the shape of a tube whose lumen remains in direct communication with the large sensory vesicle in species such as *Ciona* (Willey 1893; Satoh 1994), or with the ganglionic vesicle in others such as *Botryllus* (Fig. 17) (Manni et al. 1999). The latter consists of a small, left vesicle that communicates with the larger, right sensory vesicle (Sorrentino et al. 2000). Communication between the neurohypophyseal duct and the lumen of the larval nervous system is then lost, while anteriorly a new aperture is formed by contact and fusion of the tip of the neurohypophyseal duct with the stomodeal area (Willey 1893; Elwyn 1937; Manni et al. 1999). According to Elwyn (1937) in *Ecteinascidia turbinata* Herdman, 1880, the ciliated duct derives

Fig. 17. Schematic diagram of a natatory larva of *Botryllus* to show the relationship between the neurohypophyseal duct and its derivative, the cerebral ganglion, and the main larval organs. Arrowheads indicate oral and atrial siphon rudiments; arrow indicates ciliated duct rudiment (modified from Manni et al. 1999).

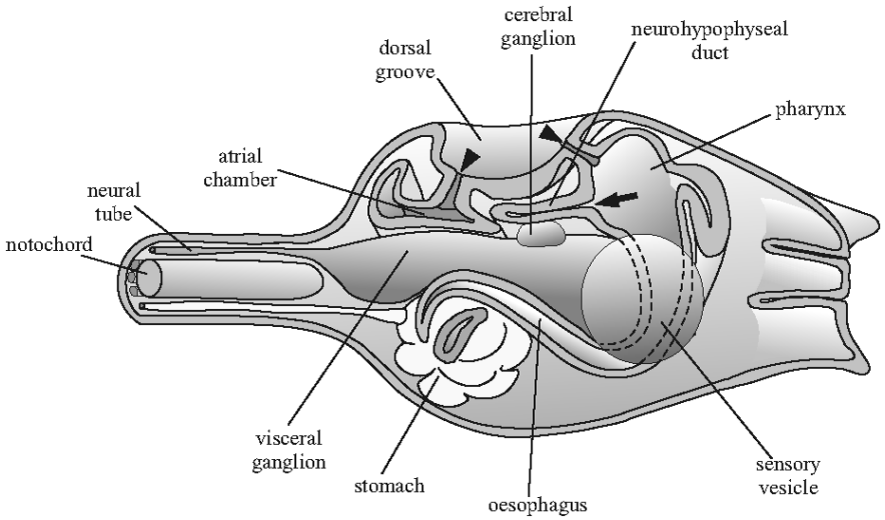
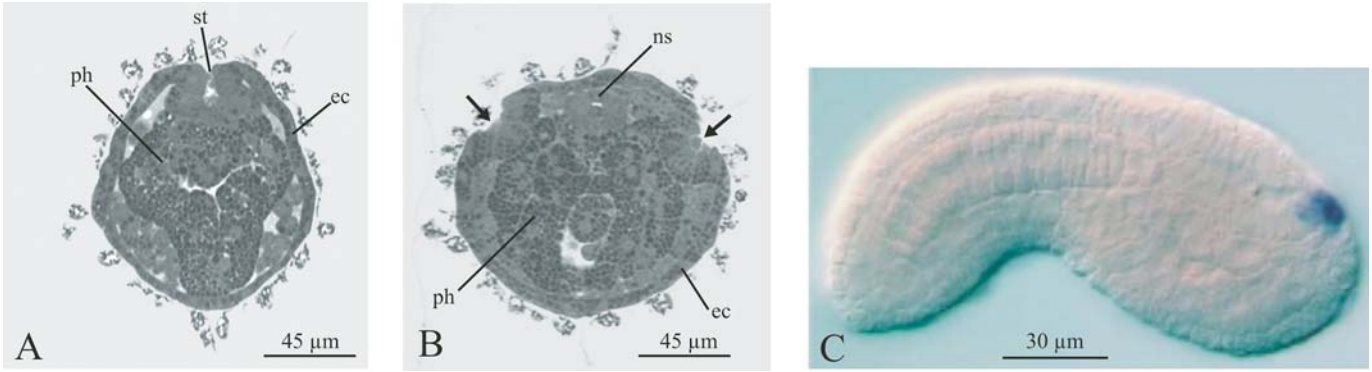


Fig. 18. *Ciona.* (A, B). Transverse sections (1 μm thick), stained with Toluidine blue, of the same larva at the levels of the stomodeal invagination (st) and of the two symmetrical atrial invaginations (arrows). ec, ectoderm; ph, pharynx; ns, larval nervous system. (C) Expression pattern of *Ci-Pitx* in an early *Ciona* embryo seen from the right side (from Christiaen et al. 2002, reproduced with permission of Gene (Amst.), Vol. 287, © 2002 Elsevier B.V.).

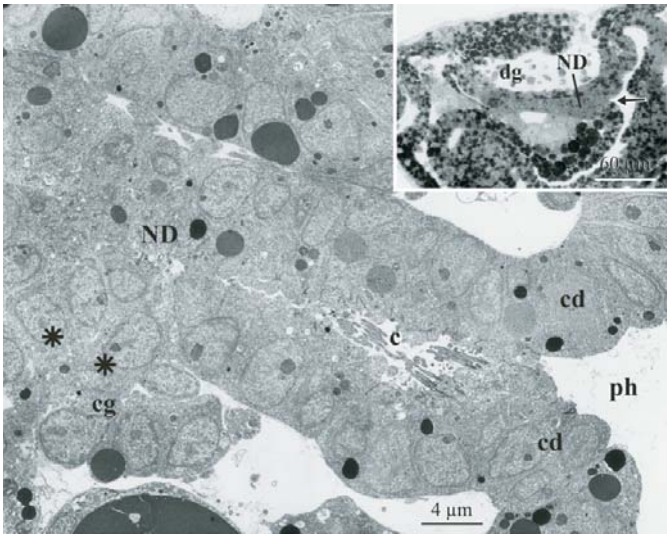


completely from the neurohypophyseal duct, whereas in *Ciona* (Willey 1893) and *Botryllus* (Manni et al. 1999), the stomodeal area evaginates and, fusing with the neurohypophyseal duct, participates in the formation of the ciliated duct (Fig. 19).

