Morphological and gene expression similarities suggest that the ascidian neural gland may be osmoregulatory and homologous to vertebrate peri-ventricular organs

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Summary
The central nervous system (cerebral ganglion) of adult ascidians is linked to the neural gland complex (NGC), which consists of a dorsal tubercle, a ciliated duct and a neural gland. The function of the NGC has been the subject of much debate. The recent publication of the complete genomic sequence of Ciona intestinalis provides new opportunities to examine the presence and distribution of protein families in this basal chordate. We focus here on the ascidian neuropeptide G-protein-coupled receptors (GPCRs), the vertebrate homologues of which are involved in homeostasis. In situ hybridization revealed that five Ciona GPCRs [vasopressin receptor, somatostatin receptor, CRH (corticotropin-releasing hormone) receptor, angiotensin receptor and tachykinin receptor] are expressed in the NGC of adult ascidians. These findings, together with histological and ultrastructural data, provide evidence to support a role for the ascidian NGC in maintaining ionic homeostasis. We further speculate about the potential similarities between the ascidian NGC and the vertebrate choroid plexus, a neural peri-ventricular organ.

Introduction
Acsidians belong to the phylum Chordata, and probably represent the closest living relatives of vertebrates (Philippe et al., 2005). Recent studies on several ascidians (e.g. Ciona and Halocynthia) and larvaceans (Oikopleura) have mostly focused on developmental genetics. However, the rapid progress made in this area has not been matched by similar advances in physiological studies. One key unresolved question in the comparative endocrinology of chordates concerns the structures involved in hormonal regulation of homeostasis in ascidians, and whether these structures are homologous to the endocrine glands of vertebrates.

We investigated whether ascidians have structures homologous to the cell systems controlling hydromineral equilibrium in vertebrates (Ruppert, 1990). Hydromineral equilibrium is of particular importance for these soft-bodied sessile animals, which live in intertidal zones in which salt concentration varies considerably. Acsidians display broad salt tolerance (from 20 to 40 g/L; Tyree, 2001). In the absence of stress, they are generally thought to be osmoconformers (Sims, 1984). Their immediate response to osmotic stress is behavioural: siphon closure and mucus secretion to thicken the integument, thereby slowing water and ion movements (Davenport, 1985). Many invertebrate species also have a brain–blood barrier (BBB), making it possible to modify the brain interstitial (extracellular) fluid.

Studies of the ascidian neural gland are therefore of particular interest. This organ, in apposition with the brain (or cerebral ganglion) and a blood sinus, is connected to the oral siphon by a narrow ciliated duct (Grasse, 1948; Georges, 1967, 1977; Chambost, 1969). Various functions have been proposed for this gland as:
1 An endocrine gland (Julin, 1881; Herdman, 1883; Willey, 1883; Pestarino, 1984), possibly homologous to the vertebrate adenohypophysis (see, for example, Gorbman, 1995; Pestarino, 1984). However, recent surveys of the Ciona genome have identified no highly conserved endocrine genes of an anterior pituitary-like type. Thus, molecular studies have provided no evidence for homology between the neural gland and the adenohypophysis (Burighel & Cloney, 1997; Campbell et al., 2004).
2 An excretory organ (Julin, 1881; Ruppert & Smith, 1988).
3 A lymph-producing organ (Herdman, 1883; Cuenot, 1891).
4 A mucus-producing organ or digestive gland (Roule, 1884).
5 An organ regulating blood volume. Ruppert (1990) provided cues that the neural gland is involved in osmoregulation, with the ciliated duct generating a unidirectional influx of seawater into the neural gland, as previously reported (Carlisle, 1951; Godeaux & Beros-Debroux, 1979).

In vertebrates, the contributions of the many organs involved in osmoregulation are coordinated by three main neuropeptide-producing systems: the neurohypophyseal system (vasopressin and oxytocin), the hypothalamic–pituitary–adrenal axis (CRH, ACTH, mineralocorticoids and corticosteroids) and the renin-angiotensin system. Gastrointestinal peptides [tachykinin/substance P, VIP (vasointestinal peptide), somatostatin] have also been reported to have pleiotropic roles in hydromineral metabolism in several target organs (intestine, renal

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tubules, gills, etc.). These peptides act on their target cells by binding to G protein-coupled receptors (GPCRs).

