A genomic view of the sea urchin nervous system

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Abstract

The sequencing of the *Strongylocentrotus purpuratus* genome provides a unique opportunity to investigate the function and evolution of neural genes. The neurobiology of sea urchins is of particular interest because they have a close phylogenetic relationship with chordates, yet a distinctive pentaradiate body plan and unusual neural organization. Orthologues of transcription factors that regulate neurogenesis in other animals have been identified and several are expressed in neurogenic domains before gastrulation indicating that they may operate near the top of a conserved neural gene regulatory network. A family of genes encoding voltage-gated ion channels is present but, surprisingly, genes encoding gap junction proteins (connexins and pannexins) appear to be absent. Genes required for synapse formation and function have been identified and genes for synthesis and transport of neurotransmitters are present. There is a large family of G-protein-coupled receptors, including 874 rhodopsin-type receptors, 28 metabotropic glutamate-like receptors and a remarkably expanded group of 161 secretin receptor-like proteins. Absence of cannabinoid, lysophospholipid and melanocortin receptors indicates that this group may be unique to chordates. There are at least 37 putative G-protein-coupled peptide receptors and precursors for several neuropeptides and peptide hormones have been identified, including SALMFamides, NGFFFamide, a vasotocin-like peptide, glycoprotein hormones and insulin/insulin-like growth factors. Identification of a neurotrophin-like gene and Trk receptor in sea urchin indicates that this neural signaling system is not unique to chordates. Several hundred chemoreceptor genes have been predicted using several approaches, a number similar to that for other animals. Intriguingly, genes encoding homologues of rhodopsin, Pax6 and several other key mammalian retinal transcription factors are expressed in tube feet, suggesting tube feet function as photosensory organs. Analysis of the sea urchin genome presents a unique perspective on the evolutionary history of deuterostome nervous systems and reveals new approaches to investigate the development and neurobiology of sea urchins.
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Introduction

Neural organization is a defining feature of animal body plans that is often emphasized in analyses of the relationships among major taxa. The arrangement of the nervous system in

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echinoderms is a feature that distinguishes them from other deuterostomes (chordates and hemichordates). Echinoderm nervous systems are dispersed, but they are not a simple nerve net. The adult is not cephalized, yet the radial nerves are segmentally organized. The larva has a nervous system, but it appears to be independent of the nervous system of the adult. Identifying neural genes in the sea urchin genome has permitted an unprecedented glimpse into how this poorly understood nervous system develops and functions. This genomic approach circumvents many of the technical problems that have hampered conventional neurobiological approaches in echinoderms. Moreover, the list of putative neural genes identified in the genome provides a unique resource for developing hypotheses of gene function and numerous opportunities for new approaches to understanding neural development and function in sea urchins.

Neural development and organization

Neurons of the larval nervous system appear as neuroblasts in the thickened ectoderm of the animal plate and in the presumptive ciliary band of the late gastrula (Bisgrove and Burke, 1986; Yaguchi et al., 2000; Nakajima et al., 2004) (Figs. 1, 2). A phase of neurite projection follows during which the tracts of axons at the base of the ciliary bands are formed. Neurons continue to be added throughout larval development and the fully formed, eight-armed pluteus has a complex array of sensory neurons, interneurons, tracts of axons and ganglia that are closely associated with the larval ciliary bands and larval muscles (Bisgrove and Burke, 1986; Beer et al., 2001). The larval nervous system has been described using ultrastructure, histochemistry, antibodies to neurotransmitters, tissue-specific monoclonal antibodies and neuron-specific, in situ hybridization probes (Burke, 1978; Bisgrove and Burke, 1986, 1987; Beer et al., 2001; Yaguchi and Katow, 2003; Nakajima et al., 2004; Thorndyke et al., 1992) (Fig. 2). Neurons and neurites are principally associated with the ciliary bands that surround the oral field and form at the rim of the larval mouth (Beer et al., 2001; Nakajima et al., 2004). Several clusters of neurons with associated neuropil are organized as ganglia. The largest of these is the apical organ, a bilaterally symmetric patch of neurons at the anterior end of the larva between the preoral arms. In the early larva, the apical organ is composed of 4–6 bilaterally positioned sensory cells containing serotonin, a central cluster of 10–12 neurons and several non-neural supporting cells. The neurons associated with the ciliary band are arrayed at intervals along the length of the ciliary band on the side of the band closest to the larval mouth. These appear to be primary sensory cells that contribute axons to a central tract that lies at the base of the ciliated cells and appears to run the length of the ciliary band. The lateral ganglia are two clusters of neurons, on the left and right sides of the larva that project axons or dendrites beneath the epidermis throughout the posterior end of the larva (Nakajima
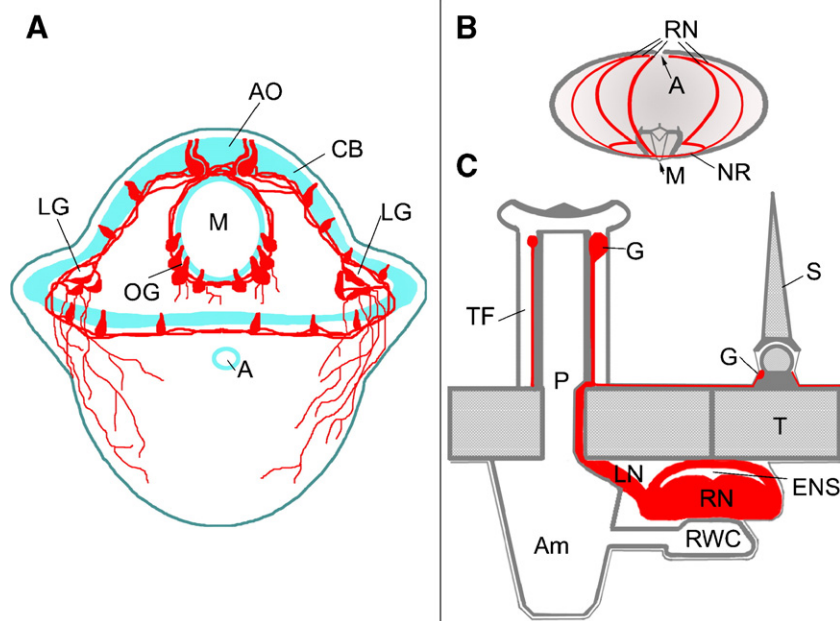

Fig. 1. Diagrams of larval and adult nervous systems. (A) Larval nervous system of an early pluteus. The nervous system is associated with the ciliary band (CB) surrounding the oral epidermis and the larval mouth (M). The apical organ (AO) is at the foremost end of the larva and there are oral ganglia (OG) in the lower lips of the larval mouth and paired lateral ganglia (LG) between the larval arms. Neurites project beneath the aboral epidermis. (B) Lateral perspective of an adult sea urchin showing the 5 radial nerves (RN), anus (A) and mouth (M). The radial nerves are joined by commissures forming a nerve ring (NR) around the base of the lantern apparatus. (C) Cross-section of adult radial nerve and test. The radial nerve (RN) and epineural sinus (ENS) lie against the inner surface of the test (T). Each segment of the radial nerve gives rise to a lateral nerve (LN) that projects through a pore (P) to connect to the base of a tube foot (TF). The water vascular system, comprised of a radial water canal (RWC) and ampulla (Am), connects to the lumen of the tube foot and overlies the radial nerve and radial hemal sinus (not shown). Each tube foot has a terminal ganglion (G) and ring of neuropil. Spines (S) all have a ganglion (G) and ring of neuropil at their base that connect to the radial nerve. Neural tissues are in red.
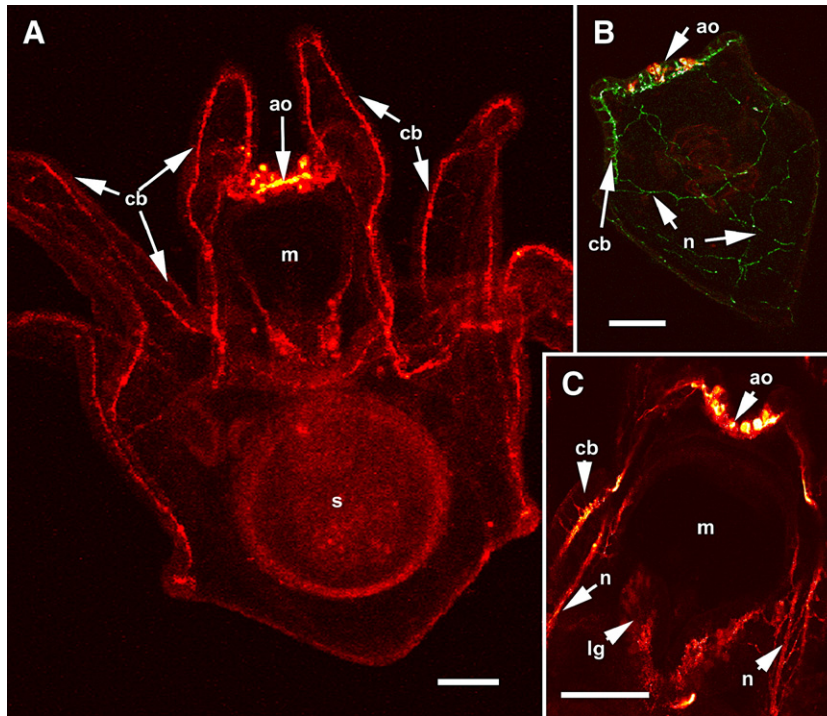
Fig. 2. The larval nervous system of *S. purpuratus* revealed with immunolocalization of anti-synaptotagmin and anti-serotonin. (A) Confocal image of an eight arm pluteus larva showing the tracts of axons that underlie the ciliated cells of the ciliary band (cb), anti-synaptotagmin. The apical organ (ao) is located on the oral hood at the foremost end of the larva and is thought to be a sensory organ. The larval mouth (m) is also innervated and nerves surround the esophagus, a tube connecting the mouth and the stomach (s). (B) In the more familiar 4 arm pluteus larva the apical organ (ao) is double labeled with anti-serotonin (red) and the ciliary band neurons are labeled with anti-synaptotagmin (green). The neural cells bodies of the ciliary bands (cb) are arrayed at intervals along the oral side of the ciliated cells. The neurons in the ciliary bands project neurites to the posterior end of the larva (n). (C) A confocal image (anti-synaptotagmin, red) showing details of the mouth (m) region. The lip ganglion (lg) contains serotonergic and non-serotonergic neurons. In addition to the ciliary band neurons and axon tracts (cb), there are bundles of neurites (n) of unknown function projecting from the apical organ, under the oral ectoderm. Scale bars=25 μm.

et al., 2004). The larval nervous system grows and becomes more complex as the larva increases in size (Fig. 2, Beer et al., 2001).

How the larval nervous system functions has been deduced from neuroanatomy and behavior. The ciliated cells of the ciliary band and musculature of the esophagus and mouth appear to be the principal effectors (Strathmann, 1975). It is assumed that coordinated reversals of cilia, reversals of peristalsis in the esophagus and contraction of the muscles that open the mouth and flex the arms are neurally controlled (Bisgrove and Burke, 1986). Mackie et al. (1969) recorded electrical activity from ciliary bands coincident with ciliary reversals, but there have been no neurophysiological investigations of larval nerves.

The oral half of the adult body develops as a rudiment on the left side of the larva, arising from a disc of ectoderm from the larval oral field that invaginates and lies against a mesodermal epithelium. This mesoderm forms a toroid and becomes the water vascular system while the overlying ectoderm makes five radially arrayed neural infoldings (Hyman, 1955 after von Ubisch, 1913). The lumen of each of the infoldings becomes an epineural sinus and the epithelium at the base of each infolding differentiates as the neural epithelium of the radial nerves (Fig. 1). Initially a single segment forms, but additional segments are added as ambulacral tube feet appear (Fig. 3). In many species metamorphosis takes place while the adult rudiment has only a

single segment. The neurons of the appendages (tube feet, spines, sphaeridia and pedicellariae, Fig. 4) appear to develop within the appendages. This is most apparent in pedicellariae that arise from separate placode-like ectodermal invaginations of the surface of the larva (Burke, 1980). The larval and adult nervous systems appear to have no direct connections and during metamorphosis the larval nervous system is largely lost (Chia and Burke, 1978).

The adult echinoid nervous system is comprised of 5 radial nerve cords, which are joined at their base by commissures that form a ring surrounding the mouth (Smith, 1965; Cobb, 1970; Cavey and Markel, 1994) (Fig. 1). Tube feet, spines and pedicellariae have ganglia and a complement of sensory and motor neurons (Figs. 1, 4). The viscera are well innervated (Garcia-Arraras et al., 2001) and there are neurons in almost all tissues. Each segment of the radial nerve cords contains a layer of neurons overlying an extensive neuropil (Fig. 3). Neurites project laterally within each segment and longitudinally to adjacent segments. Each segment gives rise to a single nerve bundle that projects laterally to a tube foot and adjacent appendages.