The neurohypophyseal duct differentiates into both glandular and neural elements: it forms the neural gland rudiment from the wall of which pioneer nerve cells proliferate and migrate and then converge to form the cerebral ganglion. The most posterior part of the neurohypophyseal duct extends backwards, differentiating into the dorsal strand. In enterogonids the cerebral ganglion is dorsal to the neural gland, whereas in most pleurogonids it is ventral.

The process of cell delamination has been followed in detail in *Botryllus* (Manni et al. 1999). The neural gland rudiment appears to be the site of intense cell proliferation, and the neuroblasts detach, especially from the ventral walls, to become migratory pioneer cells that converge on the ventral side of the neural gland (Fig. 20). The first elements of the juvenile cerebral ganglion start to reach their definitive position at the stage of the natatory larva. The neuroblasts exhibit cytoplasmic extensions that contain neurotubules and small dense granules. These extensions establish synaptic contacts with one another, distinguished by typical paramembranous densities and associated synaptic vesicles. During metamorphosis and in the early-juvenile stage, proliferation and migration continue for a time. Anteriorly, the neurohypophyseal duct contacts the stomodeal evagination (i.e., the rudiment of the ciliated duct) whose cells differentiate cilia and microvilli. The cilia appear early in the natatory larva in great numbers and their beating is directed toward the neural gland in contrast to the more external cilia, which beat toward the pharynx. Only a few, poorly differentiated cilia are present in the gland itself. The neural gland is not

Fig. 19. Natatory larva of *Botryllus*. Electron micrograph of the anterior region of the neurohypophyseal duct (ND) with the ciliated duct (cd) opening into the pharynx (ph). Several pioneer cells (*) are detaching from its ventral wall to form the rudiment of the cerebral ganglion (cg). Cilia (c) are differentiating from the neurohypophyseal duct in the lumen of the neural gland. Inset: thick section of the same larva showing the area of ND extension. The arrow indicates the ciliated duct aperture. dg, dorsal groove.

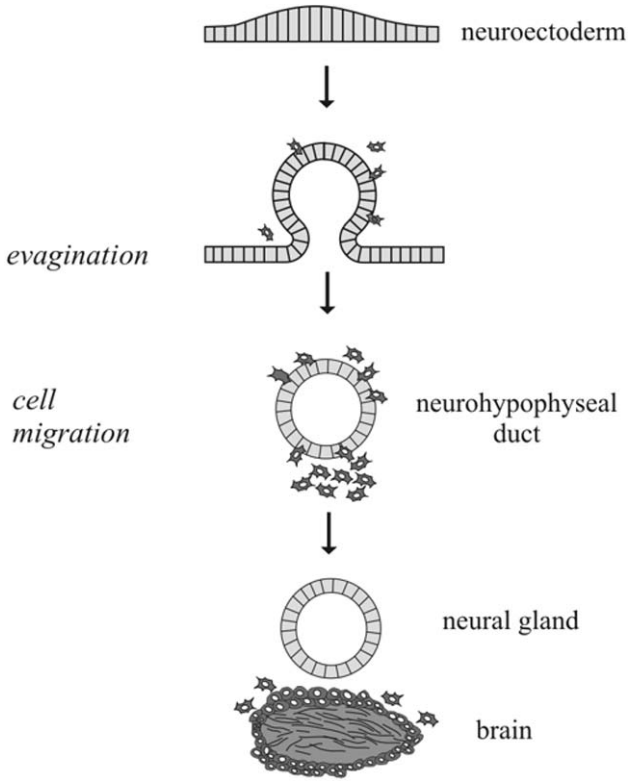


innervated and its cells show no signs of neurosecretion. The cells possess a well-developed Golgi complex and numerous vacuoles.

In the cerebral ganglion the cortical layer containing the nuclei, and the medulla, which is composed of densely packed neurites, are progressively elaborated. Nerves grow out, connecting the cerebral ganglion to organs throughout the oozooid. It has not been determined whether the peripheral nervous system of the oozooid is formed completely de novo from the cerebral ganglion, or if some elements of the larval nervous system, running among the prospective juvenile organs, are recruited during metamorphosis and participate in the new innervation pattern.

There has been much discussion of the idea that the neural gland may be homologous to the pituitary of vertebrates as proposed more than a century ago by Julin (1881). This problem has recently received renewed attention because of the hypothesis that structures equivalent to the vertebrate neural crest and placodes are present in invertebrate chordates (amphioxus and tunicates). Today, a considerable amount of data from electron microscopy and developmental gene expression studies points to the presence of populations of cells with some of the properties of placodes in these animals, and by extension in ancestral chordates (Baker and Bronner-Fraser 1997; Burighel et al. 1998, 2003; Manni et al. 1999, 2001; Graham and Begbie 2000; Shimeld and Holland 2000; Holland and Holland 2001; Boorman and Shimeld 2002; Christiaen et al. 2002). The presence of possible placodes in ascidians was recently reconsidered by Manni et al. (2004*b*), when they analysed the embryos/larvae of a solitary (*Ciona*) and a compound (*Botryllus*) ascidian.

Fig. 20. Scheme showing formation of the neurohypophyseal duct from neuroectoderm. The neurohypophyseal duct gives rise to the neural gland and also, by means of migrating neuroblasts, to the cerebral ganglion (modified from Manni et al. 2001).



These authors recognize four placodal structures: the rostral, stomodeal, neurohypophyseal, and atrial placodes. The rostral placode produces the sensory papillae and may be homologous to the placodes of the adhesive gland of vertebrates. The other three also have possible vertebrate homologs as we will now describe.

Stomodeal placode

The stomodeal area defined above can be considered a placode on the basis of its embryonic origin, morphology, and derivatives. This area is the site of origin of two important structures: the mouth with its accessory elements (velum and tentacles) and the ciliated duct of the neural gland. Both the rudiments of the oral velum and the tentacles, with associated sensory elements, and the ciliated duct have features suggesting that they are placodal derivatives. In particular, the ciliated duct rudiment is a possible homologue of Rathke's pouch in vertebrates and Hatschek's pit in amphioxus (Manni et al. 1999; Gorbman 1995). Molecular data reinforce this hypothesis: in *Ciona*, a gene homologous to the vertebrate panhypophyseal *Pituitary homeobox* (*Pitx*) gene is expressed in the anterior neural ridge (Fig. 18C) (Boorman and Shimeld 2002; Christiaen et al. 2002). In vertebrates, this same area represents the presumptive adenohypophysis whose cells migrate to form Rathke's pouch (Kawamura et al. 2002; Whitlock et al. 2003). In *Ciona*, the gene is expressed in the stomodeum and later in the ciliated duct of the neural gland of the adult (Boorman and Shimeld 2002; Christiaen et al. 2002).