We investigated whether the neural gland complex of ascidians could indeed be involved in hydromineral homeostasis regulation. We first re-evaluated the structure and organization of the neural gland complex by light and electron microscopy. We then searched the Ciona intestinalis genome for homologues of known vertebrate osmoregulatory neuropeptide receptors. Finally, we examined the patterns of expression of the corresponding genes by transcript detection by in situ hybridization (ISH), using whole-mount preparations and histological sections, to obtain further information about the neuropeptidergic systems of ascidians. Our results are consistent with a role for the neural gland complex in the maintenance of hydromineral homeostasis. This complex may play a role in the osmotic/hydric volume homeostasis of various adult compartments, and resembles the peri-ventricular organs of the vertebrate brain.

Materials and methods

Histological and ultrastructural analysis

Adult Ciona intestinalis were obtained from the zoological station at Roscoff (Brittany, France). Animals were maintained in artificial seawater at 18 °C, under constant illumination. Neural complexes were dissected out from anaesthetized (MS222, 0.4 g/L) adult Ciona specimens, fixed in 4% paraformaldehyde and embedded in Paraplast. Sections (8 μm) were cut in the transverse plane with a Leica microscope. Sections were stained with Nuclear Fast Red and observed under a Leica DRM microscope. Photomicrographs were taken with a Nikon DXM 1200 digital camera. For cryostat sectioning, fixed neural complexes were cryoprotected by incubation overnight at 4 °C in 25% sucrose, 5% polyvinylpyrrolidone (Sigma) in PBS and embedded in tissue freezing medium (Jung, Germany). Serial sections (20 μm) in the transverse plane were then cut on a cryostat (Leica) and stored at −80 °C. For transmission electron microscopy, dissected neural complexes were fixed overnight at room temperature in buffered 1.5% glutaraldehyde in 0.02 m sodium cacodylate (pH 7.4), 1% NaCl and 1.5% sucrose, and postfixed by incubation for 1 h at room temperature in a 1 : 1 mixture of 2% aqueous osmium tetroxide and 3% aqueous potassium ferrocyanide (Karnovsky, 1971). The complexes were embedded in Epon 812. Thin sections (80 nm) were prepared with an LKB ultramicrotome, counterstained by incubation for 2 min in lead citrate (0.14%), and observed in a Philips CM 12 transmission electron microscope operating at 60 kV.

Phylogenetic analysis: sequence identity and phylogenetic reconstruction

Ciona protein sequences were identified by tBlastn analysis on the JGI website (http://genome.jgi-psf.org/ciona4/ciona4.home.html; Dehal et al., 2002) and on the Ghost genome browser (http://boya.zool.kyoto-u.ac.jp/cgi-bin/gbrowse/ci), using human and fish GPCRs. In the absence of a cDNA sequence or a sufficiently large number of expressed sequence tags (ESTs), we used the peptide sequence deduced from the gene model. We checked the somatostatin, CRH, vasopressin, tachykinin and angiotensin peptide receptor sequences for conservation with Ciona savignyi, by BLAST analysis against the pre-Ensembl assembly (http://jun2006.archive.ensembl.org/ciona

Multiple sequences were aligned using ClustalX, with manual optimization (Thompson et al., 1997), using MUST software (Philippe, 1993). Regions of ambiguous homology were removed. For tree reconstruction, we first used ProTest (Abascal et al., 2005) to estimate the optimal model of amino-acid substitution (JTT + G + I + F or WAG + G + I + F depending on the alignment considered), then calculated the maximum likelihood (ML) trees using PHYML (Guindon & Gascuel, 2003) and Bayesian trees using MrBayes (Ronquist & Huelsenbeck, 2003) with these models. The robustness of the ML trees was estimated by 100 bootstrap replications. The ML trees were rooted on prior knowledge of human sequence phylogeny (Fredriksson et al., 2005).

