Characterization of Homeobox Genes Reveals Sophisticated Regionalization of the Central Nervous System in the European Cuttlefish *Sepia officinalis*

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**Abstract**

Cephalopod mollusks possess a number of anatomical traits that often parallel vertebrates in morphological complexity, including a centralized nervous system with sophisticated cognitive functionality. Very little is known about the genetic mechanisms underlying patterning of the cephalopod embryo to arrive at this anatomical structure. Homeodomain (HD) genes are transcription factors that regulate transcription of downstream genes through DNA binding, and as such are integral parts of gene regulatory networks controlling the specification and patterning of body parts across lineages. We have used a degenerate primer strategy to isolate homeobox genes active during late-organogenesis from the European cuttlefish *Sepia officinalis*. With this approach we have isolated fourteen HD gene fragments and examine the expression profiles of five of these genes during late stage (E24-28) embryonic development (*Sof-Gbx, Sof-Hox3, Sof-Arx, Sof-Lhx3/4, Sof-Vsx*). All five genes are expressed within the developing central nervous system in spatially restricted and largely non-overlapping domains. Our data provide a first glimpse into the diversity of HD genes in one of the largest, yet least studied, metazoan clades and illustrate how HD gene expression patterns reflect the functional partitioning of the cephalopod brain.

**Introduction**

Homeodomain (HD) transcription factors are important regulators of developmental patterning across animal lineages, defined by the presence of a 60aa DNA binding homeodomain. The antennapedia (ANTP) class of homeodomain transcription factors is the largest group of animal homeodomain (HD) genes (see http://homeodb.zoo.ox.ac.uk/ [1] [2]). The ANTP-class HOX sub-group genes in particular have been widely investigated since the discovery of their conserved role in axial patterning of body plans throughout metazoan lineages [3]. However, there are many other families of HD proteins which have been less well studied in a comparative, evolutionary context. The second largest homeobox gene group is the paired-class (PRD), as defined by the presence of the PRD-homeobox [4]. Where they have been studied, PRD-class genes show expression within developing nervous systems, as well as involvement in axial patterning [5] [6] [7]. The LIM-class of HD transcription factors, which includes proteins with a zinc-finger (LIM) domains, have been well studied for their role in neural specification from flies to vertebrates [7–11]. Thus, it is clear that a number HD transcription factors play a pivotal role in the generation of highly organized central nervous systems.

Contrary to the situation found in arthropods, nematodes, echinoderms, and chordates, where the genetic mechanisms that underlie embryonic development have been well studied, little is known about the genetic control of development in cephalopod mollusks. Transcriptome analysis of the adult central nervous system from an octopus reveals approximately 3% of the active transcriptome is involved in transcription factor activity [12]. Given the widespread use of HD proteins during neural specification in other metazoan lineages, we investigate HD proteins active during maturation of the central nervous system of the cephalopod mollusk *Sepia officinalis*. Development of the European cuttlefish, *Sepia officinalis*, is typical of most cephalopod molluscs with 30 morphologically described embryonic stages (E1–30: [13] [14]). Embryonic stages can be grouped into five developmental phases corresponding to cleavage and gastrulation (stages E1–15), primordial organ placode formation (disc phase: stages E15–20: [15]), organogenesis and differentiation (stage E18–27; [14]), tissue maturation (stage E28–29; [14]) and hatching (stage E30). During the straightening phase of embryonic development, when the embryonic disc elevates from the yolks (Stages E21–22: [15]), paired neural placentodes are concentrated and fuse medially to form the lobes of the adult CNS that surround the esophagus within the head. The subesophageal mass (SM) is composed from the visceral (posterior SM) and pedal (medial and anterior SM) ganglia placentodes, and the supraesophageal mass (SPM) derives from the cerebral ganglia placentodes, with optic lobes...
located laterally behind each eye (Figure 1; [16] [17]). Analysis of ELAV expression, a marker of postmitotic neural cells, has revealed asynchronous maturation of each lobe of the CNS – the pSM is the first lobe to contain mature neurons as early as stages E15/16, followed by the mSM and aSM at stage E20, whereas the SPM is the last to contain maturing neurons at stage E22 [17]. We recover homeodomain proteins of the ANTP- PRD- and LIM-classes, and find all to be expressed within the maturing central nervous system in largely un-overlapping domains during late stage embryogenesis (E24–28).

Materials and Methods

Ethics statement

The use of embryonic cephalopod material for research is currently not legislated in Europe and thus no permits were required for this work. Nonetheless, adult cephalopods are now included in European legislation in order to avoid perceived pain and suffering that may be associated with their highly functioning nervous system. See Moltschaniwskyj et al. [18] and Fiorito et al. [19] for discussion on ethical issues involving cephalopods in research. All animal material was treated with the most humane care possible – embryos used in this study were lethally anesthetized in ethanol prior to sacrifice at stages prior to yolk absorption.