The stomodeal area forming the velum and tentacles of the botryllid oral siphon could be a placodal structure because it has the ability to differentiate the secondary sensory cells of the coronal organ, a possible homologue of the vertebrate lateral-line system, and hence of other components of the acustico-lateralis system (Burighel et al. 2003; Manni et al. 2004*a*). This homology is based on morphological and molecular evidence. A considerable body of evidence regarding both the cytoarchitecture of the sensory cells and the gross anatomy of the coronal organ support the idea. Vertebrate hair cells and coronal cells are both equipped with an eccentric cilium and form afferent and efferent synapses with neurites connecting them to the brain (see above, p. 160). In some ascidians the coronal cells resemble classical hair cells with a bundle of stereovilli (microvilli) graded in length from one side to the other (Manni et al. 2004*a*). In several aquatic vertebrates the lateral-line elements extend up to and around the mouth (Coombs et al. 1988), and the coronal organ, although extending into the mouth, lies externally to the oropharyngeal border (the floor of the stomodeum/pharynx) and anteriorly to the opening of the ciliated duct. From a molecular point of view, the ascidian stomodeum expresses the developmental *Pax 2/5/8* gene (Wada et al. 1998), which marks the otic placode in vertebrates. In general, it is noteworthy that in vertebrates much of the embryonic ectoderm, including the most anterior portion, is competent to generate an otic placode if taken at an early stage (Gallagher et al. 1996; Groves and Bronner-Fraser 2000). Thus, it appears that the ancestor of chordates had a broadly diffuse capacity to form otic derivatives, in particular secondary mechanoreceptor cells, and that this capacity became restricted to the posterior epiblast of the head in vertebrates, while it was maintained in the anterior ectoderm in tunicates (Burighel et al. 2003).

Neurohypophyseal placode

The neurohypophyseal duct, because of its origin from neuroectoderm of the anterior neural plate, was regarded as the homologue of the hypothalamus and olfactory/adenohypophyseal placode of vertebrates (Manni et al. 1999). During ascidian development, the presence of the adenohypophyseal placode is recognizable in a stomodeal area that expresses the *Pitx* gene, but this gene is also expressed in a small domain in the anterior neural ridge of the early *Ciona* embryo (Fig. 18C) (Christiaen et al. 2002) (see above). In vertebrates, the olfactory placode develops adjacent to the adenohypophyseal placode and, early in development, both of them express *Pitx1*. This expression is later excluded from the olfactory placode (Lanctot et al. 1997). These placodes are grouped together with the hypothalamus in the anterior neural plate before reaching their final destinations (Couly and Le Douarin 1985; Kawamura and Kikuyama 1992; Murakami et al. 1992). This parallels the situation in ascidians, where Manni et al. (1999), taking account of evidence from study of cell lineages and embryonic origin as well as of the adult derivatives, recognized a group of structures homologous to the vertebrate hypothalamus and adenohypophyseal/olfactory placodes. At the moment, the exact extent and relationships of these components in ascidians are not firmly established, but *Pitx* expression provides a primary marker for distinguishing these areas in the early embryo. Boorman and Shimeld (2002) suggested that in early embryos of *Ciona* the domain expressing *Pitx* included the homologues of both the adenohypophyseal and olfactory placodes. It may be considered that this domain later divides into the adenohypophyseal component, which maintains *Pitx* expression, and the olfactory one that loses it, as in vertebrates (Lanctot et al. 1997), and is incorporated within the neurohypophyseal duct primordium.

The neurohypophyseal duct gives rise to a number of cell types (see Manni et al. 1999; Kawamura et al. 2002): the brain; the epithelio-mesenchymatous cells of the neural gland; the epithelial cells of the dorsal strand; and possibly the neuropeptide-secreting cells of the dorsal strand plexus. The presence of GnRH-ir cells in ascidians was first thought to provide strong evidence of a homology between the ascidian neurohypophyseal duct and the vertebrate olfactory placode, from which GnRH cells migrate into the developing brain (Manni et al. 1999). Recent data show that in zebrafish (Whitlock et al. 2003) the olfactory placode derives from a field of scattered cells before aggregating to form a discrete region and that two populations of GnRH cells (neuromodulatory and endocrine) originate from two, separate, nonolfactory regions at the border of the anterior neural plate. In ascidians there are various forms of GnRH molecules whose functions are still unclear. It seems that the cells which later express GnRH all originate in the anterior neural plate, possibly from different GnRH-cell populations. They migrate into the neurohypophyseal duct and then move into or around the brain and form the dorsal strand plexus.

In conclusion, much of the evidence covered above supports the existence of protochordate homologues of the vertebrate pituitary. The homologies are difficult to establish simply on the basis of adult structure, but other evidence strengthens them considerably. In amphioxus, a number of immunohistochemical, molecular (*Pitx* expression), and embryological data, as well as the anatomical relationships, support the homology of Hatschek's pit with the vertebrate pituitary (Gorbman 1995; Candiani and Pestarino 1998*a*, 1998*b*; Yasui et al. 2000; Boorman and Shimeld 2002). In tunicates, there appears to be a direct homology between the adenohypophysis of vertebrates and the ciliated duct of ascidians, which is based on *Pitx* expression and embryological evidence. The homologies of the neurohypophysis are rather harder to establish. The evidence suggests that the neurohypophyseal duct contains homologues of the hypothalamic rudiment of vertebrates (hence also of the neurohypophysis) and that these elements participate in the formation of derived structures (i.e., the ganglion, the neural gland, and the dorsal strand with its associated nerve plexus). It is possible that the vertebrate pituitary gland evolved from a chemoreceptive olfactory structure whose sensory receptive function was taken over by the adjacent nervous system (Nozaki and Gorbman 1992; Gorbman 1995). The primordial olfactory structure would have had a gonad-regulating role in the ancestral condition and its present-day derivatives — the neural gland of ascidians and Hatschek's pit of cephalochordates — could still play a part in sensing environmental factors that trigger reproductive events (see above, p. 161).