Neural organization is similar to the organization of the internet, an interconnected system of smaller local networks. Sensory input within tube feet, spines and pedicellariae project to ganglia within the appendages that integrate and control local motor responses (Cobb, 1987). Each of these nodes is
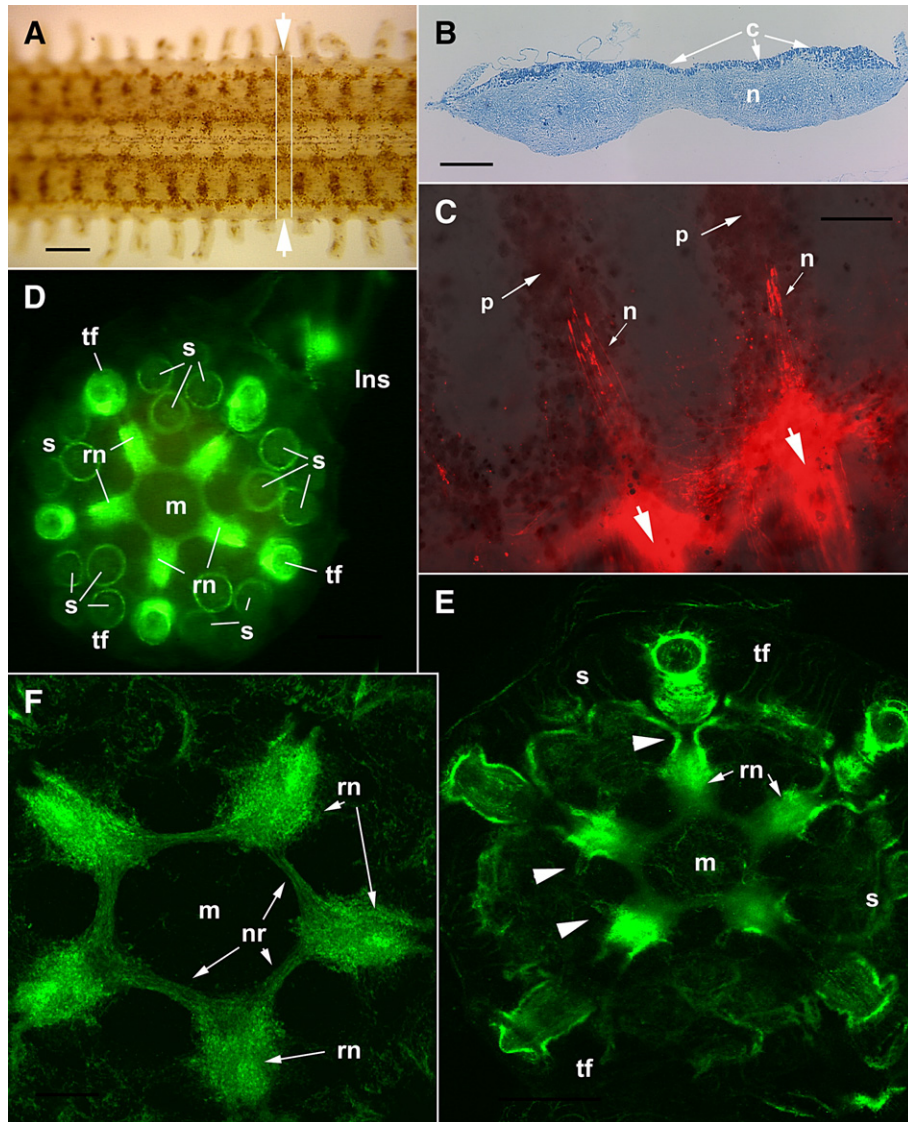
Fig. 3. The adult nervous system of *S. purpuratus*. Segmental radial nerves in adult echinoderms are known only from ophiouroids (Cobb, 1987). The adult sea urchin radial nerve appears to have a similar organization. (A) Dissected radial nerve showing the segmental organization. Each segment spans the width of the nerve and a single nerve bundle projects from each segment. The segments are arranged so that alternating segments project nerve bundles to alternating sides of the nerve. A single segment is outlined (arrows). The patterned pigmentation is in the cellular layer. Scale bar=1 cm. (B) Cross-section of a radial nerve showing that the cellular layer (c) is on the surface of the nerve and extensive neuropil (n), comprised of closely apposed neurites beneath the cellular layer. Scale bar=500 μm. (C) The segmentation of the nervous system is revealed by whole mount of a nerve in which the lipophilic tracer DiI was injected into the lateral projections of two segments and neurites were allowed to back-fill overnight. The back-filling procedure identifies a similar cluster of nerve cell bodies in both segments (n) indicating that the segments have identical organization and are repeated elements. Neurites also project between segments in longitudinal tracts that are predominantly on the lateral edges of the nerves. The darker pigmentation pattern (p) of the tissue provides orientation. Scale bar=200 μm. (D) The adult nervous system arises in the developing adult rudiment on the side of the larval body. In this immunofluorescent preparation with anti-synaptotagmin, the oral surface of the rudiment is uppermost and the larval nervous system (lns) is out of focus behind the specimen. Nerves appear in the developing appendages, spines (s) and tube feet (tf) simultaneously with the appearance of neurons in the radial nerves. Scale bar=50 μm. (E) In confocal images of later rudiments, connecting neurites from the radial nerves to appendages appear (arrowheads). Scale bar=25 μm. (F) The radial nerves (rn) first appear as a single segment and the neuronal cell bodies distinguish the radial nerves from the nerve ring (nr), which is simply a commissure of neurites. As the juvenile grows, additional segments are added to the radial nerves. Scale bar=50 μm.

interconnected to adjacent nodes and to the radial nerves. The radial nerves appear to function in overall control and coordination, but appendages have independent sensory–interneuron–motor reflex arcs and for the most part lack direct motor and sensory input from a central nervous system. Cobb (1987) emphasized that this is a highly organized, interconnected set of independent reflex pathways.

The echinoderm nervous system may be the least well studied of all those in the major metazoan phyla. Although morphological studies provide the essential neuroanatomy of the echinoid nervous system, conduction pathways are unknown because only a few neurophysiological studies using wick or metal filled electrodes have been done (Pentreath and Cobb, 1972; Binyon, 1972). The development
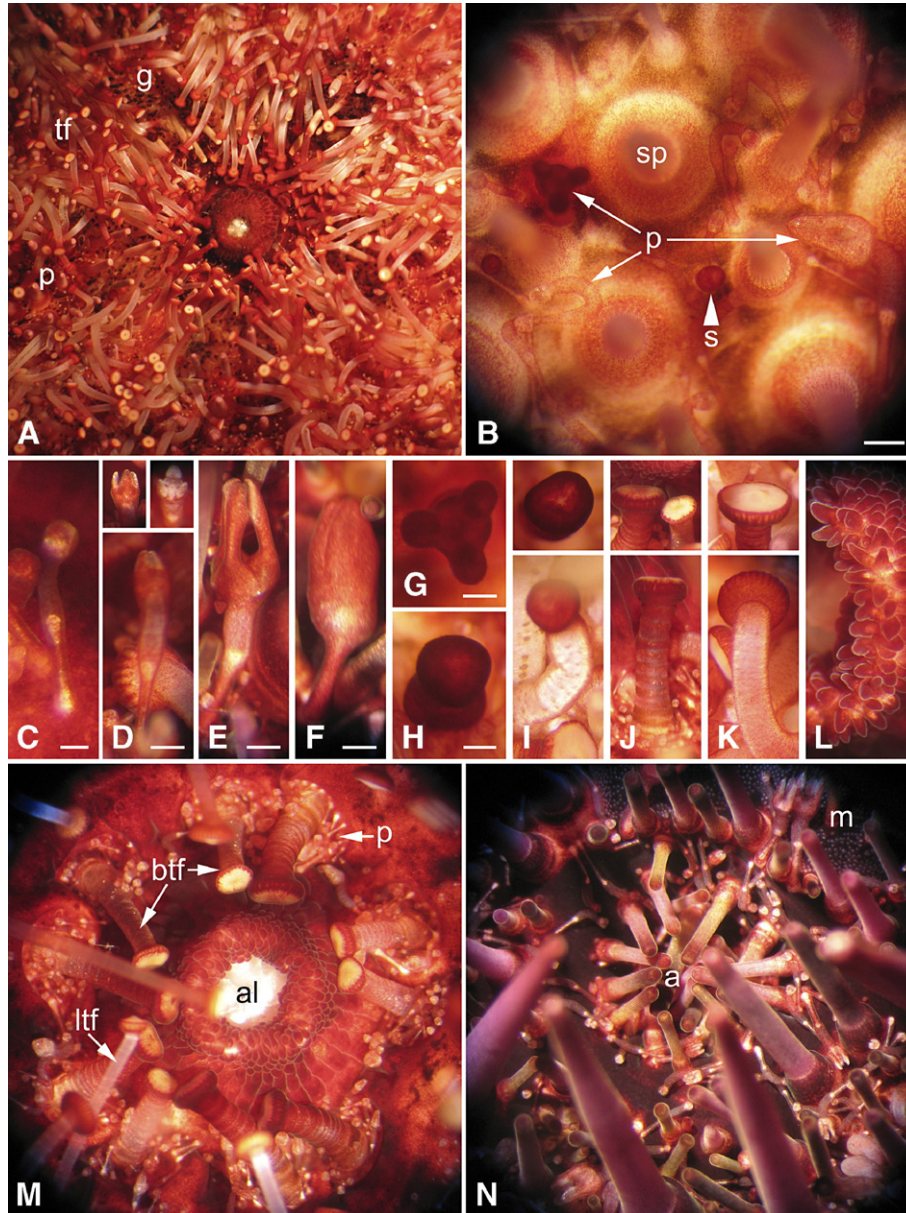
Fig. 4. Appendages of adult *S. purpuratus*. Appendage types are classified according to Hyman (1955). (A) Oral view, showing the mouth and large numbers of tube feet (tf), pedicellariae (p) and gills (g). (B) Lateral surface of the test, showing spines (sp) extending upward from tubercles, pedicellariae (p) and sphaeridium (s). Scale bar=1 mm. (C) Triphyllous pedicellaria; tiny but numerous. Scale bar=0.25 mm. (D) Ophiocephalous or trifoliate pedicellaria, with three blunt jaws (insets) that do not meet. Scale bar=0.5 mm. (E) Tridentate pedicellaria; the largest and very numerous. Scale bar=0.5 mm. (F) Globiferous pedicelleria containing poison sacs. Scale bar=0.5 mm. (G) Claviform pedicellaria, which may arise from the loss of jaws from globiferous pedicellariae. Scale bar=0.5 mm. (H) Sphaeridium. Scale bar=0.25 mm. (I) Tube foot with bulbous end (inset). (J) Buccal tubefoot from near the mouth; has a shorter, stouter stalk than locomotory tubefoot, and modified sucker (inset); a cavity surrounding the base of each stalk contains numerous tiny pedicellariae. (K) Locomotory tube foot having typical cupped sucker (inset). (L) Edge of the mouth (left) with many lobes reminiscent of taste buds on the vertebrate tongue. (M) Oral side, including the peristome, showing the teeth of Aristotle's lantern (al) protruding from the lips of the mouth, buccal tube feet (btf) on stalks stouter than locomotory tube feet (ltf), and many tiny pedicellariae (p). (N) Aboral surface, showing many spines and pedicellariae (p) surrounding the periproct and anus (a) and gonopores (not shown). The single madreporic (m) plate defines an axis of polarity and plane of bilateral symmetry.

of the echinoid nervous system is equally mysterious. Embryonic neurogenesis giving rise to larval nerves is accessible to experimental study (Yaguchi et al., 2006), but the descriptions of adult nervous system development are fragmentary and based on histological studies (Bury, 1896; von Ubisch, 1913; MacBride, 1903). Thus, while the sea urchin is used as a modern biomedical model, we have only fragmentary understanding of its neurobiology.

Analysis of the neural gene set of the sea urchin genome holds great promise for achieving a better understanding of relationships within the deuterostomes. Furthermore, a better understanding of the sea urchin nervous system permits a more incisive analysis of the features of neural development and organization shared by deuterostomes that distinguish them from other bilaterians. Identification of neural genes in the sea urchin provides a wealth of comparative data that indicate

Table 1

Gene ID	Gene name (synonym)	Gene characteristics (transcription factors)	Expression Data candidate regulatory genes	Egg	HB	MB	G	Pl	References
SPU_028148	Sp-Ac-Sc (achaete-scute)	bHLH	expressed specifically in embryonic apical ectoderm or ciliated band						1,5
SPU_023177	Sp-Hbn (homeobrain)	hbox-paired							1,5
SPU_007147	Sp-Ngn (neurogenin)	bHLH							1,5
SPU_024918	Sp-NeuroD1 (neuroD)	bHLH							12
SPU_020975	Sp-Engrailed	hbox							13
SPU_014289	Sp-Rx (retinal anterior hbx)	hbox-paired							5,6
SPU_022820	Sp-SoxB1	HMG							8
SPU_025113	Sp-SoxB2	HMG							8
SPU_004217	Sp-SoxD	HMG							8
SPU_010424	Sp-Otx (orthodenticle)	hbox							1,7
SPU_021608	Sp-Hes (hairy-related)	bHLH	expressed in embryonic apical ectoderm or ciliated band						1
SPU_027969	Sp-FoxJ1	forkhead							2
SPU_025590, SPU_025738	Sp-FoxM	forkhead							2
SPU_018908	Sp-Six3 (sine oculis)	hbox-atypical							1,5
SPU_002603	Sp-SoxC (Sox4/11/22/24)	HMG							2,5
SPU_002592	Sp-Emx (empty spiracles)	Hox							1
SPU_016449	Sp-Hnf6 (onecut2)	hbox							9,10
SPU_009771	Sp-FoxG (Brain Factor 1)	forkhead							2
SPU_002815	Sp-DLX (distal-less)	hbox-other							1,5
SPU_008936, SPU_027487	Sp-Tlx1 (tail-less)	nuclear receptor							1,5
SPU_014418, SPU_027648	Sp-FoxD	forkhead							2
SPU_000129, SPU_022971	Sp-Arnt	aryl hydrocarbon receptor nuclear translocator							1
SPU_023941	Sp-Myt1 (myelin TF1)	zinc finger							20
SPU_028583	Sp-Zic2 (odd-paired)	zinc finger							5
SPU_010351	Sp-Irx (iroquois)	hbox-atypical	expressed outside embryonic apical ectoderm or ciliated band						1,5
SPU_019290	Sp-Otp (Orthopedia)	hbox-paired							17
SPU_005435, SPU_023739	Sp-PBX (extra denticles)	hbox-atypical							1
SPU_006991	Sp-Lim1	hbox-lim							2
SPU_016443	Sp-Brn1/2/4 (Pou class 3)	Pou class3							11
SPU_004551	Sp-FoxB (Forkhead1)	forkhead							2,14
SPU_014809	Sp-FoxL2	forkhead							
SPU_002627	Sp-Beta3A (Olig3)	bHLH							1
SPU_004028	Sp-Beta3B (Olig2)	bHLH							1
SPU_011315, SPU_017983	Sp-Ato (atonal 8)	bHLH							1
SPU_022242	Sp-zfh1 (z81, Kheper)	zinc finger	embryonic expression site is unknown						3
SPU_025492	Sp-Gbx (gastrulation brain Hbx)	hox							1
SPU_011297	Sp-Mbx1 (dien-/mesencephalon Hbx)	hbox-paired							1
SPU_010438	Sp-Pou6 (Brn5)	hbox-other							1
SPU_028422	SpRXRa (retinoidX receptor)	nuclear receptor							1
SPU_013134	SpRXRb (estrogen-related receptor)	nuclear receptor							1
SPU_023730	Sp-Isl (islet)	hbox-lim							1
SPU_013464	Sp-Phox2 (Arix, Pmx2a)	hbox-paired							1
SPU_006786+006787	Sp-Pax6 (Oculorhombin)	hbox-paired							15,18
SPU_014157	Sp-Lmx1 (Lim homeobox)	hbox-lim							1
SPU_014164	Sp-BarH	hbox-Bar class							1
SPU_004702	Sp-EBF3	HLH							1
SPU_017287	Sp-Hand	bHLH							1
SPU_000255	Sp-Nurr1	nuclear receptor							21
SPU_011202	Sp-Meis	hbox-atypical	expression has not been detected in embryos						1
SPU_001975	Sp-LXH3-4 (Lim hbx)	hbox-lim							22
SPU_025632	Sp-Pou4f (Brn3b)	Pou domain							1
SPU_003681	Sp-Ato7-like (atonal7)	bHLH							4
SPU_000990	Sp-Ato2b (atonal2b)	bHLH							4
SPU_003681	Sp-Ato7-like (atonal7)	bHLH							4
SPU_014401	Sp-N-twist (nephew of atonal)	bHLH							4
SPU_022554	Sp-ac-sc1a (Achaete scute-like)	bHLH							4
SPU_006813	Sp-hairy (Hes1)	bHLH							4
SPU_009465	Sp-Hey (Hes-like)	bHLH							1
SPU_022554	Sp-Ac-Sc3 (Achaete scute-like)	bHLH							4
SPU_030153	Sp-dbx (developing brain Hbx)	Hbox							4
SPU_000757	Sp-NKX2.1	hbox							19
SPU_012384, SPU_019002	Sp-FoxQ2	forkhead	Candidates only because they are expressed in apical ectoderm						2,5
SPU_008752, SPU_011174	Sp-Nfe2 (nuclear factor, RBC)	basic-zipper							1
SPU_004414	Sp-Hlf (hepatic leukemia factor)	basic-zipper							1
SPU_006753	Sp-E2F3	miscellaneous							1
SPU_007611	Sp-Rfx3 (regulatory factor X3)	miscellaneous							1
SPU_026099	Sp-Lox (PDX1)	Parahox							16, 23
Neural regulatory genes not found	vax (ventral anterior homeobox)	hbox							
	Pax3/7	hbox-paired							

1 M. Howard (annotator, QPCR, wmish); Howard et al., 2006a, 2006b; Howard and Davidson, 2006.

2 P. Oliveri (annotator, QPCR, wmish); Tu et al., 2006.

3 S. Materna (annotator); Materna et al., 2006.

4 L. Angerer (annotator).

5 L. Angerer (RTPCR, wmish).

6 E. Arboleta (annotator).

7 Li et al., 1997.

8 Kenny et al., 1999.

9 Otim et al., 2004.

10 Poustka et al., 2004.

11 Yuh et al., 2005.

12 H. Katow, NCBI, direct submi.

13 Yaguchi et al., 2006.

14 Luke et al., 1997.

15 Czerny and Bussingler, 1995.

16 M. Arnone, personal communication.

17 Di Bernardo et al., 1999.

18 S. Liang (annotator).

19 Takacs et al., 2004.

20 M. Arnone (annotator).

21 K. Walton (annotator).

22 T. Robertson (annotator).

23 P. Martinez (annotator).

how the metazoan gene set has been adapted to construct this puzzling organism.