Animals

Embryonic material used in this study derive from fertilized eggs naturally deposited on fishing nets from the sea waters near Ancona Italy (April 2010, 2012). Deposited eggs that were normally cleaned off the nets and dumped in the sea were collected by licensed fishermen and brought to shore. The authors transported the egg capsules to the laboratory where they were kept in 20% artificial sea water (ASW: TETRA Marine SeaSalt) and 90% natural sea water (Gulf of Naples) with aeration at room temperature in a closed salt water aquarium. Embryos prior to yolk absorption were isolated from egg envelops, anesthetized in 1% EtOH, and staged according to Cole and Hall [14]. Embryos destined for RNA extraction were placed in TRIzol Reagent (Invitrogen, 15996-016) and stored at -80°C until use; embryos destined for in situ hybridization were fixed in 4% paraformaldehyde (PFA) overnight, washed in PBS and incubated in 30% sucrose in PBS overnight prior to embedding in optimum cutting temperature (OCT) compound (Killik – BioOptica, 05-9801), and stored at -80°C until sectioning.

Gene cloning

Staged embryos were homogenized in Trizol with a tissue lyser (Qiagen), and RNA extraction was performed according to manufacturer’s recommendation. An mRNA isolation kit (ROCHE) was used for the isolation of poly(A+) RNA, following the manufacturer’s instructions. Purified RNA was stored at -80°C until use. cDNA was retro-transcribed from equal quantities of purified poly(A) or total RNA extractions from embryos of stage E24-E30, using the SuperScript First-strand Synthesis System (Invitrogen) according to the manufacturer’s instructions.

Two pairs of degenerate primers, designed to amplify 96 bp between the first and third conserved helix of the homeodomain (HD), were used to amplify ANTP- (ANTfw: CCGATCCGTYT-GARYTIGARAARCART; ANTrv: GGAATTCATICKRTTYTTCRAAACCAT) and PRD- (PRDfw: CAGCTSGARGARCTGGAG; PRD: GCBCKNCGRTTYTTCRAACC) class HD fragments. Degenerate PCR was performed in a total volume of 20 µl containing 10 ng of mixed-stage cDNA (stages E24–E30) and 2 µM of each primer for 35 cycles using the following thermal profile: 30 s at 94°C, 30 s ramping from 40–55°C and 45 s at 72°C followed by a final elongation step of 10 min at 72°C. The resultant 120 bp amplicon was cloned into a pCRRI-TOPO vector (Invitrogen, K4600-01), and 50 positive colonies were sequenced. Sequenced gene fragments were identified according to BLAST similarity and orthology assignment of the genes was determined following phylogenetic analysis of the conserved HD (Figure S1). Homeodomain sequences were downloaded from the homeoDB [http://homeodb.cbi.pku.edu.cn/1] [2] and complemented with Mollusk and Annelid sequences found in GenBank. Amino acids 18–31 of the conserved 60aa HD were aligned (Supporting Information S1a) and complemented with Mollusk and Annelid sequences found in GenBank. Amino acids 18–31 of the conserved 60aa HD were aligned (Supporting Information S1a) and complemented with Mollusk and Annelid sequences found in GenBank.

Figure 1. Schematic diagram of the cuttlefish CNS. The molluscan ganglia origin of each principle lobe of the central nervous system (CNS) is color coded. On the left the head is shown from a dorsal view, with the right supraesophageal mass (SPM) removed at the midline (dashed line). The CNS is represented in lateral view on the right. The grey neuropil structure is approximate and not meant to represent the actual internal structure of the organ. a: anterior subesophageal mass; brg: branchial ganglia; m: middle subesophageal mass; ol: optic lobe; st: statocyst; p: posterior subesophageal mass; sg: stellate ganglia; spm: supraesophageal mass; wb: white bodies.

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Reverse transcription polymerase chain reaction (RT-PCR) analysis

PCR amplification was performed using 5 ng of template cDNA, derived from staged embryo total RNA extractions, for 30 cycles (So-HD genes) or 20 cycles (RPS16 internal control) with 55°C as the annealing temperature. 0.5 μM of each primer pair were used as follows: SoXLoxFw1 and SoXLoxRv1 - CGGTTTTGGACCAAAATTTTATA (304 bp); SoGbxFw2 - TCACACACGTGGACACATGTA and SoGbxRv2 - 5'GAAGCTTCCACAGCGGAAAC-3' (264 bp); SoLhx3/4Rv2 - GGTTTCAACACTCAGCTGCTCCACTGGCCACCAACATTATA and SoDrgxRv2 - TCAAGCTTGGCTGCTTGAACT (457 bp); SoDrgxFw1 - TTGACCGAGCGTTCGCAGATT.