Expression analysis

Total RNA was extracted from freshly dissected C. intestinalis tissues (neural complexes, heart, gonads, digestive organs, endostyle/branchial body), using Nucleospin RNA II columns (Macherey Nagel, Easton, PA, USA) according to the manufacturer’s instructions. The first-strand cDNA was synthesized from total RNA and polymerase chain reaction (PCR) was used for amplification with a set of specific primers. The identity of the clones was systematically checked by sequencing.

For ISH, bacterial clones were picked from the C. intestinalis gene collection release 1 (Satou et al., 2002). Clones ci0100145584 (CRHR), ci0100148068, ci0100153327, ci0100143057 (SSTR) and ci0100147553 (angiotensin receptor) were absent from this collection and were therefore not considered. DNA inserts were amplified directly by PCR, using T7 and T3 primers. Dioxigenin-labelled antisense or sense RNA probes were synthesized from PCR products (Ogasawara et al., 2001) and purified (Nucleospin mRNA purification, Macherey Nagel). Probes were hybridized to whole-mount neural complexes using an In situ Pro automat (Intavis), as described by Deyts et al. (2005). Neural complexes were dissected out directly and fixed by overnight incubation in 4% paraformaldehyde in MOPS buffer (0.1 m morpholinopropanesulphonic acid, pH 7.5, 2 m morpholinopropanesulphonic acid, pH 7.5, 2 mm MgCl2, 1 mm ethylene glycol tetra-acetic acid, 0.5 m NaCl), at 4 °C. They were treated with long proteinase K treatments (75 μg/mL, 1 h at 37 °C).

For each gene with a positive signal in whole-mount ISH, the sense probe was used as a control and additional hybridizations were carried out on cryostat sections, as described by Moret et al. (2004). Hybridized embryos, cryostat sections and whole neural complexes were mounted in 1% methylcellulose (Sigma) in PBS buffer and observed under an SV11 Zeiss dissecting microscope or a Leica DRM microscope. Images were recorded with a Nikon DXM 1200 digital camera.

Results

Morphological analysis

The terminology used to describe the ascidian neural gland and ganglia has at times been confusing. Here, we use the terms ‘neural gland’ to define the organ adjacent to the brain (or cerebral ganglion) (Fig. 1). The term ‘neural gland complex’ is used to qualify the neural gland and the attached ciliated duct, which opens into the pharynx through the ciliated funnel containing a dorsal tubercle (Grassé, 1948; Chambost, 1969; Georges, 1977). The ‘neural gland complex’ and the cerebral ganglion together comprise the ‘neural complex’, according to Ruppert (1990). Finally, the term ‘central nervous system’ (CNS) designates the cerebral ganglion, nerves of the body wall, the visceral nerves and the dorsal strand plexus in adult ascidians.
We focus here on the neural gland and its contacts with the surrounding blood sinus and the cerebral ganglion, and the epithelia of the ciliated duct.

Re-examining the borders between neural gland, cerebral ganglion and blood

Our observations showed that the neural gland abutted onto a blood sinus, which formed pouches at the basal pole of the neural gland epithelium. The sinus also surrounded the ganglion and extended within the dorsal tubercle. The neural complex and nerves were embedded in a fibrous sheath of connective tissue, facilitating extraction of the whole complex from the surrounding mantle.

The neural gland body consists of a spongy epithelium enclosing a lumen in contact with seawater via the ciliated duct (Lane, 1971). The lumen of the neural gland was found to contain numerous cells, identified as macrophages and phagocytes by Cuenot (1891) and Ruppert (1990). The neural gland epithelium consisted of cuboidal cells, devoid of cilia, with many folds extending into the inner cavity (Fig. 2). Protuberances of the apical cytoplasm, generally one per cell, were observed (Fig. 2E and F, p). The apical membrane of these cells was wavy and endowed with many microvilli forming pockets in the cells (Fig. 2G, mi). These cells were bound together by long (50 nm) apical junctions of the occludens type (Fig. 2F, j), and their lateral plasma membranes were highly folded and intermingled. The neural gland cells contained a thin granular nucleus in a latero-basal position (Fig. 2F, n) and, in most cases, at least one voluminous phagosome (Fig. 2F, ph). The endoplasmic reticulum was abundant, with numerous dilated cisternae (Fig. 2F, er). The Golgi apparatus was well developed and surrounded by numerous small vesicles.