Neural development

Neural specification

Understanding the structure and composition of gene regulatory networks that specify neurons is critical for learning how the sea urchin nervous system develops. Although little is currently known about neural specification in either larvae or adults, echinoderms are in an informative phylogenetic position and have the potential to contribute to our understanding of neural evolution. Here, our objective is to analyze the *Strongylocentrotus purpuratus* genome to define neurogenic gene regulatory networks in the larva, which we predict is likely to include core metazoan elements.

Searches of the genome have identified homologues of a number of genes known to encode transcription factors that are expressed in neural tissues in other animals (genomic methods are described in Sodergren et al., 2006). PCR, in situ hybridization or microarray analyses (Samanta et al., 2006) indicate that many of these genes are expressed during early development. Expression of several of these genes begins before gastrulation, the time when neuroblasts are first detected. We have categorized candidate neurogenic genes by what is known about their temporal and spatial patterns of expression during embryogenesis (Table 1, colored boxes). Whereas this list includes many genes, it represents only a starting point since more candidate genes will undoubtedly be found as experimentation and analysis progress.

One group comprises genes expressed exclusively in the neurogenic ectoderm at the animal pole (Table 1, blue). Two genes in this group begin to be expressed between the hatching blastula and early mesenchyme blastula stages, before the onset of morphogenesis. Transcripts from each of these genes accumulate in a nearly contiguous patch at the animal pole (Figs. 5A, C). Two of these, *Sp-Ac-Sc* (*achaete-scute*) and *Sp-Hbn* (*homeobrain*), are homologues of genes known to be involved in early neural specification in other systems: Achaete-scute is a basic-HLH atonal class factor whose function in neurogenesis is conserved in cnidarians, flies and vertebrates (Bertrand et al., 2002). In *Drosophila* embryos, *Hbn*, which encodes a paired-class transcription factor, is required for development of the brain (Davis et al., 2003) and is expressed in the presumptive brain, at the cellular blastoderm stage (Walldorf et al., 2000). Other sea urchin genes expressed exclusively in animal plate ectoderm encode the well-known proneural, atonal class factors, neurogenin and neuroD, and are expressed later in development, apparently exclusively in neural cells (LMA, unpublished; H. Katow, NCBI submission).

Genes in a second group are also expressed in the animal plate ectoderm, but expression is not confined to this region (Table 1, green). The onset of expression refers to the whole embryo and in most cases expression in the animal plate is later than in other tissues. This group includes genes widely expressed in the ectoderm (*SoxB1* and *B2*, Kenny et al., 1999)

or throughout most of the embryo (*Otx*, Li et al., 1997), but which are critical factors in neural development in other animals (Sasai, 2001; Boyl et al., 2001). Also in this group is the gene encoding *Sp-Rx* (retinal anterior homeobox), another paired-class transcription factor. In vertebrates, *Rx* is expressed in the early anterior neural plate at the gastrula stage (Furukawa et al., 1997) and subsequently in the retina where it is required for eye development (Mathers et al., 1997). The early expression domain of *Sp-Rx* is nearly identical to that of *Sp-Ac-Sc* and *Sp-Hbn* (Fig. 5E). As in *Drosophila*, the expression patterns of these three genes establish that a neurogenic territory is specified after the period of rapid cell division and before morphogenetic movements. Thus *Sp-Rx*, *Sp-Hbn* and *Sp-Ac-Sc* likely operate near the top of a neuronal gene regulatory network. As development proceeds, expression of these genes becomes confined either to a subset of cells in the apical plate of the prism-stage embryo (*Sp-Ac-Sc*; Fig. 5B; *Sp-Rx*; Fig. 5F), or to neurons in the oral ganglia and adjacent to the ciliary band of the pluteus larva (*Sp-Hbn*; Fig. 5D).

Additional candidate genes that function in neural development in other animals are not detectable in the larval nervous system (Table 1, red). However, they may function in later neural development. Genes in this group are similar in sequence to known neural factors, but either their spatial patterns of expression are not yet known (gray) or not detectable in the embryo (brown). Interestingly, additional proneural-like genes are found among those not detectably expressed during the first 2 days of embryogenesis, the period covered by the microarray analysis (Samanta et al., 2006), raising the possibility that they regulate later stages of neural development in either the larva or adult. A few genes have been included as neural candidates because they are expressed in the embryonic neurogenic region, although their homologues have not yet been shown to be expressed specifically in neural tissue (purple group). One of these (*NK2.1*) is not required for initial differentiation of embryonic neurons (Takacs et al., 2004; K. Peterson, personal communication), but a subsequent role in the nervous system remains possible. Finally, by the criterion of reciprocal BLAST searches of the genome, *ventral anterior homeobox* (*vax*) and *Pax3/7* have not been conclusively identified.

As expected, this survey has shown that the sea urchin genome contains many homologues of genes involved in neurogenesis in deuterostomes and protostomes. The neurogenic functions of several of these genes are ancient because they are conserved in cnidarians. In fact, the genes encoding transcription factors that are expressed in early phases of neurogenesis (*Sp-Rx* and *Sp-Hbn*, and *Sp-Ac-Sc*; Figs. 5A, C, E) are similar to those expressed at early stages of the neural program in *Hydra* that include the paired-class protein genes, *prdla* and *prdlb* (Miljkovic-Licina et al., 2004) and the *achaete-scute* homologue, *CnASH I* (Grens et al., 1995). This set of conserved early neurogenic factors may also include Zic, a *Drosophila odd-paired* homologue. The *Hydra* homologue, *HyZic*, is expressed in neural stem cells and is involved with the proliferation of neuroblasts (Lindgens et al., 2004); the ascidian homologue, *HrZic*, functions in early specification of
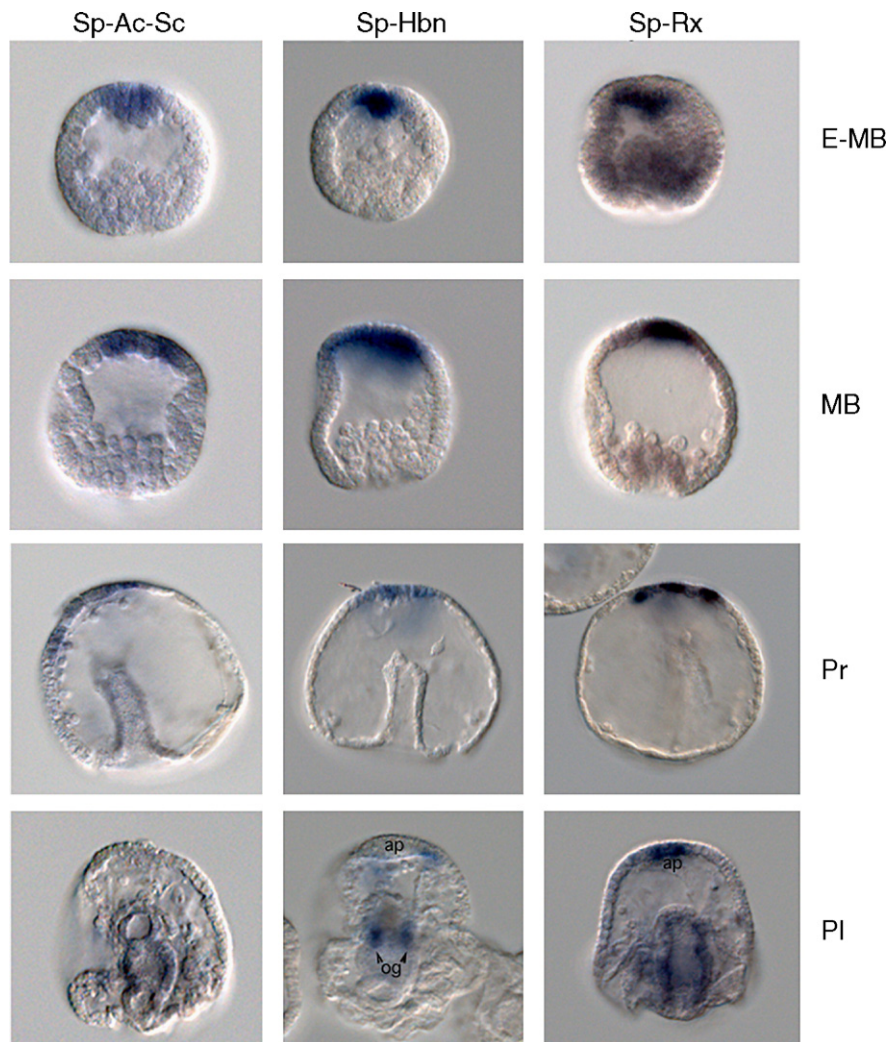
Fig. 5. *Sp-Sc-Ac* (achaete-scute), *Sp-Hbn* (homeobrain) and *Sp-Rx* (retinal anterior homeobox) transcripts accumulate in the apical ectoderm at blastula stage and in a subset of cells of the nervous system in the pluteus larva. Hybridization probes are shown in columns and stages in rows; lateral views of embryos have animal plate ectoderm at the top. *Sp-Rx* RNA accumulates in animal plate ectoderm and to lower levels in vegetal cells of blastulae (E-MB), is restricted to animal plate ectoderm in mesenchyme blastulae (MB), and is in scatted cells of the animal plate (ap) and around the gut in plutei (Pl). *Sp-Ac* is expressed in scattered cells of the animal plate ectoderm at prism stage (Pr). *Sp-Hbn* is expressed in oral ganglia (og) and scattered cells near the animal plate of plutei. Whole mount in situ hyrbridizations were carried out according to Minokawa et al. (2004). Embryos were photographed with DIC optics.

some neuronal precursors (Wada et al., 2002); and the *Xenopus* homologue is thought to function as a link between the neural inducer, chordin and the neurogenic gene, *neurogenin* (Mizuseki et al., 1998). A sea urchin Zic protein, *Sp-Zic2*, also a homologue of *odd paired*, has been shown by microarray analysis to be expressed in the embryo between egg and gastrula stages. Reciprocal BLAST identifies *Sp-Zic2* as homologous to *odd paired* and its expression begins during early blastula stage (LMA, unpublished). This conserved collection of genes, *paired-class*, *achaete-scute* and *Zic* (Table 1, yellow) appears to be expressed at the same points in the neurogenic programs of a diverse set of organisms. This supports the hypothesis that they are core components of a metazoan neurogenic gene regulatory network and its expression begins in the animal plate ectoderm of early blastulae.

Insight into the evolution of the nervous system may come from detailed phylogenetic comparisons of sea urchin, cnidarian

and protostome neurogenic gene sets leading to discovery of ancestral, deuterostome-specific genes. Shared components and pathways have the potential of revealing how metazoan neurogenic gene regulatory networks have been shaped by evolution to produce the vertebrate nervous system.

Signaling that specifies neurons

Cellular interactions mediated by ligands and receptors are known to specify and pattern the nervous systems of metazoans. The genome of *S. purpuratus* encodes ligands and receptors for all of the major signaling molecules that have previously been demonstrated to have a role in neural specification and patterning (Croce et al., 2006a; Beane et al., 2006; Walton et al., 2006; Lapraz et al., 2006). Many of these genes are expressed during early cleavage, the time when such specification must occur. Some are expressed in neurogenic domains

(Croce et al., 2006b) and there is evidence that early specification events have an impact on the abundance and patterning of larval nerves (Yaguchi et al., 2006). Access to genomic data will undoubtedly reveal approaches to investigating the signaling that underlies neural specification in sea urchin embryos. An understanding of the signaling mechanisms specifying neural tissues in urchins will provide critical data for comparison with this area of research that is well-studied in *Drosophila*, *C. elegans* and vertebrates.

Axon guidance

Neurogenesis in sea urchin embryos involves elongation of neurites that have terminal growth cones and the neurites form precise patterns in which neurons and effector cells are interconnected in a predictable pattern (Nakajima et al., 2004). The implication of these observations is that axon guidance mechanisms function during neurogenesis. The genome contains orthologues of all the deuterostome axon guidance molecules. There is a single B-type Eph receptor and a single, B-type eprhin ligand. An orthologue of semaphorin and numerous plexin receptors have been identified (Whittaker et al., 2006). There are predictions for orthologues of Netrin and the axon repulsion receptor Unc-5. A Slit orthologue and its receptor Robo have also been identified. In addition, the neural adhesion molecule L1 (Neurofascin) is present. Preliminary data indicate that *Sp-Eph* is expressed in the ciliary bands of the larva and in adult radial nerves (RDB, preliminary data) and L1 appears to be expressed in larval nerves (D. McClay, personal communication). The small number of paralogues may permit incisive determinations of how this set of metazoan axon guidance molecules functions in neurogenesis in sea urchins.

Synapse formation

Echinoderm synaptic morphology is reported to be unusual in that morphological studies have failed to identify direct synaptic contacts and pre- and post-synaptic specializations. Pre-synaptic terminals appear as swellings, but they do not appear to directly contact post-synaptic membranes (Cobb and Laverack, 1967; Cobb and Pentreath, 1976, 1977; Pentreath and Cobb, 1972). In some situations, it has been proposed that substantial distances separate neurons and effector cells (Florey and Cahill, 1977). The genome encodes several genes that in other metazoans mediate the processes of synapse formation. Orthologues for neuroligin, β-neurexin, agrin, MUSK and thrombospondin have been identified by reciprocal BLAST searches, PFAM and Smart searches (Whittaker et al., 2006). In vertebrates, β-neurexin binds neuroligin, which appears to function in recruiting post-synaptic components (Washbourne et al., 2004). Agrin is a nerve derived, heparan sulfate proteoglycan extracellular matrix component that interacts with MUSK to aggregate acetycholine receptors in post-synaptic terminals (Gautam et al., 1999). Echinoids appear to have these molecules that are specialized for roles in synapse formation in vertebrates, but it is not known if they are involved in synapse formation in urchins. EST's encoding Agrin (SPU_0 22633) and thrombospondin

(SPU_017370) have been identified in primary mesenchyme libraries suggesting a more generalized role in cell adhesion.