Atrial placode

The possibility that the atrial primordia of *Ciona* are ho-

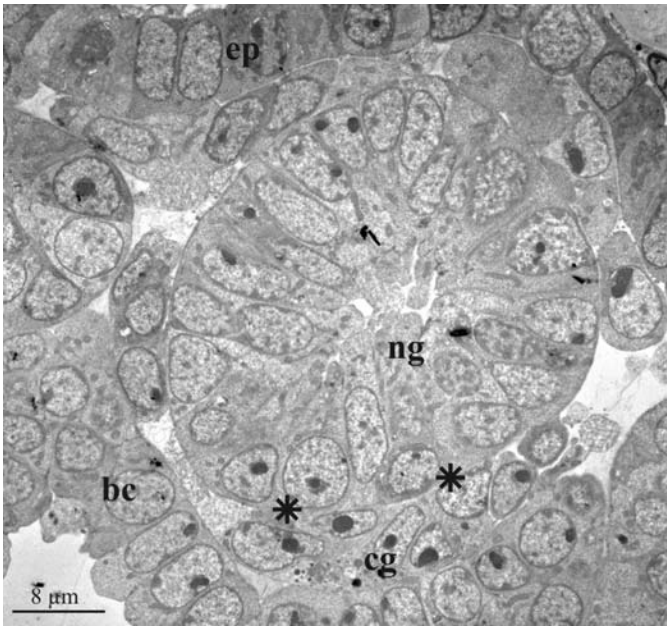
mologous to the otic placode of vertebrates was proposed by Katz (1983) on the basis of the topological relationship of the two atrial invaginations and the brain and the presence in the atrial chamber of cupular organs, which are reminiscent of neuromasts (Fedele 1923; Bone and Ryan 1978). The homology was further elaborated by other authors taking into account palaeontological evidence (Jefferies 2001) and data from molecular biology (Baker and Bronner-Fraser 1997; Wada et al. 1998; Shimeld and Holland 2000). Doubts have recently been raised about the proposed homology between cupular sensory cells, which are primary sensory neurons, and vertebrate hair cells, which are secondary sensory cells (Mackie and Singla 2003). The finding of new hydrodynamic sense organs in the atrium of ascidians (the capsular organs and the cupular strand, which are also based on primary sensory neurons) suggests that these animals have a broad ability to differentiate mechanoreceptors in the atrium. In any event, the embryological origin (Fig. 18B), structure and morphogenetic capability of the paired atrial rudiments qualify them for consideration as placodes homologous with the vertebrate otic placodes, and this should be further investigated.

Formation of the adult ascidian innervation

Formation of the nervous system during asexual reproduction

The process of development of the neural complex has been followed in detail during the vegetative reproduction of the colonial ascidian *Botryllus* (Burighel et al. 1998). In this ascidian, the bud arises from the atrial mantle of the parent giving rise to a new zooid with its own neural complex. Early in bud formation, an evagination of the dorsal mantle wall produces a blind tubular structure, the dorsal tube, which as in the case of the neurohypophyseal duct in the embryo opens into the prospective prebranchial region. As in the embryo, the cerebral ganglion is formed in the bud by pioneer cells, which detach from the walls of the dorsal tube to reach their final position beneath the neural gland (Fig. 21). In the blastozooid, the cerebral ganglion assumes the same form as in the oozooid, with an outer cortex of nerve cell bodies and an internal medulla consisting of a neuropile of neuronal processes making classical synaptic contacts with one another. The dorsal tube soon shows the characteristics of the rudiment of the neural gland, from the wall of which neuroblasts continue to delaminate (Fig. 21). Contact is maintained for a lengthy period between the gland and brain rudiments. The gland differentiates into the ciliated duct that opens into the branchial chamber, the body of the neural gland, and the dorsal organ, as in the juvenile. Thus, a number of corresponding events are apparent during development of the neural complex in the oozooid and in the blastozooid (Fig. 22) (Manni et al. 1999). The similarity of the two processes suggests that a similar process of gene activation and cell patterning regulates neurogenesis in both cases. *Botryllus* expresses a *Pitx* (*Bs-pitx*) gene whose pattern of expression during embryogenesis and blastogenesis shows a correspondence of the expression domains and their fates (Tiozzo et al. 2005): in both developmental pathways, *Bs-pitx* is expressed in specific rudimental regions (ciliated duct, cerebral ganglion, and stomodeum) that acquire corresponding neurogenic potentiality.

Fig. 21. Detail of a bud of *Botryllus*. The thin section passes transverally through the rudiment of the neural gland (ng), which is derived from the dorsal tube. Pioneer cells (*) migrate from its wall and start to form the cerebral ganglion (cg). bc, branchial chamber wall; ep, epidermis (modified from Burighel et al. 1998).