We focused in particular on the interface between the neural gland and the cerebral ganglion. In the anterior half of the cerebral ganglion, the ciliated duct epithelium was found to line the ventral surface of the ganglion, and a thick basal lamina systematically separated the ganglion and the neural gland (Fig. 2E, Lb). In the posterior part of the cerebral ganglion, the basal lamina became much thinner. The basal part of the neural gland epithelium was sometimes found tightly apposed to this lamina, and sometimes separated from it by a thin blood sinus, as described by Ruppert (1990). There thus appears to be no direct communication between the neural gland epithelium and the cerebral ganglion.

Two distinct ciliated epithelia in the ciliated funnel

On transverse wax sections, the ciliated organ of the neural gland appeared to consist of a ciliated epithelium lining a connective tissue, as observed by Ruppert (1990). However, these cells were heterogeneous in terms of the cilia they bore. The cilia in the internal folds of the ciliated organ were longer than those in external areas, including the dorsal tubercle (Fig. 2A). We studied the ciliated duct epithelium in more detail by transmission electron microscopy (TEM). TEM analysis confirmed the presence of two types of epithelial cell. Both had apical cilia anchored on a basal body. The ‘long-cilia epithelium’, in the internal parts of the organ, consisted of tall, narrow, dense cells (Fig. 2A and B). These cells had an ovoid nucleus at the basal pole containing fine granules with a dense nucleolus. The apical cytoplasm of these cells contained numerous glycogen particles (Fig. 2B, gl) and large numbers of mitochondria with densely packed cristae. Each cell had several (4–5 per cell) long apical cilia (about 500 nm long), and this epithelium had numerous (5–10 per cell), long (1 μm), external apical microvilli (Fig. 2B, mv).

The ‘short-cilia epithelium’ was found in the external parts of the organ (Figs 2A and 3C, c). The apical cilia of the cells in this epithelium were shorter (about 300 nm) and less numerous (2–4 per cell). These cells were also characterized by the presence of densely stained spherical granules (100–200 nm in diameter), concentrated at the apical pole, the surface of which appeared wavy (Fig. 2C and D, gr). They had an abundant endoplasmic reticulum, forming distended cisternae (Fig. 2C and D, er). Progranules with irregular contours were found near the Golgi apparatus (Fig. 2D, Ga). Mitochondria were scarce and dispersed throughout the cell.

A survey of C. intestinalis GPCRs for neuropeptides associated with osmoregulation and phylogenetic analysis

We searched for GPCRs in C. intestinalis by first inferring phylogenetic relationships based on BLAST sequence comparisons on the JGI (http://genome.jgi-psf.org/cgi-bin/runAlignment?db = ciona4 &advanced = 1; Dehal et al., 2002) and GHOST Ciona genome databases (http://ghost.zool.kyoto-u.ac.jp/index1.html; Satou et al., 2002) (Table 1). ClustalX (Thompson et al., 1997) was used to produce alignments, which were then refined manually. Molecular phylogenetic analyses were carried out, using ML and Bayesian methods. These two methods gave very similar tree topologies for each set of sequences (Fig. 3).
We investigated the phylogenetic relationships between a given ascidian gene and its human counterparts, by building trees including subfamilies of potentially orthologous human GPCRs, together with neighbouring human subfamilies, as identified by Fredriksson et al. (2003). Only the seven-transmembrane core region was used for the subclassification of these proteins (between 150 and 300 conserved positions), because the N- and C-terminal (non-transmembrane) sequences are poorly conserved.

Fig. 2. (A–D) Micrographs of the ciliated organ of the neural gland complex. (A) Light (B): Electron micrograph of apical region of cells with long cilia (×12 000). (C) Electron micrograph of cells with short cilia (×11 000). (D) Golgi apparatus surrounded by granules (×60 000). (E–G) Electron micrographs of transverse sections through the neural gland. (E) Low-magnification micrograph. The neural gland is separated from the cerebral ganglion by a basal lamina (×2000). (F) The epithelium cells of the neural gland present apical protuberances and contain voluminous phagosomes. The lateral plasma membranes are interdigitated (×3000). (G) The apical plasma membrane has inner microvilli (mi) (×60 000). Lb, basal lamina; C, cilium; E, epithelium of the neural gland; er, endoplasmic reticulum; gg, cerebral ganglion; gl, glycogen particle; Ga, Golgi apparatus; gr, granules; j, adherens junction; Lc, long cilia; lm, lateral plasma membrane; mv, microvilli; n, nucleus; p, protuberance; ph, phagosome; Sc, short cilia.
Table 1. A short list of Ciona intestinalis neuropeptide G protein-coupled receptors potentially involved in osmoregulation