Electrical signaling

The ability of neurons to generate and transmit electrical signals is dependent on expression of voltage-gated ion channels (VGICs) that are permeable to ions including potassium (K^+), calcium (Ca^{++}) and sodium (Na^+). The proteins that form the ion pore of VGICs (alpha subunit) have sequence similarity consistent with a common evolutionary ancestry and members of this family have also been identified in prokaryotes (Hille, 2001). Relatively little is known about the electro-physiological properties of neurons in echinoderms, although there is evidence for calcium-based action potentials (Cobb and Moore, 1989). No VGIC molecules have previously been identified in echinoderms, but our analysis of the sea urchin genome has uncovered 11 K+ channels, 4 Ca++ channels and 1 Na+ channel (Table S1). Voltage-gated sodium channels in animals probably evolved from T-type calcium channels prior to the radiation of the bilateria because they share more sequence similarity with T-type calcium channel alpha subunits than with other VGICs and are present in protostomes and deuterostomes. However, subtle changes in the sequences of voltage-gated ion channels can switch ion selectivity and therefore it is possible that the single putative Na^+ channel in *S. purpuratus* actually functions as a calcium channel. Experimental studies are required to address this issue.

Gap junction proteins

Gap junctions are intercellular channels derived from multimeric channel proteins that attach head to head to form a pore that permits the passage of small molecules between adjacent cells. Gap junctions provide ionic or electrical coupling between neurons and in vertebrates gap junction proteins are expressed in all tissues. Two unrelated families of proteins are known to form gap junctions: connexins, which have only been found in chordates, and pannexins, which include innexins and are found throughout metazoans (Panchin, 2005). Searches with BLAST, pfam and IPRSCAN have failed to find representative genes for any of these proteins in the urchin genome. Gap junctions have distinctive ultrastructural characteristics and several morphological studies have searched systematically for gap junctions without success (Cobb, 1987; Cavey and Wood, 1991; Florey and Cahill, 1977, 1980). Thus, sea urchins appear to lack gap junction proteins, which implies that their neurons lack ionic coupling and must rely on chemical synapses for communication. Some classes of cnidarians appear also not to have gap junction proteins, suggesting that this condition may have arisen more than once in metazoan evolution (Mackie et al., 1984; Mire et al., 2000).

Neurotransmitter release

Neurotransmitter release at nerve terminals is mediated by calcium-dependent fusion of synaptic vesicles (SV) with the

plasma membrane. Synaptic vesicle trafficking has been studied in a few model organisms: rodents, *Drosophila* and *C. elegans* (Sudhof, 2004; Richmond and Broadie, 2002). In the SV trafficking cycle, vesicles are filled with neurotransmitters, dock at the active zone and are primed to become calcium sensitive. Arrival of action potentials in the axon terminal causes voltage-gated calcium channels to open and trigger fusion of SVs with the plasma membrane, releasing neurotransmitter into the synaptic cleft. Following fusion, empty SVs are recycled and refilled for another round of neurotransmitter release.

Regulated secretion (exocytosis) appears to proceed through common mechanisms in eukaryotes, mediated by highly conserved proteins; some exist as single copy genes and others belong to multigene families (Jahn et al., 2003). These proteins are generally conserved in the *S. purpuratus* genome, with numbers of isoforms that are similar to other invertebrate metazoans (Table S2). There are no published studies on the SV cycle in *S. purpuratus*; however, homologues of several critical proteins have been characterized in studies on the role of calcium-triggered exocytosis in egg maturation and fertilization (Avery et al., 1997; Conner et al., 1997; Leguia and Wessel, 2004; Schulz et al., 1998; Tahara et al., 1998; Whalley et al., 2004).

Membrane attachment

The Rab GTPases are a multigene family that mediates targeting of intracellular vesicles to target membranes (Pereira-Leal and Seabra, 2001). *S. purpuratus* has 33 Rab genes, similar to *C. elegans* and *Drosophila melanogaster* (29) and fewer than human (~50). Rab3 is the major isoform bound to SVs. The *S. purpuratus* homologue has 47% identity to vertebrate and mammalian Rab3 (Table S2). The GTP-bound form of Rab3 binds to specific effectors in the active zone: Unc-13 and RIMs (Rab3-interacting molecules). *S. purpuratus* Unc-13 is smaller (898aa) than its homologues in zebrafish (1121aa) or mouse (1797aa). These proteins have highly conserved (50–80% identity) C2 domains at their amino- and carboxy-termini. The size difference is due to expansion of the central region of Unc-13 in mouse and zebrafish. In vertebrates, RIMs are large, multidomain proteins (~1500 aa) with a Rab3 binding site near the amino-terminus. The single *S. purpuratus* RIM homologue (*Sp-RIM2*) is a small protein (305 aa) similar to the C-terminal region of mammalian RIM2 (55% identity) and lacking the Rab3 binding site (Table S2). These differences in Rab3 effectors suggest that the mechanism of SV docking to the active zone may differ between echinoderms and vertebrates.

The exocyst complex is another highly conserved Rab effector, consisting of eight subunits (*Sp-Exoc1-8*). It mediates insertion of proteins into the plasma membrane, and possibly formation of synaptic junctions (Clandinin, 2005).

SNARE and SM proteins

The SNAREs are small, membrane-anchored proteins with one or two 70-residue SNARE domains. Genetic studies indicate that SNAREs are required for both intracellular vesicle trafficking and secretion in eukaryotes (Sudhof, 2004; Jahn et al., 2003). The four classes of SNARE domains, Qa, Qb, Qc and R, form a stable alpha helical bundle (core complex) comprised of 3–4 SNAREs (some SNAREs contain both a Qb and a Qc domain) anchored in the vesicle and target membranes. *S. purpuratus* has at least 17 SNAREs including eight syntaxins (Stx 1, 5, 6, 8, 12, 16, 17, 18) (Table S2). Syntaxins are highly conserved among *S. purpuratus*, vertebrates (*Danio*) and mammals, having 30–70% identity over their entire length. It is interesting to note that *S. purpuratus* is apparently missing a node from the mammalian syntaxin family tree comprising syntaxins 2, 3, 4 and 11 (Teng et al., 2001). Syntaxins 2–4 are located in the plasma membrane and mediate transport of proteins to the cell surface and protein secretion.

In SV fusion, VAMP1 (vesicle-associated membrane protein)/synaptobrevin (R-SNARE) on the vesicle, forms the core complex with SNAP25 (Qb, Qc-SNARE) and syntaxin 1 (Qa-SNARE) on the target membrane. *S. purpuratus* appears to have single-copy genes coding for VAMP1, SNAP25 and syntaxin 1 homologues (Table S2).

SM proteins bind to SNAREs and may regulate core complex assembly (Sudhof, 2004; Jahn et al., 2003). Genetic studies in rodents indicate that these proteins are required for SV fusion. *S. purpuratus* has a single gene encoding a homologue of the mammalian Sec1/munc18 family, *Sp-Stxbp* with 60% identity to vertebrate and mammalian Stxbp1.

Following vesicle fusion with the target membrane, the SNARE complex is disassembled through the action of the NSF ATPase (*N*-ethyl maleimide-sensitive factor) and soluble NSF-attachment proteins (SNAPs). *S. purpuratus* has single genes encoding NSF (*Sp-Nsf*) and alphaSNAP (*Sp-Napa*) homologues with >60% identity the vertebrate and mammalian proteins.

The expression patterns of these SV-specific SNAREs and associated proteins in echinoderm neural tissue have not yet been described. However, these proteins are also expressed in echinoderm eggs and early embryos where they are localized to the cortical region of the cell and may mediate exocytosis required for early embryogenesis (Conner et al., 1997; Leguia and Wessel, 2004; Tahara et al., 1998).

Synaptotagmins

Synaptotagmins (Syts) are a multiprotein family that have attracted considerable interest as potential sensors for calcium-triggered neurotransmitter release (Sudhof, 2002). Synaptotagmins are anchored in vesicle and target membranes at their amino-terminus, and have two tandem C2 domains (termed C2A and C2B) which bind to phospholipid in the presence of calcium. Membrane fusion may be triggered by binding of the C2 domains to the target membranes, in cooperation with the SNARE complex (Arac et al., 2006). Synaptotagmins bind to SNAREs in vitro and co-localize with SNAREs and SM proteins in vivo.

There are 17 Syt isoforms in human and rodents, expressed mainly in neural tissue (a few isoforms are non-neuronal). Genetic studies indicate that Syts 1 and 2 are required for the fast, synchronous component of neurotransmitter release (Sudhof,

2002, 2004; Jahn et al., 2003). Other Syts may mediate the slow, asynchronous component of release, and secretion from neurendocrine cells. Some isoforms (Syts 8, 12–16) lack calcium-binding activity; their functions are unknown.

S. purpuratus has 14 synaptotagmin isoforms (Table S2). These proteins have tandem C2 domains with greater sequence similarity to the C2A–C2B domains of Syts than to other C2 domain proteins (>30% identity, Expect value $<1e^{-15}$). We have adopted a provisional nomenclature for *S. purpuratus* syts, based on the most similar mammalian isoform. Nine isoforms exhibit a high degree of similarity to a particular mammalian isoform (>50% identity, Expect value $<1e^{-50}$) and may be considered to be orthologues: Syt1-1, Syt5, Syt6-1, Syt7, Syt9, Syt12, Syt14, Syt15-1, Syt17. The other isoforms: Syt1-2, Syt4, Syt6-2, Syt15-2 have similar degrees of similarity to two or more mammalian syts and are thus difficult to classify according to the mammalian tree. Three of these isoforms have a tandem duplication of the N-terminal 1/2 (Syt1-2, Syt12) or whole C2A domain (Syt15-1). Such duplications have not been described in syts from other species. This may reflect problems with the current genomic assembly; hopefully, cDNA cloning of these genes will resolve this issue.

A pairwise comparison of the predicted C2A–C2B domains, of the 14 *S. purpuratus* isoforms indicates that Syts 1–1, 5, 6–1, 7 and 9 comprise a closely related group (Fig. 6). These are all orthologues of mammalian syts known to have calcium–phospholipid binding activity and to be calcium sensors for regulated exocytosis (Chenna et al., 2003). The others, including orthologues of mammalian syts which lack this activity (Syts 12, 14, 17) are more distantly related (Fig. 6).

Syt1-1 (also termed Synaptotagmin B) appears to be the orthologue of mammalian Syt1, the calcium sensor for SV fusion. Both Syt1-1 mRNA and protein are expressed in the early pluteus (72–96 h) in the apical organ, the ciliary band and the adoral ciliary band surrounding the mouth, in cells resembling neurons and tracts of neurites, and also in adult neural tissues, consistent with a role in neurotransmitter release (Burke et al., 2006).

Neurotransmitters, transporters and receptors

Early studies of echinoid neurotransmitters used biochemical methods to detect neurotransmitters extracted from tissues and this provided good evidence that acetylcholine and some associated enzymes are present (Pentreath and Cottrell, 1968). Applications of neurotransmitters and inhibitors and histochemical methods have provided inconclusive information on other putative transmitters (Binyon, 1972; Cobb, 1987). Immunocytochemical methods have been helpful, but they have not been systematically applied. Thus, the full spectrum of neurotransmitters employed by echinoids is not known (Welsh, 1966; Cobb, 1987). We have analyzed the sea urchin genome for orthologues of proteins involved in the biosynthesis, reception or inactivation of neurotransmitters in other animals (Table S3).

Serotonin is synthesized from tryptophan by tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase

(AADC). Sea urchin TPH is known from *Hemicentrotus pulcherrimus*, (Yaguchi and Katow, 2003) and there is a single TPH homologue in the *Stronglylocentrotus* genome that is almost identical to HpTPH (93% amino acid identity). We identified 1 AADC gene from the *S. purpuratus* genome and 1 serotonin transporter gene that is most similar to serotonin transporters of other invertebrates (Table S3). Melatonin is derived from serotonin in a pathway that includes Serotonin *N*-acetyltransferase and acetylserotonin *O*-methyltransferase. However, neither gene has been found in either ascidians or sea urchins, suggesting that this may be a pathway unique to vertebrates.

Monoamine neurotransmitters, such as serotonin and dopamine are inactivated/degraded in the synaptic cleft and presynaptic terminal by the activities of monoamine oxydase (MOA) and catechol *O*-metyltransferase (COMT). Several MOAs have been identified in the sea urchin genome, and they include both types of MOA-a and MOA-b (Table S3). One COMT was also found in the genome.

Serotonin receptors are categorized into 7 types and 18 subtypes based on their amino acid sequences and responses to agonists and antagonists (Adayev et al., 2005). To date, invertebrate genomes have revealed only a few types of serotonin receptors. In the sea urchin genome, 4 serotonin receptors were found searching with extracellular and cytoplasmic domains of all known types of serotonin receptor (Fig. 7A). Although the BLAST results are strong for all of these predictions, in neighbor joining trees of aligned sequences clear relationships cannot be readily resolved. Type-3 serotonin receptors differ from other serotonin receptors in not being G-protein-coupled receptors. Urchins appear not to have type-3 receptors, suggesting that these might be a chordate innovation (Table S3).

The catecholamine dopamine is synthesized from tyrosine in a pathway including tyrosine hydroxylase (TH) and AADC. We have identified both genes in the sea urchin genome (Table S3). In the *Drosophila* genome, there are two types of dopamine receptor, whereas we found 7 putative dopamine receptors in the *S. purpuratus* genome. In a phylogenetic analysis, the sea urchin and *Drosophila* genes are most similar to the human D1-type dopamine receptor.

Noradrenaline is derived from dopamine by dopamine β-hydroxylase (DBH). Phenylethanolamine *N*-methyltransferase (PNMT) catalyzes the conversion of noradrenaline to adrenaline. A DBH-like gene is present in the sea urchin genome but PNMT genes have not been identified in either the tunicate or the sea urchin genome, suggesting that adrenaline may be specific to vertebrates. We identified 1 adrenergic receptor from the sea urchin genome that clusters weakly with *Ciona* and human adrenergic receptors (Table S3; Fig. S1).

GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD). *Drosophila* and human genomes have two types of GAD and there is a single GAD gene in the *Ciona* genome. In the sea urchin genome, we found 1 GAD that is most similar to *Drosophila* GAD1 (Table S3). We found 1 ionotropic (A-type) GABA receptor gene in the sea urchin
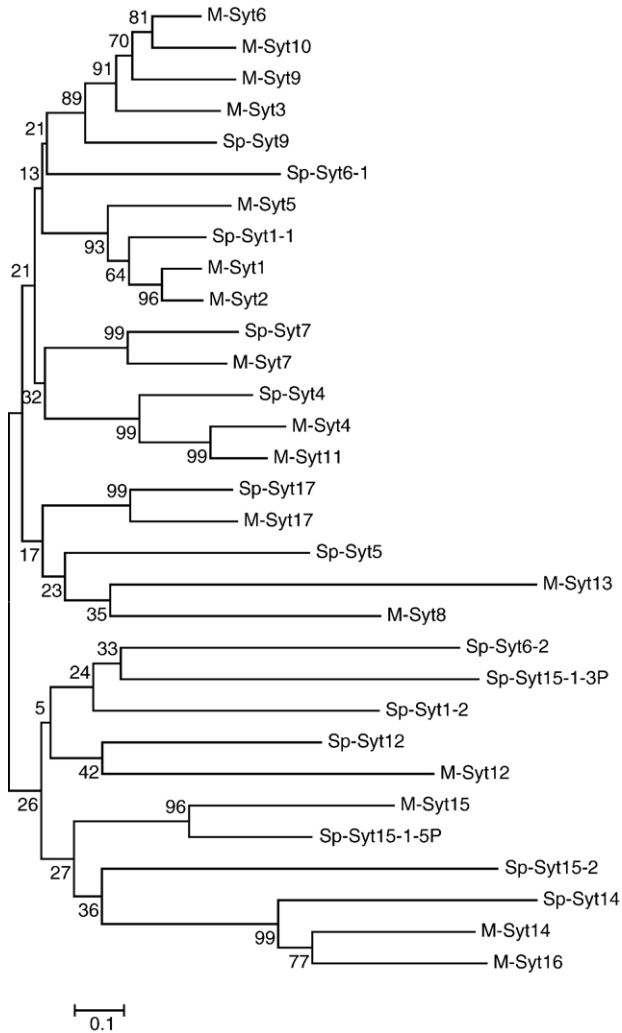
Fig. 6. Neighbor joining tree of the 14 synaptotagmin isoforms in *S. purpuratus*. Alignments of the C2A–C2B domains were made using ClustalW, numbers indicate bootstrap values (1000 replicates). For this analysis, the extra N-terminal C2 domain in SPU_16698 (*Sp-Syt15-1-5P*) and the two C-terminal C2 domains in SPU_28132 (*Sp-Syt15-1-3P*) were treated as different genes rather than alleles. The predicted amino acid sequence for *Sp-Syt1-1* derived from cDNA (AAB67801) was used instead of SPU_005854, as this gene model is missing part of the C2B region.

genome, whereas *Drosophila* and humans have several. In phylogenetic analysis, the sea urchin GABA receptor is most similar to the human GABA receptor type E (Fig. 7B).