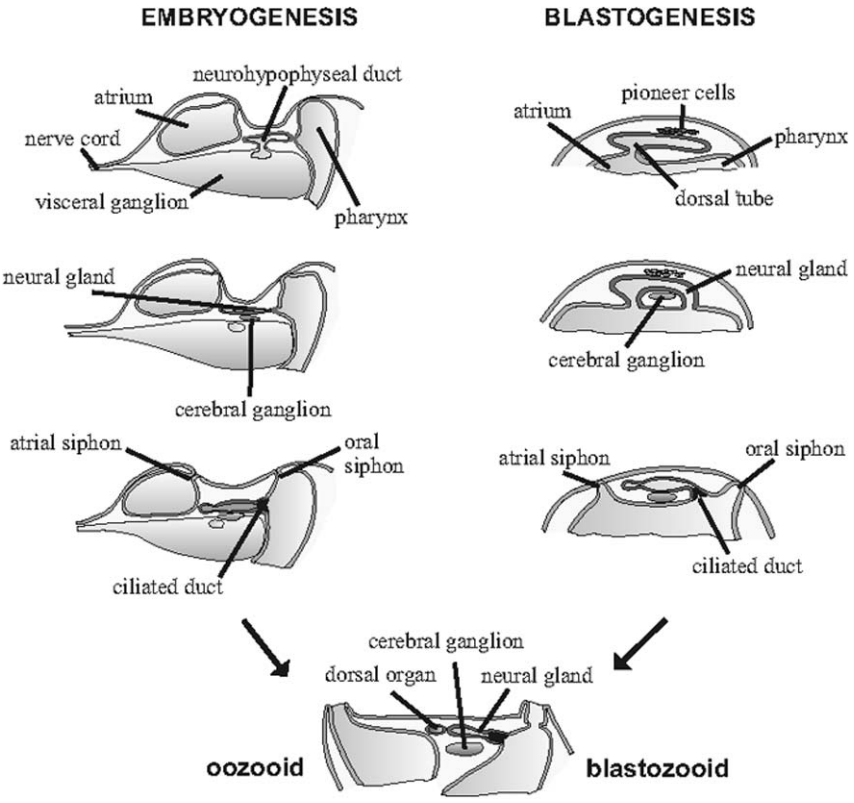


Formation of the peripheral nervous system

The establishment of the adult pattern of peripheral innervation has received little attention. One detailed paper (Zaniolo et al. 2002) is devoted to the development of the motor nervous system in blastozooids of *Botryllus*, but there are few comparable observations on juveniles of solitary and compound ascidians. In particular, it is not known if neurons can be recruited from the larval nervous system to participate in the formation of the juvenile nervous system. Markman (1958) gave a brief account of the development of the peripheral nervous system in living, postmetamorphic individuals of *Ciona*, based on phase-contrast microscopy, and giving a general picture of the pattern of nerves running out from the anterior and posterior roots of the brain. The study by Zaniolo et al. (2002) used the reaction product of acetylcholine esterase histochemistry, which labels entire nerves including their thin terminals, making them identifiable within the tissues by both optical and electron microscopies (Fig. 23A). The blastozooid nervous system consists of neurons in the cerebral ganglion whose axons run out directly to effectors, i.e., muscular and ciliated cells (Fig. 11A). Other fibres having somata in the ganglion travel as efferent nerves to the hair cells of the coronal organ and may modulate the responsivity of the latter (Burighel et al. 2003).

The nerves start to grow out early in development, just at the time when the main organs are forming. In general, it seems that the innervation is established as in other invertebrates and vertebrates: immature neurons extend their axons toward the target, forming fascicles, and synapsing with specific target cells. In *Botryllus* there is a strict temporal rela-

Fig. 22. Scheme comparing neural complex development during embryogenesis and blastogenesis in *Botryllus*. Despite profound differences in the starting points of the two pathways, similar zooids are produced, and the cerebral ganglion is formed through comparable mechanisms of pioneer cell proliferation and migration from the rudiment of the neural gland (modified from Manni et al. 1999).



tionship between the outgrowth of nerves and the growth of the target organs, but the innervation is well advanced before the effector cells are fully differentiated. Initially, it seems that the target tissue is not involved in axon guidance and that the axons elongate following stereotypical pathways to reach the target. This applies to the innervation of the oral and cloacal siphons, branchial basket, gut, and heart. For example, before the primordia of the branchial stigmata are recognizable, the innervation of the branchial basket is confined to nerves running along the dorsal and ventral sides (the visceral and subendostylar nerves); the nerves then subdivide into branches that run out into the developing interstigmatic blood vessels. Eventually, these branches subdivide further and extend among the stigmatal rudiments. Compared with other organs, the heart becomes functional and acquires its pericardial innervation rather precociously.

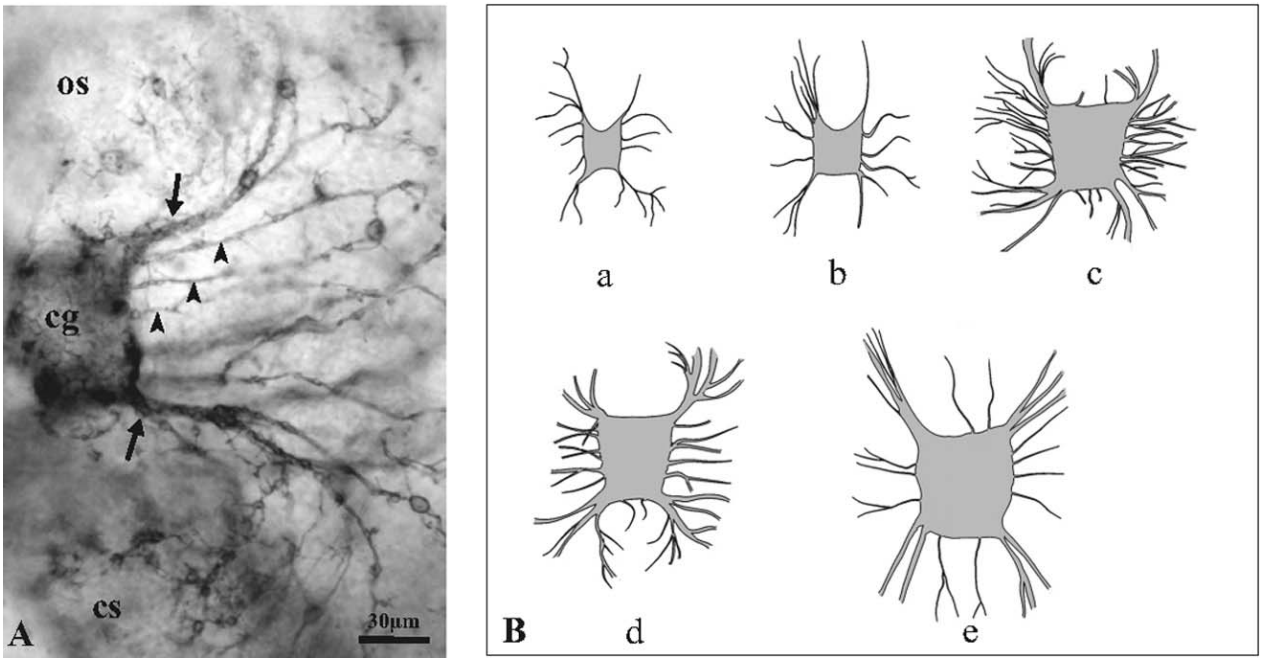
Growing axons can penetrate the extracellular matrix and arrive at their targets from different directions. In some cases, the axons appear to use blood sinuses as their preferred routes, navigating by what may be contact guidance or stereotropism. Substrate factors associated with the extracellular matrix also probably play a role in axon guidance as in other systems (Goodman and Shatz 1993), but this has not been investigated experimentally. The innervation undergoes progressive remodelling: at first, few fibres emerge from ganglion, but then they increase in number and thickness (Fig. 23). Finally, in the last stages of blastogenesis, a marked process of elimination of axons is apparent. No signs of apoptosis were observed by light and electron microscopies, and the final patterning of the motor innervation seems to be achieved mainly by axon withdrawal.