<table>
<thead>
<tr>
<th>Group/gene name</th>
<th>The best gene model in the version 4 assembly</th>
<th>cDNA cluster</th>
<th>The best hit protein in the human proteome</th>
<th>Best hit analysis</th>
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<td>Somatostatin receptor (GC25G15)</td>
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<td>04726</td>
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<td>37017</td>
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<td>Somatostatin receptor (not in GC)</td>
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<td>Somatostatin receptor (GC38A13)</td>
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In our search for osmoregulatory neuropeptide receptors, we identified 19 Ciona genes belonging to three monophyletic vertebrate GPCR subgroups: secretins, and the beta and gamma families of rhodopsin (Fig. 3). These groups are unlikely to be the only such genes in ascidians (Fredriksson et al., 2005; Sherwood et al., 2005). Most of the subfamilies of osmolarity-associated GPCRs are represented in the Ciona genome, with only VIP receptor homologues remaining undetected.

These Ciona genes were found to diverge markedly from their vertebrate counterparts (Fig. 3), although it was possible, in most cases, to assign them unambiguously to GPCR subfamilies. We therefore named the Ciona genes following the human nomenclature proposed by Fredriksson et al. (2003).

Surprisingly, BLAST searches identified a group of 12 genes associated with the somatostatin (SSTR) receptor family (Fig. 3D), which were investigated further. They could not be assigned to the somatostatin or opioid receptor subfamilies based on phylogeny alone. However, as these genes had the clear molecular signatures of SSTRs, we assigned them to this subfamily. Urochordates may therefore have experienced the independent expansion of this gene family.

Ciona GPCR gene expression

We checked EST profiles from extensive Ciona cDNA sequencing projects (Dehal et al., 2002; Satou et al., 2002). In all cases, only a few ESTs (three at most; data not shown) corresponded to these receptor sequences, suggesting that these genes are weakly expressed, or expressed in only a small proportion of cells.

We then analysed the distribution of the GPCR transcripts identified with gene collection release 1 in whole neural complexes, by automated ISH. Signals were detected for only five genes, and were not correlated with EST profiles. The neural gland is an adult organ with many epithelial cell sheets. We therefore adjusted the experimental conditions, varying proteinase K treatments, and using sense probes as controls. However, it remains possible that we failed to detect the discrete or weak expression of some receptors. Positive signal detection was confirmed by ISH on cryostat sections.

The C. intestinalis genome contains a single gene (GC05O02) related to vertebrate vasopressin receptors (Family A/group V), which have been implicated in hydromineral balance. Two isoforms are present in all vertebrates (Vaso-R1 and Vaso-R2). We studied GC05O02 expression in various tissues (gonad, endostyle, neural complex, digestive organs and heart) by reverse transcription PCR (RT-PCR), and obtained positive signals in the neural complex and heart (data not shown), consistent with predicted EST profiles (neural complex, 1/10029; hear,: 5/12414). No embryonic expression was detected. In dissected neural complexes from adult ascidians, vasopressin receptor transcripts were detected in the body of the neural gland and in the cerebral ganglion (Fig. 4A and B). No staining was detected in the ciliated organ.
In vertebrates, osmotic regulation also involves corticotropin-releasing hormone (CRH), the main regulator of the adrenal system, which binds to a specific receptor, the CRH receptor (CRHR). Two isoforms of this receptor, CRHR1 and CRHR2, are present in mammals. Two CRHR receptors not orthologous to either of the two vertebrate paralogues are present in the *Ciona* genome. We arbitrarily named these receptors Ci-CRHRa (cii010011455584) and Ci-CRHRb (GC45K20). We analysed the expression only of Ci-CRHRb, which was present in the gene collection. RT-PCR (data not shown) showed that Ci-CRHRb was expressed in the neural complex (EST count: 2/10029 in the neural complex, zero elsewhere). This expression was restricted to the body of the neural gland, on whole-mount (Fig. 4C) and cryostat (Fig. 4D) sections.