Histamine is synthesized from histidine by histidine decarboxylase (HDC). HDC genes are known from *Drosophila* and humans, but in ascidian genomes HDC and histamine receptors have not been identified (Dehal et al., 2002). There are two HDC gene predictions in the sea urchin genome and three histamine receptors (Table S3). Phylogenetic analysis of the receptors suggests that two of these form a clade with human HRH3. Another appears to be most similar to a *Drosophila* histamine receptor. Thus, histamine appears to have a bilaterian origin with subsequent loss in ascidians.

Drosophila, ascidians and humans have a single choline acetyltransferase (ChAT) and a single acetylcholinesterase

(AChE). In the sea urchin genome, we found 1 ChAT gene, and several AChE genes (Table S3). Vesicular acetylcholine transporter was also found in the sea urchin genome as in *Drosophila* and human.

Acetylcholine receptors are classified into two major groups, muscarinic (mAChR) and nicotinic (nAChR). In humans, there are 5 types of mAChR receptors (M1–M5). We identified four mAChR in the sea urchin genome (Table S3). Phylogenetic analysis shows two of these form 1 group with human mAChR-M4. The other two position weakly within the same clade. The sea urchin genome also contains 12 genes for nAChR. In phylogenetic analyses, four of these form clades with human nAChRs, and the other four group with non-vertebrate clades. The remainder has unresolved affinities (Fig. S1).

Glycine is known to function as a neurotransmitter through the glycine receptor (GLR). Humans have 4 A-type and 1 B-type glycine receptors. In the sea urchin genome, we found two glycine receptors (Table S3). One is similar to human GLRA1 and the other has no clear affinity to vertebrate glycine receptors.

These gene predictions indicate that sea urchins are likely to use a broad range of neurotransmitters. However, there is no evidence that they are able to use either adrenalin or melatonin, as is the case for *Ciona* (Dehal et al., 2002). These data indicate that although the organization of the echinoid nervous system differs from that of other deuterostomes, we can anticipate that the neurons are similar with respect to synthesis and perception of neurotransmitters.

Neuromodulators

Adenosine, cannabinoids, lysophospholipids and melanocortins

The G-protein-coupled receptors of classical neurotransmitters and neuromodulators, such as acetylcholine, catecholamines and serotonin are rhodopsin-like receptors known as α-type receptors (Fredriksson et al., 2003). A sub-group of mammalian α-type receptors includes adenosine, cannabinoid, lysophospholipid and melanocortin receptors. Orthologues of adenosine receptors are present in *D. melanogaster* and other protostomes, indicating a bilaterian ancestry of adenosine receptors. Consistent with this, we have identified an adenosine receptor gene in *S. purpuratus* (SPU_008789). Orthologues of mammalian cannabinoid, lysophospholipid and melanocortin receptors are not present in *Drosophila* and *C. elegans* (Elphick and Egertova, 2001, 2005). However, the *Ciona* genome contains an orthologue of mammalian CB1/CB2-type cannabinoid receptors and genes encoding receptors related to mammalian lysophospholipid receptors (Elphick et al., 2003; MRE, unpublished). Orthologues of mammalian CB1/CB2 cannabinoid receptors, lysophospholipid receptors and melanocortin receptors were not identified in the *S. purpuratus* genome, suggesting that this group of receptors is unique to chordates. However, cannabinoid binding sites have been detected on sea urchin sperm and endocannabinoids may prevent of polyspermy by blocking the acrosome reaction
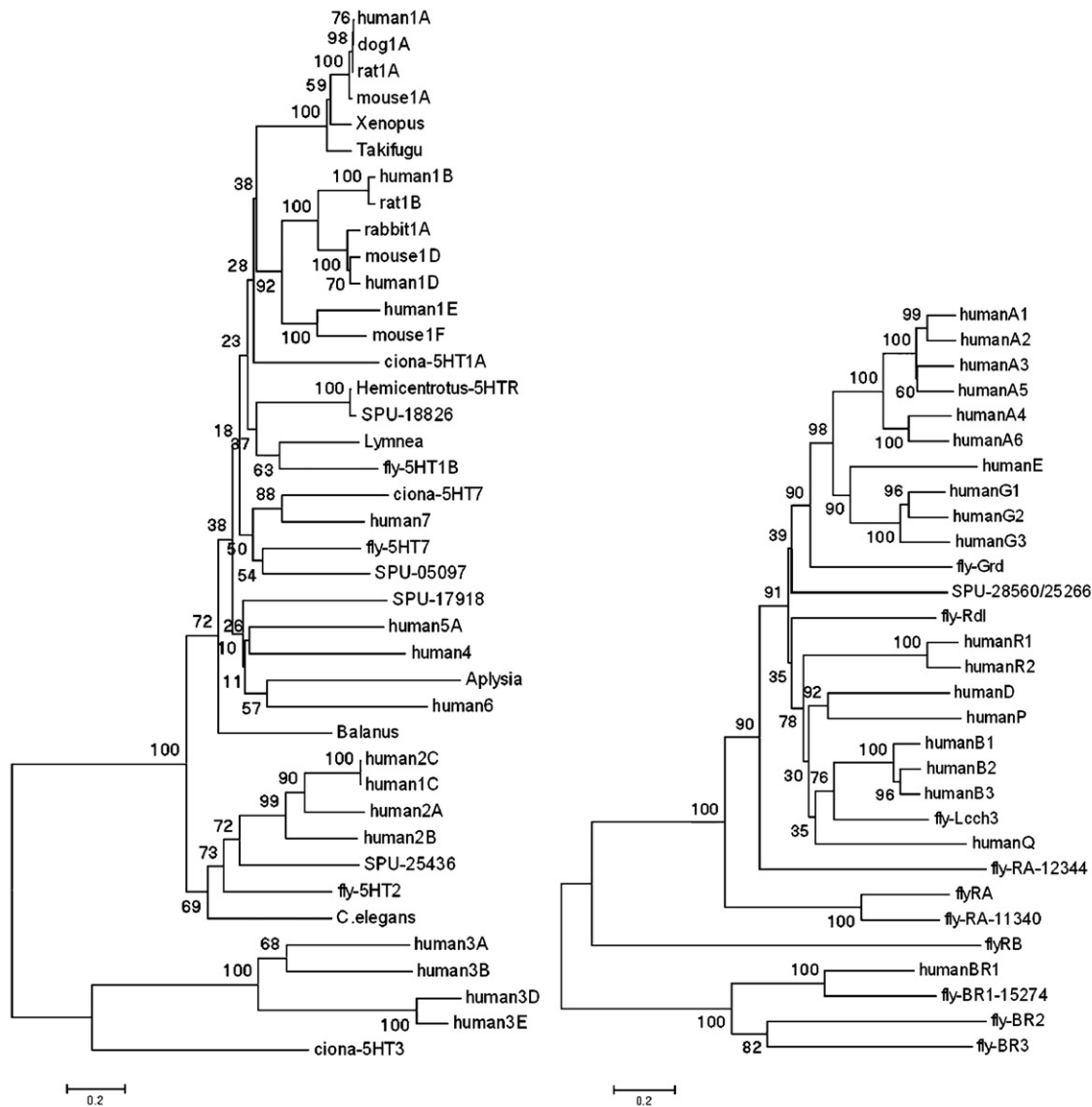
Fig. 7. Neighbor joining trees based on alignment of full length sequences and predictions for serotonin receptors indicate there are 4 sea urchin homologues that can be associated with known families of vertebrate and invertebrate receptors. (B) Neighbor joining tree based on alignments of full length cDNA sequences or predictions for GABA receptors. Numbers indicate bootstrap values (1000 replicates).

(Chang et al., 1993; Schuel et al., 1994). Further studies are required to determine the identity of the putative cannabinoid receptor detected on sea urchin sperm.

Nitric oxide

Nitric oxide (NO) modulates many physiological processes in animals. NO is produced by nitric oxide synthase (NOS) and often operates by stimulating the soluble guanylyl cyclase (sGC) receptor to produce cGMP. NO causes SGC/cGMP-dependent relaxation of the cardiac stomach in the starfish *Asterias rubens* (Elphick and Melarange, 1998) and subsequent studies indicate that NO acts as a general muscle relaxant in echinoderms (Elphick and Melarange, 2001; Melarange and

Elphick, 2003). Metamorphosis of the urchin *Lythechinus pictus* is negatively regulated by NO and cGMP (Bishop and Brandhorst, 2001). Immunostaining indicates that NOS is expressed in the larval nervous system in the apical organ, oral ganglion, gut and arms of plutei and a few ciliated neuronal cell bodies in the ventral transverse ciliary band that appear during larval development (Bishop and Brandhorst, 2001; and unpublished). These latter cells have projections to the apical organ that stain for NOS and microsurgical experiments are consistent with the hypothesis that these cells mediate the response of the larva to chemical cues for metamorphosis.

Analysis of the *S. purpuratus* genome reveals the presence of a gene that encodes an orthologue of the NOS proteins that have been identified in other metazoans (Table S4). However,

there are also 4 genes encoding NOS-like proteins and further studies are needed to determine the functions of these proteins. A family of genes encoding putative sGC subunits is present in *S. purpuratus*, which include 1 α subunit, 2 β1 subunits, 1 β2 subunit and several sGC-like proteins (Table S4).

Neuropeptides, peptide hormones and G-protein-coupled receptors

Secreted peptides generated by cleavage of larger precursor proteins are important neuroendocrine signaling molecules throughout the metazoans (Strand, 1999). There is evidence for a neuroendocrine system in echinoderms and several neuropeptides have been identified in starfish and sea cucumbers. However, neuropeptides have not been identified in sea urchins and it is not known if peptide hormones are produced by non-neural endocrine organs.

Most neuropeptides and many peptide hormones exert their effects by activating G-protein-coupled receptors (GPCRs). To obtain insight into the potential diversity of peptide signaling, we searched the *S. purpuratus* genome for orthologues of neuropeptide receptors. There are five families of GPCRs (Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin) and most peptide receptors belong to the rhodopsin or secretin families (Fredriksson et al., 2003). There are 37 *S. purpuratus* G-protein-coupled peptide receptors in the rhodopsin family (Table 2). In a similar analysis of the *Drosophila* genome, 32 putative G-protein-coupled peptide receptors were identified (Hewes and Taghert, 2001). The identities of the peptides that activate many of these *Drosophila* GPCRs have subsequently been identified (Cazzamali et al., 2002). Based on orthology with human GPCRs, we predict genes for a vasopressin/oxytocin receptor and a cholecystokinin/gastrin receptor (Table 2). The family of secretin receptors is exceptionally large with 161 members compared to only 15 in the human genome. Although genes that are related to the human corticotrophin-releasing hormone (CRH) and calcitonin receptors were identified, most sea urchin receptors in this family are not closely related to known vertebrate receptors. The expanded secretin family of GPCRs may be unique to sea urchins.

Although 37 sea urchin GPCRs related to human neuropeptide or peptide hormone receptors were identified (Table 2), it was more difficult to identify genes encoding precursors of the putative peptide ligands for these receptors. However, we have identified a gene encoding a peptide (echinotocin, Table 2) related to vasopressin/oxytocin/vasotocin peptides of vertebrates and protostomes (van Kesteren et al., 1992). Echinotocin may act as the ligand for a sea urchin orthologue of human vasopressin and oxytocin receptors.

Glycoprotein hormones occur throughout bilaterians and include the mammalian hormones luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone (Hsu et al., 2002; Luo et al., 2005). The sea urchin genome contains members of this family of hormones and GPCRs related to human glycoprotein hormone receptors (Table 2). Putative sea urchin glycoprotein hormones are of particular interest because they may function as gonadotropins. Gonadotropins in echino-

derms were first discovered in extracts of starfish nerve cords nearly 50 years ago (Chaet and McConnaughy, 1959), but their molecular identity remains elusive. Two other members of the glycoprotein hormone family in *S. purpuratus* appear to be orthologues of the alpha and beta subunits of the insect hormone bursicon (Table 2; Luo et al., 2005).

The sequences of several neuropeptides have been determined in starfish and sea cucumbers. The first to be identified in echinoderms were SALMFamides, which act as muscle relaxants (Elphick et al., 1991; Elphick and Melarange, 2001). Two related peptides, S1 and S2, were isolated from *Asterias* and subsequently other SALMFamides were found in holothurians. A SALMFamide precursor gene in *S. purpuratus* contains two exons encoding a signal peptide and seven putative SALMFamides (Elphick and Thorndyke, 2005). Although a number of other echinoderm neuropeptides have been identified in the sea cucumber *Stichopus japonicus*, based on a muscle activity assay (Iwakoshi et al., 1995; Ohtani et al., 1999), homologues of most of these could not be identified in the sea urchin genome. One predicted gene encoding two copies of a putative neuropeptide (NGFFFamide) does appear to be related to a *Stichopus* peptide (NGIWYamide), which causes muscle contraction (Inoue et al., 1999). A promising approach for further studies of many of the putative neuroendocrine peptides will be to determine if they have activity in standard bioassays using echinoid tissues.

Growth factors and growth factor receptors

Growth factors that bind to non-G-protein-coupled receptors include insulin, neurotrophins and the ependymins. From a structural and functional perspective, the distinction between peptide hormones and peptide/protein growth factors is arbitrary. Although often products of the nervous system, several are also expressed in non-neural tissues.