Neurogenesis during regeneration

The adults of several ascidians have the unusual ability to survive for long periods — over 1 year in *Chelyosoma* (Hisaw et al. 1996) — after extirpation of the brain, and to maintain spontaneous activity and responses to stimulation in the brainless state. Spawning occurs on a seasonal basis as in normal animals. Other species, such as the widely studied *Ciona*, have the ability to regenerate the ablated neural complex completely within a few weeks. This phenomenon, reported many years ago (Schultze 1900) and initially studied in the context of the regenerative capacities of ascidians (for review see Berrill 1951), was more recently investigated in *Ciona* by Lender and Bouchard-Madrelle (1964) and by Bollner and collaborators (Bollner et al. 1992, 1993*a*, 1993*b*, 1995, 1997; Thorndyke et al. 2001) to determine what cells were involved in the formation of the new organs and how they differentiate into neurons.

The regenerated neural complex, although smaller than the original, resembles it in all essential respects. Interestingly, a series of immunological studies with antibodies directed against selected neuropeptides and other neural products showed that the normal pattern of distribution of a variety of molecules was restored in the regenerated brain. In particular, these neurons expressed transmitters or modu-

Fig. 23. (A) AChE reaction on a whole mount bud of *Botryllus*. The main anterior and posterior nerves (arrows) are shown arising from the right side of the cerebral ganglion (cg). Arrowheads, lateral nerves; cs, cloacal siphon; os, oral siphon. (B) Schematic drawing illustrating different stages of development of the cerebral ganglion and its main nerves from the young bud (*a*) to the adult (*e*). Anterior is up. (Modified from Zaniolo et al. 2002.)

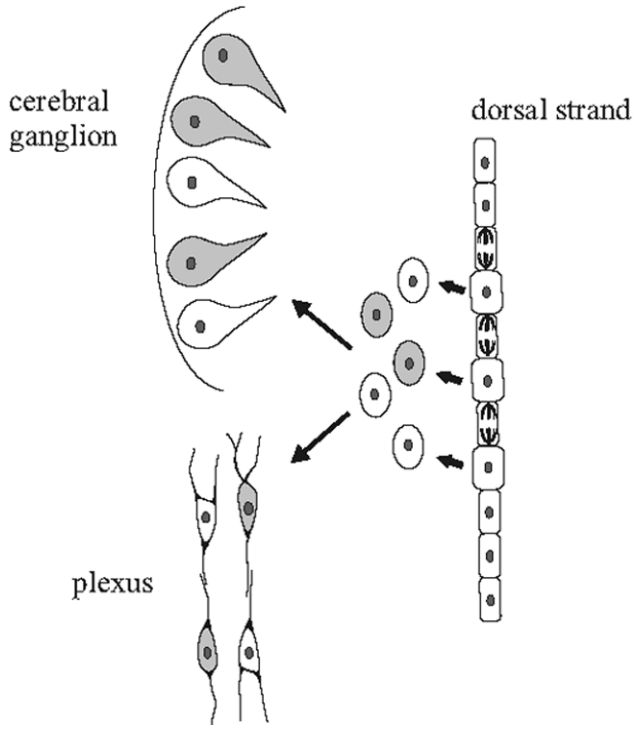


lators such as CCK, substance P, GnRH, and GABA (Bollner et al. 1992, 1993*a*, 1993*b*, 1995, 1997). The origins of the new neurons have not yet been completely established, but a series of data suggest that the ciliated duct, the dorsal strand, and haemoblasts are sources of cells present in the regenerating region. However, differentiation and transdifferentiation occur during regeneration (Brien 1933; Lender and Bouchard-Madrelle 1964; Bollner et al. 1993*a*, 1993*b*, 1995). Experiments with BrdU, along with immunohistochemical tests by Bollner and collaborators, suggest that the new neurons derive from at least two populations of progenitor cells. Some cells do not show incorporation of BrdU, indicating that they underwent their last mitotic division prior to the ablation, but other cells are labelled by BrdU, and hence were clearly born in response to the ablation (Bollner et al. 1995). The former consist of large neurons localized in the cortical rind of the ganglion. They presumably derive from circulating pluripotent haemocytes that accumulate in the wound healing zone (Bollner et al. 1993*a*, 1993*b*). The latter consist of neurons scattered throughout the ganglion and there is some evidence that they originate from the dorsal strand (Lender and Bouchard-Madrelle 1964; Bollner 1993*a*, 1993*b*, 1995). In particular, Bollner et al. (1997) showed by double-labelling with BrdU and anti-GnRH that GnRH-ir neurons appeared early on (2 days after ablation) and then increased in the regenerating brain. These cells appeared to derive by delamination and migration from the stem cells of the dorsal strand, which shows signs of proliferation (Fedele 1937; Bollner et al. 1997). The dorsal strand may also be the source of the GnRH-ir neurons of the dorsal strand plexus (Mackie 1995*a*). Thus, Bollner et al. (1997) proposed that the precursors of GnRH-containing neuroblasts, recruited for both the dorsal strand plexus and the regenerating brain, delaminated from the dorsal strand epithelium, which replaces them by mitosis (Fig. 24). This proposal is of particular interest because it supports the idea that the basic mechanisms of proliferation, delamination, and migration of neuroblasts operate in production of nervous structures not only during embryogenesis and blastogenesis but also during regeneration. Moreover, it strongly supports the view that ascidians possess epithelial domains derived from neural ectoderm, of which the dorsal strand is an example, that have some properties in common with vertebrate placodes and neural crest.