Renin and angiotensin exert their effects on hydromineral homeostasis in vertebrates by binding to different, specific receptors. These receptors are located in the kidney, the choroid plexus and the hypothalamus. In the two *Ciona* genome sequences available, genes encoding two peptides related to angiotensin receptors were identified. Only one of the two transcripts (clone GC24N16) was detected in the cerebral ganglion and neural gland (Fig. 4E and F).

Somatostatin is a hypothalamic neurohormone. Somatostatin-producing fibres, originating from paraventricular neurons, are abundant in the median eminence of the mammalian brain. Somatostatin is not specific to the brain. This hormone is also found in the intestine and stomach walls, along with somatostatin receptors. Somatostatin regulates ACTH secretion by the pituitary gland; it is also involved in osmotic regulation in the intestine and stomach walls. Five subtypes of somatostatin receptor have been identified in mammals: SS1R, SS2R, SS3R, SS4R and SS5R. In the *Ciona* genome, 12 putative somatostatin receptors were identified by BLAST searches, eight of which were present in the gene collection (GC07F01, GC08A11, GC25G15, GC38I03, GC37F23, GC38A13, GC38F09, GC45E04). However, branching patterns are unstable in this subgroup and the evolutionary relationships between these receptors are unclear. ISH showed that only one gene (GC38A13) was strongly expressed in the neural gland complex, in the body of the neural gland and in the ciliated organ (Fig. 4G–I). In the ciliated organ, GC38A13 was found to be expressed preferentially in one of the cell types identified by TEM: ‘long cilia epithelial’ cells (Fig. 4G and I).

Tachykinins are vertebrate multifunctional brain/gut peptides involved in various central and peripheral functions, including smooth muscle contraction, and the regulation of blood volume and hydro-mineral balance. RT-PCR detected the expression of a single *Ci-TK* receptor in the dissected neural complex, digestive organ, gonads and endostyle (not shown). The expression of this receptor was restricted to the body of the neural gland complex (Fig. 4J and K).

**Discussion**

We have compared the ascidian neural gland with the dorsal and ventral peri-ventricular organs found in the vertebrate CNS. These structures include the saccus vasculosus, present in cartilaginous and teleostean fish, of unknown function, and the choroid plexus (CP), present in all craniate species. The CP produces large quantities of cerebrospinal fluid (CSF) from blood in vertebrates, along an osmotic gradient or via specific water channels, such as aquaporin (Oshio et al., 2003). About 500 mL of CSF is produced per day in humans, and total CSF volume is about 80–150 mL. The CP plays a key role in human health, and changes in CP function have been associated with migraine, ageing and neurodegeneration (Emerich et al., 2005). We evaluated whether the function of the neural gland complex (neural osmoregulation) was similar to that of the CP, by studying ascidian neuropeptidergic systems, focusing on receptors because it is difficult to obtain short divergent peptide sequences. The entire genomes of two ascidian species (*Ciona intestinalis* and *Ciona savignyi*) have recently been sequenced (Dehal et al., 2002; Vinson et al., 2005). We carried out data mining to identify genes encoding GPCRs in the *Ciona* genome.

In *C. intestinalis*, the substance P/tachykinin neuropeptide is the best known neuropeptide system. O’Neill et al. (1987) and Bollner et al. (1992) detected these neuropeptides in extracts of pharynx/body wall and in the cerebral ganglion and ciliated duct epithelium. Satake et al. (2004) identified two *C. intestinalis* genes encoding proteins related to the vertebrate tachykinins, *Ci-TK-1* and *Ci-TK-2*, and a tachykinin receptor (*Ci-TK-R*). We found that this tachykinin receptor was expressed in the body of the neural gland, suggesting possible neuroendocrine links between the neural gland and either the ganglion or the ciliated duct.