Insulin and IGF family

The insulin peptide family is one of the best-known neuro-endocrine systems of chordates. In vertebrates, insulin (INS) is a pancreatic endocrine hormone with a role in regulating of carbohydrate metabolism. Other members of this family include the insulin-like growth factors (IGFs), relaxins (RLNs) and insulin-like factors (INSLs) (Chan and Steiner, 2000; Conlon, 2000, 2001; Reinecke and Collett, 1998). IGFs are ubiquitously expressed and regulate cell proliferation and plasticity (Reinecke and Collett, 1998). RLN is a mammalian placental hormone important in angiogenesis and softening of ligaments during parturition (Wilkinson et al., 2005). Mature RLN and INS are derived from single chain precursors in which B and A domains are linked by a connecting C-peptide. During processing, the amino-terminal signal sequence is removed and the intervening C-peptide is cleaved leaving B and A domains linked by disulphide bridges. In contrast, IGFs not only retain the C-peptide but also contain D and E domains. This family of peptides interacts with tyrosine kinase receptors (INS/IGFs) or GPCRs (INSLs/RLNs) and there are binding proteins responsible for their transport. Invertebrate insulin-like peptides constitute large,

Table 2
Gene predictions for G-protein-coupled peptide receptors and peptide precursors

Rhodopsin-type G-protein-coupled peptide receptors	
Vasopressin/Oxytocin/Gonadotropin-releasing hormone-like receptors	SPU_021291, SPU_028207, SPU_001850, SPU_026012
Vasopressin/Oxytocin receptors	SPU_021290
Endothelin/Bombesin-like/Gastrin-releasing peptide/Neuromedin-B receptors	SPU_009901, SPU_027116, SPU_010606, SPU_011360, SPU_021160, SPU_008003
Neuromedin U/Neurotensin/Growth hormone secretagogue/Ghrelin receptors	SPU_004603, SPU_015957, SPU_016898
Cholecystokinin/Gastrin receptors	SPU_026458
Orexin receptors	SPU_023223, SPU_012799
Thyrotropin-releasing hormone receptor	SPU_010167
Tachykinin receptors	SPU_015140, SPU_015139
Galanin receptors	SPU_024231, SPU_022317
RFamide receptor (GPR54)	SPU_021219, SPU_007422, SPU_012318, SPU_022376, SPU_022468
RFamide receptor (GPR103)	SPU_006927, SPU_024924
Prolactin-releasing hormone receptor	SPU_020376
Leucine-rich-repeat-containing receptors 7 and 8	SPU_012382, SPU_005162, SPU_012383
Gonadotropin receptors	SPU_027375, SPU_000995, SPU_004389

Neuropeptide and peptide hormone precursors	
Echinotocin	SPU_006899
SALMFamides	SPU_021555
NGFFFamide	SPU_030074
Glycoprotein hormone β subunit	SPU_004405, SPU_005842, SPU_011451
Bursicon-α	SPU_003984
Bursicon-β	SPU_017707

divergent multi-gene families that share the basic insulin structure, including the conserved cysteines essential for formation of inter- and intra-chain disulphide bonds. Most invertebrate insulin-like peptides are expressed in the nervous system and act as neurohormonal growth regulators (Smit et al., 1998).

The *S. purpuratus* genome has two genes encoding members of the insulin family with paired cysteines, a propeptide containing the amino-terminal signal sequence and B, C and A domains (Table S5; Fig. S2). *Sp-IGF1* has characteristics of both the INSLNs and IGFs, including dibasic processing sites at the carboxy- and amino-termini of B and A domains, that may allow removal of the C domain, as in vertebrate insulin and relaxin. However, the A domain extends to D and E domains, as in the IGFs. In contrast, *Sp-IGF2*, is more IGF-like as predicted cleavage sites suggest that the C-peptide is not processed for removal. Gene predictions also exist for appropriate processing enzymes, receptors and binding proteins for the *Sp-IGF*s (Lapraz et al., 2006).

Both amphioxus and *Ciona intestinalis* have genes encoding proteins similar to *Sp-IGF1* and *Sp-IGF2*. Amphi ILP has been variously interpreted as being derived from a common ancestor of both INSLN/IGF or derived from an ancestral IGF (Chan et al., 1990). However, like *Sp-IGF1*, Amphi ILP has potential processing sites at the borders of B–C and C–A domains so the relationship is not straightforward. In *Ciona* Ci INS-L2 shares with *Sp-IGF1* processing sites for removal of the C domain

while Ci-INS-L1 and 3 are likely processed in a more IGF-like fashion and thus resemble *Sp-IGF2* (Olinski et al., 2006) (Table S5). The *S. purpuratus* INSLN and IGFs appear more similar to chordate peptides than to invertebrate INSLN and IGF.

Previous work suggests that *Sp-IGF*s may be expressed in the sea urchin larval gut (DePablo et al., 1988) and in adult starfish gut (Wilson and Falkmer, 1965). Microarray data confirm expression in the sea urchin embryo (Samanta et al., 2006).

Neurotrophins and Trk receptors

Neurotrophins regulate survival, growth and plasticity of neurons. Neurotrophins have two transmembrane–receptor systems: the Trk receptor tyrosine kinases and the common p75NTR. In tetrapods, neurotrophins and Trks include nerve growth factor (NGF) for which TrkA is a receptor, brain derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) for which TrkB is a receptor, and neurotrophin-3 (NT-3) for which TrkC is a receptor. Phylogenetic analysis suggests that the four neurotrophins and three Trks in extant vertebrates are each derived from an ancestral neurotrophin and Trk gene that was duplicated in concert with two whole genome duplications, on the lineage leading to the gnathostomes (Hallböök, 1999).

The neurotrophin/Trk signaling system has been proposed to be a vertebrate innovation (Satou et al., 2003) because no genes encoding neurotrophophins, Trks or p75 receptors have been found in the *C. intestinalis* genome (Dehal et al., 2002). However, the recent discovery of a Trk receptor in amphioxus established that Trk receptors originated prior to the cephalochordate/vertebrate split (Benito-Gutierrez et al., 2005). Furthermore, there appears to be a molluscan Trk-like receptor (LTrk) (van Kesteren et al., 1998), raising the possibility that this system is much older, present in the common ancestor to deuterostomes and protostomes. Analysis of the *S. purpuratus* genome provides further support for this idea because neurotrophin-, Trk- and p75NTR genes are *bona fide* orthologues of vertebrate genes, as shown by phylogenetic analysis (Fig. 8). *Sp-NT* protein is encoded within 1 exon, as are its vertebrate counterparts, and contains all the cysteines necessary to form a characteristic neurotrophin cysteine knot. *Sp-Trk* has a potential signal peptide and a canonical vertebrate domain structure (Fig. 9). The extracellular region of *Sp-Trk* shares with vertebrate Trk receptors two immunoglobulin (Ig)-like domains following a leucine-rich repeat. The most carboxy-terminal Ig domain contains conserved asparagine residues with structural roles for ligand–receptor interactions. On the cytoplasmic side, *Sp-Trk* includes a tyrosine kinase (TK) domain belonging to class II tyrosine kinase receptors (Prosite pattern PS00239 [DVYstdYYR]). *Sp-NT* does not cluster with any 1 vertebrate neurotrophin, implying a pro-orthologous ancestor (Fig. 8).

The neurotrophin/Trk system in *S. purpuratus* suggests that neurotrophin/Trk is a shared deuterostome feature. An important question is whether the urchin neurotrophin system is involved in neurotrophic regulation in a similar manner to the vertebrates.

Ependymins

Ependymin is a secreted glycoprotein implicated in a variety of cellular actions that are hallmarks of the complex neurotrophic factor family of proteins (Shashoua, 1991). Evidence links ependymin to consolidation of long-term memory, neuronal regeneration, learning and neuronal growth (Shashoua, 1985, 1991; Piront and Schmidt, 1988; Schmidt and Shashoua, 1988; Shashoua and Hesse, 1989; Schmidt et al., 1992). Ependymins have been postulated to play a role in establishing specific cell contacts (Hoffmann and Schwarz, 1996; Nimmrich et al., 2001). Recently, short peptides from goldfish ependymins were shown to function like neurotrophins (NGF and BDNF) during neuronal survival, proliferation and axon guidance (Gaiddon et al., 1996; Marsh and Palfrey, 1996; Adams et al., 2003; Shashoua et al., 2001). Ependymins, like neurotrophins,
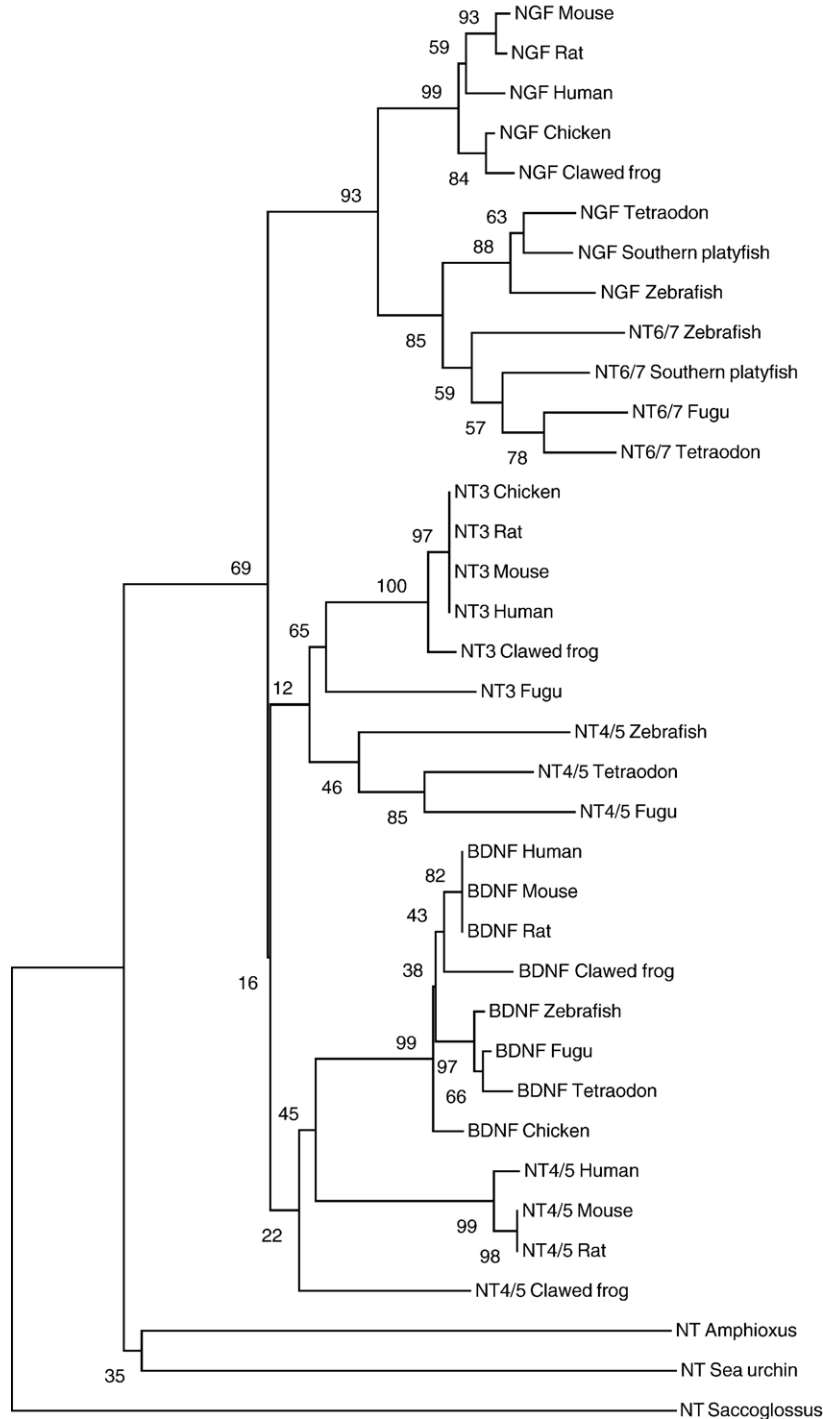

Fig. 8. Neighbor joining tree of ClustalW aligned mature neurotrophin protein sequences, numbers indicate bootstrap values (1000 replicates).

were proposed to be vertebrate-specific molecules. Recently, an ependymin-related gene was found in two holothurian species and in the sea urchin *Lytechinus variegatus* (Suarez-Castillo et al., 2004).

Predicted *S. purpuratus* ependymin genes encode peptides that contain four conserved cysteines crucial for dimeration, the strictly conserved residues D59, L107, P117, G129 and W140, and have sequence similarity to vertebrate ependymin-related proteins (Fig. 10). *Sp-Epen* is expressed during early larval development (Poustka et al., 2003) and transcripts have been localized to the intestine, esophagus, and gonads of *Lytechinus variegates* and in regenerating intestine of sea cucumbers (Suarez-Castillo et al., 2004). The expression of ependymin in these tissues may be neural since in fish, it is mainly expressed in the brain.

Other endocrine systems

Classic endocrine pathways in vertebrates and invertebrates include the steroid and thyroid nuclear receptors and their ligands, such as estrogens, progestogens, androgens, thyroxine and tri-iodothyronine in chordates and ecdysteroids in protostomes. Nuclear hormone receptors are transcriptional regulators involved in development, differentiation and endocrine hormone activity (Mangelsdorf et al., 1995). Studies of steroid hormones in echinoderms have focused on their roles in the gonads (Janer et al., 2005; Wasson and Watts, 2000), and recently on environmental aspects (Roepke et al., 2005). Only 1 member of the steroid/thyroid hormone receptor family has previously been characterized in *S. purpuratus* (Chan et al., 1992), but analysis of the urchin genome has now revealed genes for estrogen, androgen, glucocorticoid and retinoic acid receptors. A more complete analysis of nuclear receptors and transcription factors may be found in Howard-Ashby et al. (2006a,b).

The chordate thyroid gland is a compact mass of endocrine cells derived from the pharynx. At least some thyroid functions are conserved throughout deuterostomes. In fishes, endocrine cells are scattered ventrally around the aorta, and in tunicates, ancestral thyroidal tissue is a subset of endostyle cells. A thyroidal system is present in tunicates, although thyroglobulin itself has not been identified (Campbell et al., 2004). In echinoderms thyroxine plays a role in metamorphosis (Heyland and Hodin, 2004; Heyland et al., 2004). The sea urchin genome contains predictions for several thyroidal proteins including a sodium iodide transporter, a thyroid peroxidase for activating and coupling iodide to thyroglobulin, thyroglobulin and thyroid hormone nuclear receptors. Expression of the putative urchin thyroidal system remains to be determined. The key role of thyroid hormones in chordate development and metamorphosis raises the intriguing possibility that these regulators may play a similar role in echinoderms.

Sensory biology and behavior

Adult sea urchins detect and integrate a range of external stimuli using several sensory systems. They can discriminate among different foods; their spines respond to touch; they are probably thermotactic; and some are negatively geotactic (Lindahl and Runnstrom, 1929). Spines, tube feet and pedicellariae are extensively innervated, presumably for both motor and sensory purposes. The test and spines have a ciliated epithelium including interspersed cells with sensory hairs suggested to have a mechanosensory role (Hyman, 1955). Sphaeridia on the surface of their tests are thought to function as balance organs (Hyman, 1955; Fell and Pawson, 1966).

Echinoderm larvae also have a range of behaviors dependent upon sensory systems. Plutei use cilia to sweep currents of water over their bodies and redirect food particles toward the mouth where it is swallowed using coordinated muscular activity (Strathmann, 1975). Plutei quickly change their direction of swimming and move their arms in an evasive response to chemicals or touch. Plutei can maintain a fixed orientation with their arms up and exhibit phototactic and geotactic responses, including an ability to influence their position in the water column (Yoshida, 1966). Echinoderm larvae competent to metamorphose often select a substrate based on chemical cues prior to settling and initiating metamorphosis (Cameron and Hinegardner, 1974; Burke, 1983; Bishop and Brandhorst, 2001).