Neural development in salps

Information regarding development of the nervous system in pyrosomes and doliolids is old or fragmentary, but salps have received detailed attention recently, with an electron microscope study of the development of *Thalia democratica* by Lacalli and Holland (1998). These animals show an alternation of generations in which the sexually produced individual (oozooid or the solitary stage) develops within the parent body, being nourished through a placenta, and then reproduces asexually by budding a chain of blastozooids. Each blastozooid becomes sexually mature and produces a single egg that is fertilized and develops into a new oozooid. The development of the salp embryo shows what are probably derived features: lack of typical gastrulation and neurulation and lack of a free-swimming chordate-like larva. Instead, the blastomeres coalesce to form the rudiments of the oozooid organs directly. The early rudiment of the central nervous system appears as a solid mass of cells above

Fig. 24. Pluripotent cells delaminate from the dorsal strand to form GnRH-like immunoreactive neurons for brain regeneration and for the dorsal strand plexus. Delaminated cells are replaced by mitosis in the dorsal strand (modified from Bollner et al. 1997).



the anterior part of the pharynx. A neural canal is then formed in the rudiment, which becomes a sort of "neural tube", whose wall extends anteriorly to contact and fuse with the pharyngeal epithelium to form the rudiment of the ciliated duct that opens into the pharynx. The ciliated duct cells derive from the neural tube wall, but a possible contribution from the pharyngeal epithelium cannot be excluded. The lumen of the ciliated duct connects secondarily with that of the neural canal so that the latter for a time communicates with the pharynx. The neural canal then becomes progressively obliterated by nerve fibers. A typical neural gland is absent in the mature solitary stage.

The early neural tube of salps never acquires features comparable with those of the neural tube of the other chordates, but grows and differentiates to form the dorsal ganglion of the oozooid. Thus, it can be better compared with the ascidian neurohypophyseal duct. Development progresses through the following phases: (*i*) formation of a thick dorsal mantle of neuroblasts from which two paired dorso-lateral (paraxial) neuropiles, running parallel to the ganglion axis, arise; (*ii*) formation of a central fibrous neuropile obliterating the central canal; the paraxial neuropiles do not link with each other, instead they link with the central neuropile; (*iii*) differentiation of three paired clusters of primary motor neurons along the ventral margin of the mantle. These clusters differentiate around the equatorial margin of the mantle and are called C1, C2, and C3, going from front to back, respectively. The motor neurons radiate from them to form the series of peripheral nerves running out into the body and going in particular to the muscle bands. The axons emerging from C1 and C2 mainly innervate the muscle bands lying rostral and lateral to the ganglion, whereas those emerging from C3 run directly to the posterior region of the body (Fig. 25A).

According to Lacalli and Holland (1998), although the salp ganglion shows no sign of being subdivided along the anteroposterior axis and is severely truncated in comparison with more typical neural tubes, it shows patterns of dorsoventral differentiation that resemble those found in nerve cords of more advanced chordates. The dorsal paraxial centres resemble the dorsolateral tectal centres in amphioxus in both position and organization, and the central neuropile resembles the translumenal system of amphioxus.

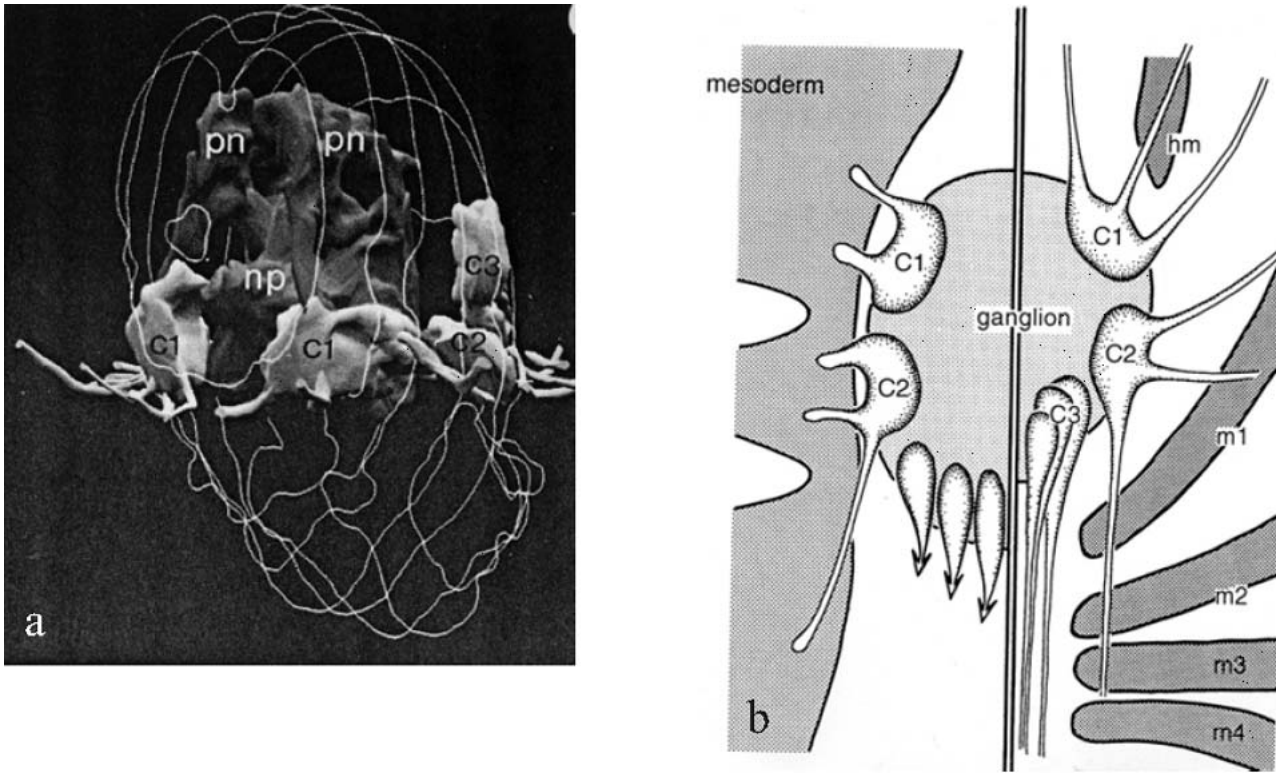
Lacalli and Holland (1998) have tried to clarify the process of patterning and registering of nerves and musculature during development (Fig. 25B). The muscle bands develop close to the neural tube from an initially continuous sheet of lateral mesoderm (Berrill 1950), which then becomes segmented into distinct bands. A reciprocal shifting of the neural and muscular components occurs: the ganglion shortens posteriorly, while the muscle bands shift backwards as the body elongates. Direct contact between neuroblasts of the anterior clusters (C1 and C2) and contiguous muscle appears necessary for registering and development of anterior nerves. In contrast, the caudal nerves of the third cluster (C3) responsible for innervation of more distant, posterior targets develop without such contacts. This suggests that a different patterning mechanism is employed in different regions of the neuromuscular system. In the early embryos examined, Lacalli and Holland did not encounter specific stages of eye differentiation. The cerebral eye develops from a dorsal rudiment above the paraxial neuropiles, but it forms after the paraxial and central neuropiles have formed.