Many other neuropeptide GPCRs are expressed in both the neural gland and the CP: angiotensin, CRH, vasopressin, and somatostatin receptors. VIP receptors are also expressed in the vertebrate CP, but no VIP receptor genes have been identified in the *Ciona* genome. The common expression of polypeptide receptors suggests that these structures may be involved in neural gland/choroidal haemodynamics, transport, secretion and clearance of seawater or CSF.

Morphological analysis indicated that the cellular features of the neural gland strongly resembled those of peri-ventricular organs. These organs, which are also apposed to the CNS, have a spongy appearance similar to that of the neural gland (Emerich et al., 2005). They consist of cuboidal epithelial cells resting on a basal lamina and an inner core of highly vascularized connective tissue. Their cells have a large central nucleus, abundant cytoplasm, numerous invaginations, and scattered internal and external villi, and are linked together by tight junctions. They also have large numbers of mitochondria, which are probably required to maintain high levels of respiratory metabolism and to fulfil large energy requirements. Are there similarities between the ascidian neural gland and the saccus vasculosus? Coronet cells, which have several external tubulated bulbs, are characterized in the saccus vasculosus (Follenius, 1982; Svane, 1982), and are thought to be part of a sensory complex sensitive to hydrostatic pressure. Coronet cells bearing a single bulb have been identified in the ascidian sensory vesicle (Olsson, 1975; Svane, 1982; Moret et al., 2005). Their presence is consistent with equivalence between the tunicate ventral sensory vesicle and the vertebrate hypothalamus. However, despite exhaustive microscopic searches, including the use of TEM, we found no such cells in the ascidian neural gland (C. Deyts, unpublished data). Ontogenetic position also argues against the presence of these cells: in vertebrates, the saccus vasculosus develops ventrally to the CNS, together with the hypothalamus. Svane (1982) reported a possible ascidian counterpart to the...
Fig. 4. Expression pattern of GPCR genes in the neural complex of adult *Ciona intestinalis* (ISH on whole neural complex and on cryostat sections). GC05O02, vasopressin receptor (VASR) (A and B); GC45K20, corticotropin-releasing hormone receptor (CRHR) (C and D); GC24N16, angiotensin receptor (AGR) (E and F); GC38A13, somatostatin receptors (SSR) (G, H and I); GC13F01, tachykinin receptor (TAKR) (J and K). gg, cerebral ganglion; NG, neural gland; Lc, long cilia.

Fig. 5. (A) Dorsal schematics for brains of E13.5 (left) and E10 (right) mice embryos. Rostral is towards the top left and caudal is towards the bottom right. The choroid plexus (blue) forms at or near the dorsal midline (DM) in the hindbrain (HBCP, 4th ventricle), diencephalon (DICP, 3rd ventricle) and on both sides of the dorsomedial telencephalon (TELCP, lateral ventricles: LV). (B) Left: dorsal schematics of the neural gland complex and ganglion at V protostigmata stage (day 8, drawn from Fig. 4D in Chiba et al., 2004). The neural gland primordium (NGP, blue) becomes visible dorsally to the neural tube, caudally to the former sensory vesicle. Right: side view of the embryonic CNS. The shaded area indicates the region degenerating during metamorphosis. The blue line shows the position of the hypothetical presumptive neural gland domain. Schematics of C: the choroid plexus and associated SNC; schematics of D: neural gland and associated SNC. The choroid epithelium and neural gland epithelium (1) lie on the basal lamina, (2) delimiting the inner connective core (3). This stroma is highly vascularized and contains a large number of dilated fenestrated capillaries (4) in vertebrates and in the blood sinus (4) of ascidians (4). B, blood; CSF, cerebrospinal fluid; SW, seawater; EC, ependymal cells; CE, ciliated epithelium.
vertebrate saccus vasculosus in *Pyura tessellata*. This structure consists of an accessory brain vesicle developing ventro-laterally to the sensory vesicle, with a single globular structure resembling the coronet cells found in sharks and teleosts. *Pyura* larvae have a dorsal neural complex in their heads, suggesting that the neural gland is unlikely to be homologous to the saccus vasculosus. So, as the lack of coronet cells and developmental location seem to rule out homology between the neural gland and the saccus vasculosus, could the neural gland be homologous to the vertebrate choroid plexus?