qPCR

To analyze the temporal expression profile of So-HD genes, qPCR experiments were carried out as described below, using the following gene specific primers: SoArxFw2 - CGTCCACTGGTTATCCCTATGGTAAC (264 bp). RTP16Rv1 - AGGCCCATACCAGGGATGTITATGC and SoVsxRv2 - CCCAGCAAGACCAGCTTGAGGCTTTT; SoGbxFw1 – TCTAGGCCCATTACCCGGATGTTTATGC and SoVsxFw2 – AGAGTGCATTTCAAGAGGGACTGG (145 bp); RPS16Fw2 - AAAAAGAAGTTTTAGTTGGGGTGA and SoHox1Rv3 TTGCCATGTCCACCGTTT (171 bp); SoHox1Fw2 - ACGAGACGCACACTGTTGTG (134 bp); SoGbxFw3 - TGGCAAAATCCGCTTGTTGAACT (133 bp) and SoVsxFw1 - TACACTGGTAAAGAACAACTAT (457 bp); SoDrgxFw2 – AGAGTGTTGTG (171 bp); SoHox1Fw2 - ACGAGACGCACACTGTGTTG and SoVsxFw2 - CTGGTTATACACTACGCTGCTC (215 bp); RPS16Fw2 - GTGTTTGACGAGGATTTACCTG and RPS16Rv1 - CGCCGTATTCCTATGTTAGA (264 bp).

To visualize expression profiles (RPS16 internal control) or 20 cycles (RPS16 internal control) with 55°C as the annealing temperature. 0.5 μM of each primer pair were used as follows: SoXLoxFw1 and SoXLoxRv1 - CGGTTTTGGACCAAAATTTTATA (304 bp); SoGbxFw2 - TCACACACGTGGACACATGTA and SoGbxRv2 - 5'GAAGCTTCCACAGCGGAAAC-3' (264 bp); SoLhx3/4Rv2 - GGTTTCAACACTCAGCTGCTCCACTGGCCACCAACATTATA and SoDrgxRv2 - TCAAGCTTGGCTGCTTGAACT (457 bp); SoDrgxFw1 - TTGACCGAGCGTTCGCAGATT.

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Total RNA extracts from 3 same-staged individuals was pooled and cDNA was retro-transcribed using the SuperScrip First-strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. PCR amplifications were performed using 5 ng of cDNA as template with 0.3 μM primer and 1X FastStart Universal SYBR Green Master (RoX); each assay included a no-template control for each primer pair and were carried out in duplicate. All genes were included in at least three independent assays. Data were normalized to RPS16 levels as an endogenous control. Relative expression levels were calculated as the ratio of each sample to the lowest expression level using the 2-DDCt method; standard errors in fold-change values were calculated with respect to standard deviations in the raw data according to [21].

In situ hybridization

RNA probes corresponding to 5' RACE products were transcribed with the Sp6-T7 kit from Roche as recommended by the manufacturer: DIG RNA Labelling Kit (Roche 11 175 025 910) was used for the in vitro transcription: Sof-Arx1 (533 bp); Sof-Gbx (571 bp); Sof-Lhx3/4 (254 bp); Sof-Hox3 (402 bp); Sof-Fsx (390 bp). 10 μm frozen sections were cut with a cryostat (MICROM HM500) under RNAase-free conditions, and mounted on SUPERFROST PLUS slides (Menzel-Gläser, J1800.AMNZ). These prepared slides were processed for in situ hybridization following the protocol of Little et al. [22] with the following modification: Proteinase K was used at a concentration of 1 μg/ml in PBS. Probe localization was visualized using incubation with BM Purple (Roche, 1142074001) until the signal reached satisfactory intensity and mounted with Glycergel (Dako - C0563). Mounted slides were digitally imaged with a Zeiss Axioplan2 microscope using the Axiosio REL 4.8 software. All images presented in this work were processed in Adobe Photoshop CS5 using a High Pass filter (250 pixel radius) to remove lighting artefacts introduced by the imaging system, and subsequently adjusted for Brightness and Contrast (see Figure S2 for an example of a pre- and post-processed image). Schematic illustrations of expression domains were generated with Adobe Illustrator CS5.1 based upon analysis of serially sectioned material and relevant data from the literature.