Chemosensory receptors

Although the behavior of urchin larvae and adults indicates that they respond to chemicals, olfactory organs have not been identified with any certainty. Chemoreceptors are likely included in the large family of GCPRs. Because of rapid divergence, genes encoding chemoreceptors are difficult to identify (Clyne et al., 1999). We used two approaches to identify putative chemoreceptors genes. In the first, termed the 7TM/PFAM/*C. elegans* search, we identified GLEAN3 predictions that contain the 7tm_1 Pfam domain (rhodopsin-like domain). Genes with significant similarity (e-value $\leq 1e^{-20}$) to any non-chemoreceptor
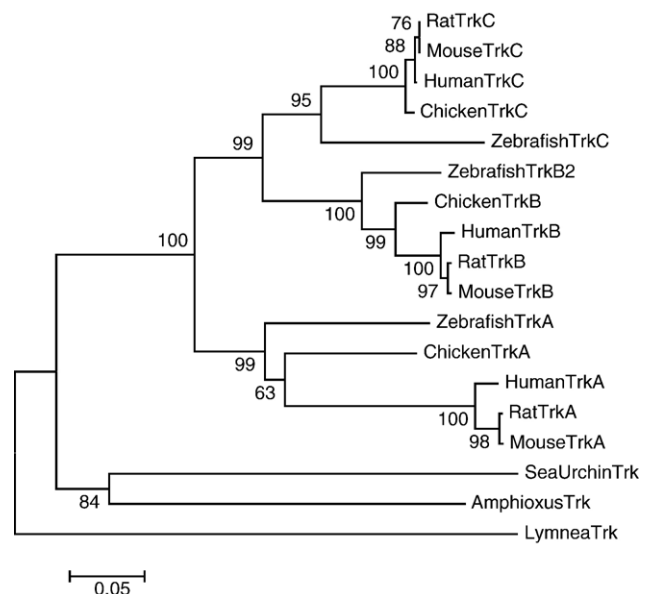

Fig. 9. Neighbor joining tree of the Trk receptor protein sequences with 500 bootstrap replicates.
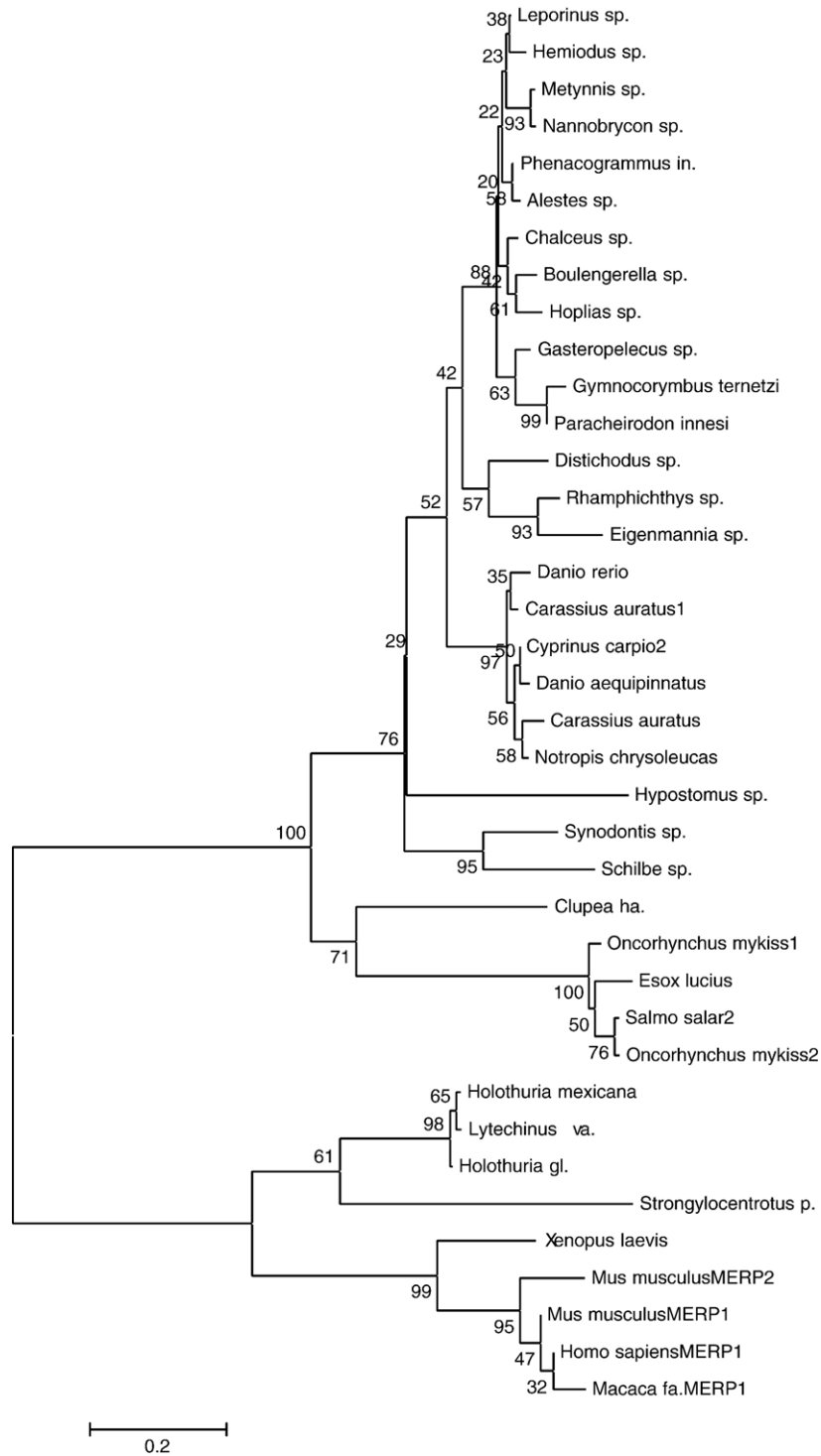
Fig. 10. Neighbor joining tree of ClustalW aligned vertebrate and echinoderm ependymin-related protein sequence, numbers indicate bootstrap values (1000 replicates).

in *C. elegans* were excluded. This yielded 678 putative chemoreceptor genes (Table S6). Many of these genes form tandem clusters, a property shared by chemoreceptor genes in all sequenced eukaryotic genomes. For example, 12 genes in a large tandem gene cluster consist of two sub-clusters, with a gap of 120 kb between them. Genes within each sub-cluster share considerable similarity, suggesting that repetitive tandem duplica-

tion has occurred (Fig. 11). The majority of these predicted genes lack introns, a property shared by essentially all chemoreceptor genes in vertebrates, but not those in the protostomes, *C. elegans* or *Drosophila*. Nevertheless, some of these putative echinoid chemoreceptors are similar to known chemoreceptors in protostomes, such as *C. elegans*. Raible et al. (2006) have also performed a 7TM screen and identified 979 putative

chemoreceptors after selecting full, non-overlapping domains at an e-value cutoff of 0.001. The sets of 678 and 979 putative chemoreceptors genes each contain many closely related genes that are arranged in clusters and many of the genes lack introns. Raible et al. (2006) have gone on to demonstrate that some of these genes are expressed in larvae and adult tissues including tube feet.

The second approach combined two strategies—an odorant-specific motif-based search and searches with three hidden Markov models (profile HMMs). The motif, LxxxxxxRxxAlxxPL, occurs in most zebrafish and mouse odorant receptors (ORs) (Alioto and Ngai, 2005) and was found in 43 GLEAN3 models. Blastp searches identified 11 additional closely related (≥ 70% identity) protein sequences that lack the motif. Five genes were subsequently removed from the set because they appear to have a non-homologous LxxxxxxRxxAIxxPL sequence or were ≥ 70% identical to a sequence with a non-homologous motif. However, not all mouse or zebrafish ORs possess the motif described above. To expand the list of candidate sea urchin chemoreceptors, we constructed profile hidden Markov models using HMMER (http://hmmer.wustl.edu) to query GLEAN3 peptides. The three profile HMMs used were based upon alignments of 12 sea lamprey odorant receptors (Berghard and Dryer, 1998), 16 fish T1R taste receptors (Ishimaru et al., 2005) and 239 fish odorant receptors (Alioto and Ngai, 2005). The lamprey odorant profile identified 91 sea urchin genes, the fish T1R profile identified 15 sea urchin genes and the fish odorant receptor profile identified 64 sea urchin genes (e-values ≤ 10^{-10}) (Table S7). Together the motif- and HMM-based searches identified 192 genes encoding putative sea urchin chemoreceptors. Of these, 74 can also be found in the set of 678 generated in the 7TM/PFAM/*C. elegans* search. The several hundred putative urchin chemoreceptors predicted by our approaches and by Raible et al. (2006) are similar to the numbers of chemoreceptors identified in many other animals, suggesting an elaborate chemosensory system operates in sea urchins.

Precursors of sensory placodes

The visual, olfactory, acoustic and gustatory systems of the peripheral nervous system of vertebrates develop from cells of the neural crest and several pairs of neurogenic cranial placodes that initiate as patches of thickened ectoderm (reviewed by Baker and Bronner-Fraser, 2001). Gans and Northcutt (1983) proposed that the neural crest and cranial placodes are distinctive innovations of vertebrate embryos that allowed for the extensive remodeling of the head during early vertebrate evolution. They also speculated that placodes evolved from a diffuse ectodermal nerve plexus similar to that in echinoderms and hemichordates (Lowe et al., 2003). However, whether the chordate ancestor had placodes or neural crest is controversial. On the one hand, marker gene expression and anatomical features suggest that embryos of the ascidian *C. intestinalis* (Mazet et al., 2005; Mazet and Shimeld, 2005) and the larvacean *Oikopleura dioica* (Bassham and Postlethwait, 2005) possess placode-like ectodermal specializations that form sensory organs. Furthermore, migratory neural crest-like cells have been detected by Jeffery et al. (2004) in ascidian

embryos. However, Holland and Holland (2001) argue that the sensory organ primordia derived from ectoderm in amphioxus are not placodes, but may have evolved into placodes and the neural crest. Schlosser (2005) agrees that sensory primordia in urochordates and cephalochordates are not the integrated placodes characteristic of vertebrates. These considerations raise the interesting question of whether basal deuterostomes, like echinoderms, have primordial patches of ectodermal cells resembling placodes that give rise to sensory organs.

To address this question, we used RT-PCR to assess the expression of members of gene families that are associated with placode development in vertebrates (Table 3). We compared expression levels of genes in sea urchin gastrula-stage embryos, early larvae, late larvae containing rudiments, adult radial nerves and adult tube feet. All the genes were expressed, often differentially. Transcript levels for *Sp-Eya1*, *Sp-Phox2*, *Sp-Pax1-9*, *Sp-EBF3* and *Sp-Pitx3* were relatively high in adult radial nerves compared to tube feet or larvae. *Sp-PaxB* was expressed at very high levels in all samples tested. The high level of expression in gastrulae presumably reflects involvement of PaxB with late histone gene regulation (Czerny et al., 1997). These observations indicate that genes collectively associated with placode formation are expressed in urchins when sensory organ primordia may be forming. It will be informative to determine the spatial patterns of expression of these genes in larvae and adults, particularly in the tube feet and pedicellariae that are derived from placode-like structures.

Photoreception

It is clear from their behavior that echinoderms are photo-sensitive. Although most echinoderms lack obvious light-sensitive organs, they nevertheless respond to light, photoperiod and lunar cycles (Boolootian, 1966; Yoshida, 1966). *S. purpuratus* adults are negatively phototactic (Giese and Farmanfarmanian, 1963) and photoperiod influences their reproductive cycle (Boolootian, 1963). Most urchins will quickly initiate a righting reaction to keep their oral surface in contact with the substratum and this behavior can be influenced by light (Reese, 1966; Yoshida, 1966). Many urchin species will select opaque rather than transparent objects to cover themselves in response to lighting from above (Millott, 1966; Yoshida, 1966).

Although sea urchins lack conventional eyes, they probably evolved from organisms that collected light through an aperture and focused it with a lens onto photoreceptor cells specialized to convert photons into neural signals (Fernald, 2000). All echinoderms display dermal photosensitivity based on diffuse dermal light receptors (Yoshida, 1966). Both surfaces of the calcitic endoskeleton (the test) and the pigmented radial nerves are photosensitive as are tube feet, which exhibit phototropic behavior and reflexive responses to light (Yoshida, 1966).

The translucent echinoderm test lined with photosensitive tissue provides an opportunity for lenses and filters to evolve. Single calcite crystals in brittle star skeletal ossicles can serve as microlenses in specialized photosensory organs that may collectively function as a compound eye (Aizenberg et al., 2001). A diffuse photoreceptor system with directional sensitivity may
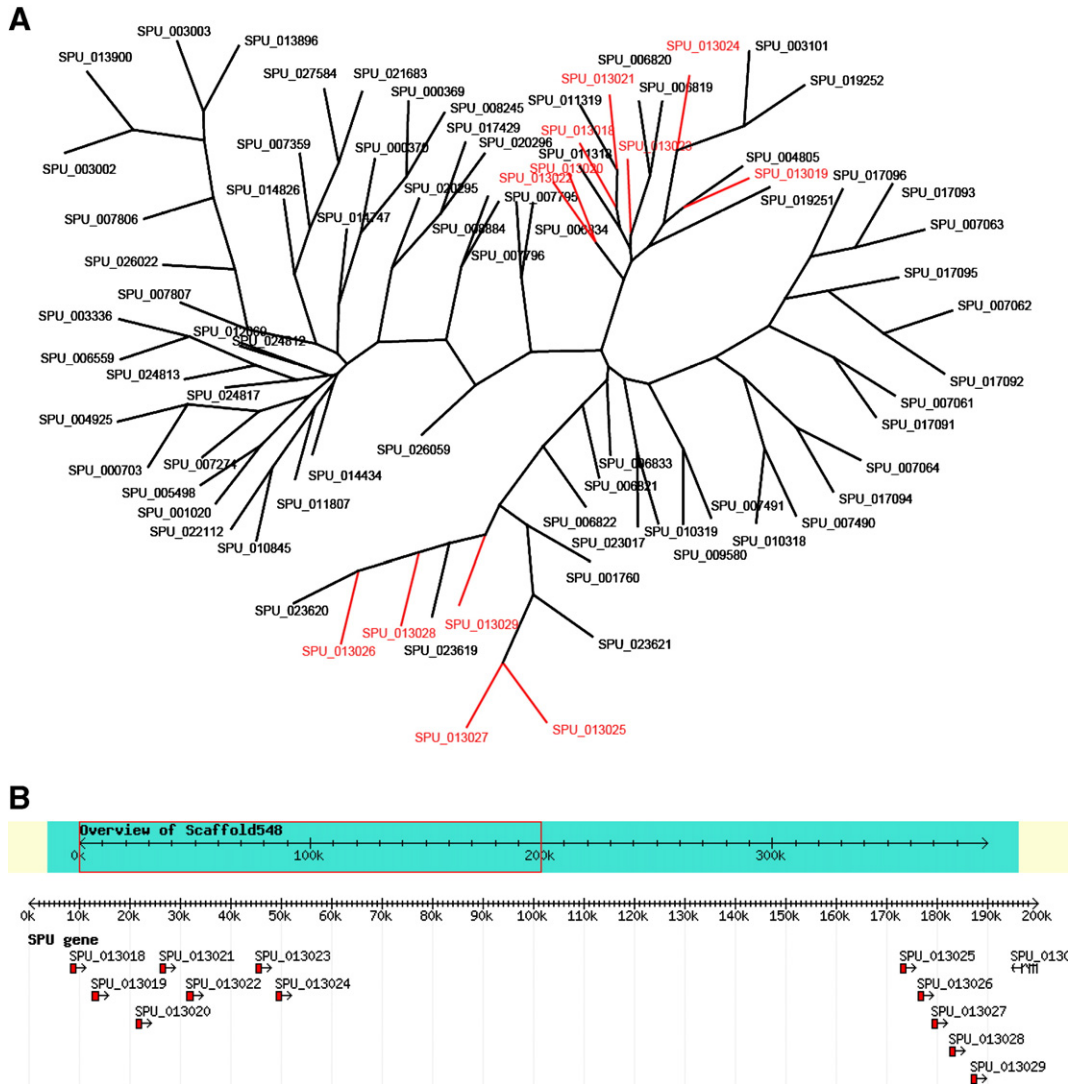
Fig. 11. Putative sea urchin chemosensory genes form clusters in genome. (A) Genes that show significant similarity (e-value $\leq e^{-100}$) to the putative chemosensory gene G13018 were pooled and aligned with ClustalW (Thompson et al., 1994). Alignment in the PHYLIP format was then analyzed in sequence with seqboot, protdist, neighbor, consense (PHYLIP, Felsenstein, 1988) with 100 bootstrap iterations. The resulting tree was then viewed (treeview) and edited (Adobe Illustrator). Genes coded in red are physically clustered on a single scaffold. (B) An example of the gene clustering found within putative chemosensory genes. Individual gene predictions that appear to be paralogous are found on the same scaffold. Genes in the cluster on the left are shown in the upper right of the tree in panel A, while those clustered on the right are shown on the lower left.

perceive images, albeit with limited resolution. Woodley (1982) suggested that the opaque spines of urchins might restrict the direction and angle of light reaching the photosensitive test, with the diffuse photoreception system of the test becoming a large compound eye. Consistent with this prediction, Blevins and Johnsen (2004) reported that urchins could locate and move toward dark targets and locate hiding places, apparently using their spatial vision.