At the moment nothing is known about developmental genes in thaliaceans. In general, it appears that the formation of the adult central nervous system is abbreviated in comparison with that of ascidians, and no typical neural tube with a larval brain is ever developed. Viviparity and direct development are presumably advantageous in allowing the embryo to rapidly produce the adult organs. The early central nervous system rudiment, the "neural tube", appears to be homologous to the ascidian neurohypophyseal duct. Both structures have neurogenic potential and give rise to the ganglion of the adult. Although a typical neural gland is not present in salps, a ciliated duct is formed and it forms in a way reminiscent of the situation in ascidians (see above).

Concluding remarks

In 1982, when the last comprehensive review of this field appeared, it could not have been foreseen that understanding of early-tunicate development and of the homologies linking tunicate and higher chordate nervous systems would be so dramatically transformed by the application of molecular biological techniques, in particular gene-expression studies. The analysis is far from complete and a more complete picture of the place of tunicates in chordate evolution can be expected within the next decade, including a better understanding of neural crest, placode, and pituitary homologies. Now that the *Ciona* genome has been sequenced, molecular approaches are likely to bear fruit in many other areas. Just

Fig. 25. (A) *Thalia democratica* (Thaliacea). Reconstruction of a developing ganglion in oblique frontal view. Peripheral nerves arise from three paired clusters of motor neurons (C1–C3). The central neuropile (np) and the two paired, dorsal paraxial neuropiles (pn) are indicated. (B) Diagram to show the proposed nerve–muscle register changes during development of oozooid. At first (on the left) the developing muscle bands approach the anterior and middle part of the neural tube. Then, the muscle bands are displaced and the nerves are towed along with the muscles. Compare this figure with the adult pattern of muscle and nerve arrangement reproduced in Fig. 1A (from Lacalli and Holland 1998, reproduced with permission of Philos. Trans. R. Soc. Lond. B Biol. Sci., Vol. 356, © 2001 The Royal Society).



as this paper was going to press, a report appeared in *Nature* (Jeffery et al. 2004) demonstrating migration of pigment cells from the neural tube to destinations in the body wall and siphons. This process is reminiscent of the migration of neural crest cells, and the cells do indeed express gene markers for vertebrate neural crest. We cannot predict where advances will come, but some areas of tunicate neurobiology seem to be particularly ripe for further research.

Immunocytochemistry continues to provide valuable clues regarding the presence and origins of chordate regulatory peptides. This work, however, has only rarely been matched with new physiological work on the functions of the various peptides shown to be present. Thus, we have long lists of peptides and very little information about what they are doing in the animal.

Recent studies of sense organs have shown that, though they lack eyes, adult ascidians have photosensitive neurons in the brain (and possibly elsewhere). Cells containing GnRH occur very close to the photoreceptor cells. Photic regulation of reproductive activities is well documented in ascidians, and regulation of these activities is known to be mediated by GnRH. Finally, GnRH receptors are present in the gonoducts. All we need to complete the picture is evidence of a direct functional link between the photoreceptors and the GnRH-containing neurons.

Several new hydrodynamic sense organs have recently come to light. One of these, the capsular organ, is extraordinarily sensitive to vibration. The functions of others (cupular and coronal organs) are less clear, but they may function in sensing water-flow variations or the presence of particulates in the incurrent stream. Coronal organs are the closest thing yet found in tunicates to an evolutionary starting point for the vertebrate acustico-lateralis system, and physiological analysis of their function will be of particular interest. Equally intriguing is the evidence reviewed here that the coronal organ derives embryologically from a cell population showing the characteristics of a neurogenic placode, probably homologous to vertebrate acustico-lateralis placodes. Thus, neurogenic placodes and the acustico-lateralis system may both have originated in protochordates (Manni et al. 2004*a*).

One of the most curious features of ascidians is that they have the functional equivalent of a peripheral nerve net, capable of generating local activity and mediating simple reflexes that continue after brain removal. We have summarized the considerable body of evidence suggesting that the "nerve net" is not a separate system of peripheral neurons but that it consists of the terminals of the motor neurons (whose cell bodies lie in the brain) interlinked synaptically in the periphery. This arrangement is unique in the animal kingdom. If we have understood the situation correctly, a motor neuron can therefore both innervate effectors in con-

ventional, centrally mediated reflexes (when the brain is present) and at the same time its terminals can link with others to provide a peripheral network coordinating local activities on a semi-autonomous basis. The existence of the "nerve net" is most obvious after brain removal, but presumably the net also functions in the normal animal and evolved as an adaptation of normal animals with intact brains. It would be of considerable interest from the viewpoint of comparative neurobiology to gain a better understanding of the role of this "nerve net" in normal ascidian behaviour.

Finally, ascidians are increasingly being raised in Japan and elsewhere for human consumption. As such, they have a commercial importance that is likely to lead to new research not only in aquaculture techniques, nutrition, etc., but on many other aspects of their biology and behaviour. This is already apparent in some of the work dealing with squirting behaviour and pumping rates covered in this review. Like bivalve molluscs, which have also benefitted from aquaculture-oriented research, ascidians are filter feeders. The feeding mechanisms of the two groups show interesting parallels. It now seems likely, for instance, that the branchial cilia creating the ascidian feeding current are capable not only of arrests but of varying their beating rate. This may mean that here, as in bivalves, the cilia are under excitatory as well as inhibitory nervous control. If so, the motor innervation may be a good deal more sophisticated than previously suspected.

Acknowledgements

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