Ontogenetic evidence is consistent with homology between the neural gland and the CP. In the order Enterogona, in which the species studied are thought to have retained ancestral characters (Berrill, 1948, 1950; Svane, 1982), the neural gland has been described, by all authors, including Willey (1893), as developing dorsally to the larval CNS (Takamura, 2002; Chiba et al., 2004), from the larval ciliated duct (Manni et al., 2005). The ventral part of the neural tube is thought to degenerate during ascidian metamorphosis (Fig. 5) (Willey, 1883). The CP also develops early, mainly from thin sheets of cells located at the rooves of several brain structures in vertebrates. For example, in the rhombencephalon, the CP develops from a thin sheet of cells called the tela choroidea located in the hindbrain roof.

Cell types with highly specific characteristics are found in both the neural gland and the CP. The ascidian neural gland has long been thought to comprise macrophage-like cells (Herdmann, 1883; Cuénot, 1891). Moreover, Georges (1977) observed cyclical variation in the number of these immune cells in animals collected around the Roscoff marine station. This variation was part of a more general cyclical pattern of cell proliferation, which appeared to be related to tides. The immune cells may protect the neural gland against compounds or microorganisms present in seawater, in the ciliated duct or the neural gland lumen. Similarly, numerous macrophages, dendritic cells and fibroblasts are found in the CP stroma, and are thought to provide the first line of defence for the brain, via the neuroimmune system. The CP is ideally located for monitoring for harmful compounds or potentially damaging cellular invasion in the CSF.

Ruppert et al. (1990) suggested that the neural gland is involved in regulating blood volume, by transporting seawater into the blood. This would place neural gland cells at the interface between the external medium (seawater) and the internal medium (Gorbman, 1995; Ruppert, 1990). We suggest that the neural gland may be involved solely in regulating nervous system homeostasis, as with the CP. Indeed, it seems unlikely that this small gland, bearing a fairly limited epithelial surface, could regulate the volume of blood in an entire animal up to 15 cm in size. It also seems unlikely that ascidians, which have an open circulatory system, could easily maintain their global osmolarity. Abbott et al. (1986) suggested that the evolution of invertebrate nervous systems was accompanied by an increase in the control of ionic homeostasis around central synapses. Many invertebrate species have a BBM, making it possible to modulate brain intestinal (extracellular) fluid. According to this hypothesis, the neural gland probably regulates peri-ganglionic ionic homeostasis by liquid pumping, ionic exchange and amino-acid transport (from blood or seawater), and the osmolarity/ionic composition of the rest of the body fluctuates to a greater extent, in response to environmental conditions. In *C. intestinalis*, the oval-shaped cerebral ganglion is embedded in a fibrous sheath of connective tissue that probably isolates it from the surrounding mantle. Two nerve fascicles, also surrounded by a dense collagen matrix, leave the ganglion at its anterior and posterior poles (proximal and distal, respectively, to the ciliated funnel; Arkett et al., 1989). On the ventral side, there seems to be no direct communication between the neural gland and the cerebral ganglion. This situation is similar to that in vertebrates, in which the CP is separated from the nervous tissue by a basal lamina. The possible existence of other types of BBM in tunicates requires further investigation.

In conclusion, the results presented here highlight similarities between the ascidian neural gland and the vertebrate peri-ventricular organs. These structures have genetic components, cytological types and morphological/macroscopic aspects in common. They seem to act as an interface between two media with different ionic and amino-acid compositions, although this remains to be verified experimentally. More precise hypotheses concerning the homology between the neural gland and the CP may emerge following the completion of comparative analyses of the ontogenesis of the ascidian neural complex, including late lineage analyses.

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**Abbreviations**

BBB, brain–blood barrier; CNS, central nervous system; CP, choroid plexus; CRH, corticotropin-releasing hormone; CRHR, corticotropin-releasing hormone receptor; CSF, cerebrospinal fluid; EST, expressed sequence tag; GPCRs, G-protein-coupled receptors; ISH, *in situ* hybridization; ML, maximum likelihood; RT-PCR, reverse transcription-polymerase chain reaction; TEM, transmission electron microscopy; VIP, vasoactive intestinal peptide.

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