Molecular evidence for photosensory organs in echinoderms is sparse, but the case is strongest for tube feet. There is a terminal nerve ring and a ganglion in each tube foot. As well, tube feet also connect directly with the radial nerves underneath the test. The buccal tube feet surrounding the mouth have modified suckers and are thought to be sensory appendages (Fig. 4; Hyman, 1955). The most intriguing observation is that cells adjacent to the longitudinal muscle fibers express a

homologue of *Pax6* (Czerny and Busslinger, 1995), a critical transcriptional regulator of vertebrate and invertebrate eye development (Gehring, 2005).

Retinal gene homologues

To investigate the cellular and genetic basis of echinoderm photosensitivity, we searched the *S. purpuratus* genome for genes homologous to known mammalian retinal genes because the retina is the neural tissue responsible for phototransduction and for making connections with other neurons required for visual responses. The first approach was based on 228 Math5-dependent mouse genes identified by microarray analysis, which have roles in specifying retinal cell fates (Mu et al., 2005). *Math5* is a mouse homologue of *Drosophila atonal*, a gene encoding a proneural bHLH transcription factor required for development of

Table 3
Expression of placode gene markers

Predicted gene	Gene name or synonym	Domain structure or orthology	Gastrula	Early larva	Late larva	Adult tube foot	Adult radial nerve
SPU_002815	Sp-Dlx	Homeobox TF	0.04	0.02	0.01	ND	ND
SPU_013869	Sp-Eya1	Tyrosine phosphatase	2.10	0.65	0.09	0.55	17.19
SPU_013464	Sp-Phox2	Fork head TF	0.02	0.17	0.23	0.86	13.92
SPU_004702	Sp-EBF3	COE family TF	2.12	1.48	0.89	0.24	8.11
SPU_014461 (contains SPU_024163)	Sp-Pitx1 (primer set1)	Homeobox TF	0.03	0.16	0.78	0.02	0.04
SPU_014461 (contains SPU_024163)	Sp-Pitx1 (primer set2)	Homeobox TF	0.52	0.25	1.73	0.08	0.02
SPU_004599	Sp-Pitx2	Homeobox TF	0.14	0.34	0.42	0.01	0.17
SPU_006159 (contains SPU_004598)	Sp-Pitx3	Homeobox TF	0.01	0.01	0.91	0.01	1.90
SPU_023894	Sp-FoxI	Fork head TF	0.68	0.39	0.89	0.00	0.02
SPU_027334	Sp-PaxA	Paired box TF	0.02	0.15	0.10	0.07	0.04
SPU_018351	Sp-PaxB	Paired box TF	138.99	18.95	6.34	4.80	5.89
SPU_006683	Sp-Pax1-9	Paired box TF	2.38	3.54	2.46	1.62	25.35
SPU_014539	Sp-Pax2-5-8	Paired box TF	7.83	0.70	0.55	0.16	0.06

Expression for each gene is based on a qRT-PCR assay and is shown as the percentage of the level of expression of Sp-ubiquitin in that sample, based on average of two determinations for each sample.

sensory organs and retinal ganglion cells (Wang et al., 2001; Brown et al., 2001). We used 228 protein sequences to BLAST search the GLEAN3 gene predictions at a threshold of e^{-20}, and found 117 putative homologues. In a second approach, we searched for candidate genes encoding transcription factors known to play important roles in mammalian eye and retinal development (Hatakeyama and Kageyama, 2004; Mu and Klein, 2004).

These searches identified 20 genes encoding transcription factors (Table 4). Some of these are involved in early retinal patterning and specification events include *Pax6* (Gehring, 2005), three members of the *Six* gene family (Zhu et al., 2002) (Donner and Maas, 2004) and *Rx*, a homeodomain gene essential for vertebrate eye development (Bailey et al., 2004). Genes involved in the later events of cell type specification and differentiation include three members of the *atonal* family, *NeuroD*, *POU4f* and *POU6f* homologues, several homeodomain-containing genes, and genes for zinc finger transcription factors. Sequence alignments suggest that there are single genes equally similar to mouse POU4F1 or POU4f2, NeuroD1 or NeuroD4, and Six3 or Six6 (Fig. S3).

In addition, we searched for genes encoding photo opsins because they are critical components in phototransduction. Four opsin homologues were identified with many signature features indicative of a prototypical photopigment (Table 4; Fig. S4). These include a highly conserved lysine (K398) that binds 11-*cis*-retinal (Wang et al., 1980), which absorbs photons causing a protein conformational change essential for phototransduction (Hargrave et al., 1984a,b), a conserved aromatic amino acid (Y170) in the third trans-membrane domain that stabilizes the 11-*cis*-retinal attachment and two critical cysteines (C167 and C245), essential for correct tertiary structure (Karnik et al., 1988). Three of the four *S. purpuratus* homologues contain a conserved arginine–tyrosine (R192–Y193) required for opsin-mediated G-protein activation, although only one has the preceding conserved acidic amino acid. Raible et al. (2006)

independently report finding two additional opsin homologues in the *S. purpuratus* genome, including rhabdomeric and ciliary opsins. Genes encoding a serine/threonine phosphatase (phosphatase 2A) and a tyrosine phosphatase (phosphatase 4) associated with phototransduction events downstream of opsin were also identified as Math5-dependent homologues in the urchin genome (Selke et al., 1998; Ensslen and Brady-Kalnay, 2004), as well as genes associated with neural transmission (a voltage-gated potassium channel) and with neuronal microtubules (Tubb2/Tubb3, Tubb4) (Table 4).

The fact that the sea urchin genome contains many genes encoding homologues of retinal transcription factors, opsins and phototransduction proteins provides strong evidence for a phototransduction system. Because tube feet are excellent candidates to have such systems, we tested whether other retinal gene homologues were expressed in this tissue using real time RT-PCR (Table 3, Table S8 for primers). In addition to Pax6, *Sp-NeuroD*, *Sp-Blimp1*, *Sp-Six3*, *Sp-BarH1*, *Sp-Rx* and *Sp-Ato* are all expressed in tube feet at levels ranging from 0.0015% to 2% of levels for *Sp-ubiquitin* transcripts (Table 4). Detectable PCR products were not obtained for *Sp-Ato2b*, *Sp-Ato7* and an *Sp-opsin* (SPU_027634). However, there are 3 *Sp-opsins* that are expressed in tube feet. Raible et al. (2006) also report that opsin genes are expressed in this tissue as well as in growing larvae. Although we have not shown that expression is specific to tube foot neurons, or that the genes are expressed together in the same cells, the results support the hypothesis that tube feet contain cells that express many of the genes expressed in mammalian retinas.

We also tested whether the set of retinal genes expressed in adult tube feet are also expressed during embryogenesis (Table 4). Two opsins, *Sp-Opsins* and *Sp-BarH1*, are expressed only in tube feet, whereas 1 *Sp-opsin*, *SpPou4f1* and *Sp-NeuroD* are expressed in 72 h embryos and tube feet but not in 24 h embryos. Furthermore, the relative expression levels were quite different among the three samples. For example, *Sp-Blimp1* and

Sp-Six3 are expressed at relatively high levels in embryos while the same genes are expressed at lower relative levels in tube feet. Raible et al. (2006) report that opsin genes are expressed in growing larvae and adult tube feet. These results suggest that the retinal gene set may be used for distinct purposes in different cells.

Circadian genes

Diurnal rhythms are widespread in metazoans, and usually linked to daily and seasonal cycles that are a feature of feeding, reproduction and migration (Merrow et al., 2005). Circadian rhythms are not well known in echinoids although photoperiodic activity has been recorded for both feeding and reproductive behaviors (Rosenberg and Lundberg, 2004; Coppard and Campbell, 2005) and temporal variability in larval settlement is also noted (Hereu et al., 2004).

Studies in flies and fish show that the gene regulatory network that controls circadian rhythms includes transcription factors and inhibitory and enhancing pathways. The central clock genes of this network include Period (*Per*), the first clock gene identified (Konopka and Benzer, 1971), Cryptochrome (*Cry*), Clock (*CLK*), Timeless (*Tim*) and Brain and muscle Arnt-like protein (*BMAL*) (Glaser and Stanewsky, 2005; Hirayama

and Sassone-Corsi, 2005). Per proteins appear to be a central component of the circadian clock across the animal kingdom and together with Cry and Tim proteins are fundamental to circadian behavior pathways.

Analysis of the urchin genome using reciprocal BLAST searches has revealed the presence of at least 4 genes including *Sp-Clock*, *Sp-CRY*, *Sp-Timeless1* and *Sp-Timeless2* that may belong to a Clock gene network (Table S9). There is also evidence for urchin genes encoding other transcription factors that may be part of a Clock network, such as *Sp-Arnts* and *Sp-Arnt*-like genes (Table S9). At present, we know little about the temporal or spatial expression patterns of these key genes although the tiling data (Samanta et al., 2006) suggests that except for one of the Arnt-like genes, all are expressed in embryos. It is clear that one of the most exciting challenges of sea urchin genomics will be an analysis of this putative network.

Conclusions

Our analysis of the genome has provided a rich source of information that has opened several completely new lines of investigation into the neural development and neurobiology of sea urchins. It appears that sea urchins share with other

Table 4
S. purpuratus homologues of mammalian retinal genes

Predicted gene	Mouse gene name (*S. purpuratus* gene name)	Gene function (domain class)	Expression[a] 24 h/72 h/tube foot 10^4
SPU_000990	Atonal (Sp-Ato2b)	bHLH transcription factor	ND/ND/ND
SPU_003681	Atonal7	bHLH transcription factor	ND/ND/ND
SPU_011351/	Atonal	bHLH transcription factor	3.2/16.8/2.3
SPU_014289	Rx	Homeobox transcription	2.5/7.4/0.43
SPU_010438	Pou6f2	POU domain transcription	
SPU_025632	Pou4f1/Pou4f2	POU domain transcription	ND/1.9/3.1
SPU_004702	Ebf1/Ebf3/Olf-1	Zinc finger transcription factor	
SPU_000237	Islet1	LIM homeobox transcription	0.95/4.6/3.1
SPU_017627	Gli1	Zinc finger transcription factor	
SPU_010351	Irx2/Irx5	Homeobox transcription	
SPU_024918	NeuroD1/NeuroD4	bHLH transcription factor	ND/1.8/5.0
SPU_017348	Atbf1	Zinc finger homeobox	
SPU_027235	Blimp1	Zinc finger transcription factor	46.6/25.0/3.5
SPU_006786+SPU_006787	Pax6	Paired class homeobox transcription factor	1.2/6.3/20.8
SPU_017379	Six1/Six2	Homeobox transcription	
SPU_018908	Six3/Six6	Homeobox transcription	35.8/17.6/1.6
SPU_017380	Six4/Six5	Homeobox transcription	
SPU_007599	GLASS 2	Zinc finger transcription factor	
SPU_012645	GfI1	Zinc finger transcription factor	
SPU_014164	BarH1	Homeobox transcription	ND/ND/8.3
SPU_005569	Rhodopsin (Sp-opsin1)	Photo pigment	ND/4.0/0.60
SPU_006737	Rhodopsin (Sp-opsin5	Photo pigment	ND/ND/0.15
SPU_027634	Rhodopsin (Sp-opsin3.1)	Photo pigment	ND/ND/ND
SPU_022851	Melanopsin (Sp-opsin4)	Photo pigment	ND/ND/5.6
SPU_000178	Phosphatase 2a	Photo transduction	
SPU_008502	Phosphatase 4 (receptor	Photo transduction	
SPU_019810	Notch	Notch signaling	
SPU_000092	K voltage-gated channel	Nerve transmission	
SPU_000062	Tubb2/Tubb3/Tubb4	Tubulin cytoskeleton	
SPU_009708	Trim2	Not assigned	

ND, not detected.
[a] Expression values are from qRT-PCR using the procedures of Revilla-i-Domingo et al. (2004). RNA from 500 embryos (24 h, 72 h) or from 5 tube feet was used as template.

metazoans the gene regulatory programs that specify embryonic neurons. The experimental dissection of the sea urchin neurogenic network is now possible. How this network is deployed will prove particularly important in resolving how the distinctive pentaradial, yet segmented, echinoid nervous system relates to that of other deuterostomes and how it was derived from the deuterostome ancestor. The unusual synaptic connections between neurons and between neurons and effectors in echinoids can now be approached with molecular and cellular studies. In addition, investigations of the mechanisms underlying embryonic axon guidance and neural adhesion appear tractable. The array of neuromodulators, neuropeptides and growth factors that have been identified in the genome suggests that the echinoid nervous system fully utilizes these modes of communication and regulation. Investigation of the physiological roles of these molecules will provide fascinating insights into the evolution of chemical signaling systems of animals. The expression of a set of retinal genes in tube feet, which are non-ocular structures, provides a novel framework for understanding how organisms perceive light. Similarly, the characterization of chemoreceptors will provide novel tools for an analysis of sea urchin sensory modalities. Analysis of the sea urchin genome has facilitated a revitalization of sea urchin neurobiology that will improve on its status of being among the least studied metazoan nervous systems.

The list of neural gene predictions provides a resource for developing hypotheses of gene function in the nervous system. Most of the gene predictions will need to be verified with full-length cDNAs and expression studies and experimental perturbations will be necessary to determine if gene functions are conserved. The separate larval and adult phases of sea urchin life history provide two distinct body plans with what appear to be separate nervous systems. A more complete analysis of the development and organization of echinoderm nervous systems is central to the problem of fitting these two body plans into the evolutionary history of deuterostomes as well as helping to resolve longstanding questions of deuterostome ancestry.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.007.

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