Results

We used degenerate primers specific for the conserved portion of the paired-class (PRD) and antennapedia-class (ANTP) homeo-domain to identify homeodomain (HD) proteins expressed during late-stage organogenesis in the cuttlefish Sepia officinalis. Using this strategy we identified 28 amino acids that correspond to amino acids 19–46 of the conserved 60aa homeodomain for four PRD-class HD proteins: dorsal root ganglia homeobox (Sof-Drgx), visual system homeobox (Sof-Vsx), prophet of Pit1 homeobox (Sof-Propx), and the aristal-less-like homeobox (Sof-Arx); and nine ANTP-class HD proteins: gastrulation brain homeobox (Sof-Gbx) new, distalless-like homeobox (Sof-Dlx: new), paralogy group labial/Hox1 (Sof-Hox1: identical to CAI77461.1), paralogy group Hox5/Scr (Sof-Ser: identical to CAI77463.1), paralogy group Hox3 (Sof-Hox3: identical to CAI77462.1), paralogy group ubx/Lox2/Hox7 (Sof-Lox2: new), paralogy group Lox3/Hox8 (Sof-Lox4: identical to CAI77464.1), paralogy group ant/Lox5/Hox6/7 (Sof-Lox5: new), and the ParaHox protein xLox/Pdx (Sof-xLox: new). We also recovered one LIM-class homeodomain of the Lhx3/4 group (Sof-Lhx3/4: new). Neighbour-joining analysis based upon alignment of aa19–46 of the homeodomain is illustrated in Figure S1. The Sepia HD fragments group with other molluscan sequences where available, and are clearly identifiable as orthologs of the identified gene families.

Of these HD gene fragments, seven sequences were successfully extended towards the 5prime end (Sof-Arx; Sof-Drgx, Sof-Vsx, Sof-Gbx, Sof-Lhx3/4, Sof-Hox1, and Sof-Hox3) and two towards the three prime end (Sof-Gbx, and Sof-xLox) using RACE amplification with gene specific primers. Analysis of the coding sequence reveals no identifiable conservation outside of the HD for Sof-Arx (data not shown). The recovered Sof-Vsx sequence shows similarity in the 5' end with the Grassoseta Vx2 sequence (EKC18872.1; data not shown), which suggests that this gene
extends beyond the sequence recovered here. In contrast, 5’ extension of _Drgx, Gbx, Lhx3/4, Hox1, xLox and Hox3_ gene sequences reveals a high degree of protein sequence conservation with available molluscan sequences (Table 1). Interestingly, the Sof-xLox protein has two isoforms, the longer of the two containing an additional 28 amino acids upstream of the HD (GenBank accession n. KJ467080), in the same position as a similarly sized exon identified from genomic DNA in the sea star [23].

We proceeded to investigate the temporal expression dynamics for these eight HD transcription factors from stages E15–30. Sof-Arx, Sof-Drgx, and Sof-xLox are nearly undetectable with RT-PCR prior to stage E22, whereas all other genes are detectable with this highly sensitive method in all stages investigated (Figure 2A); qPCR analysis confirms this expression profile for all of these eight genes (Figure 2B) in three independent assays. Overall, the relative gene expression profiles highlight the oscillatory nature of ANTP-class genes with respect to PRD-family genes (Figure 2B). All genes examined show the observed peak in relative embryonic expression seen at this stage (Figure 2B), whose expression may contribute to patterning along the anterior-posterior axis, and a large number of non-neural tissues from stage E24, including the gills, the epithelium of the beak, the medial portion of the external wall of the funnel tube, the white bodies, and the epithelium of the arms (Figure 4B). These expression patterns remain stable stage E27, with the following exceptions: expression in the epithelium of the beak and the gills disappears by stage E26, and is no longer detectable within the funnel by stage E27.

The single LIM-class gene identified here, _Sof-Lhx3/4_, is expressed in all lobes of the CNS in spatially restricted domains in stages E24–28. It is detected in a population of cells scattered throughout the OL, in a small domain in the dorsal-posterior SPM (vertical lobe), in the posterior-most pSM, in the mid-anterior mSM, the anterior aSM, the brachial ganglia and nerve cord of the arms, and within the SG where the cell population is restricted to the dorsal portion of the ganglia, absent from the interior-most ventral zones (Figure 5). _Sof-Lhx3/4_ is also expressed within the statocyst epithelium, the buccal ganglion, and the sensory epithelium of the presumptive olfactory pit (Figure 5). At stages E25/E26 expression is found within the arm epithelium; this expression domain was not detected in any other stage investigated here.

### Discussion

Homeodomain proteins are most well-known for their role in patterning along the anterior-posterior axis, and a large number of

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**Table 1. Details of recovered HD gene sequences from the European cuttlefish _Sepia officinalis_.**

| Gene name | Recovered Sequence length (nt) | GenBank accession number | Gene portion covered | Best BLAST Hit* | % identity* |
|-----------|-------------------------------|--------------------------|---------------------|-----------------|-------------|
| Sof-Arx   | 653                           | KJ467073                 | 5 prime + HD        | Capitella (ELT87482.1) | 96% (aa167–217) |
| Sof-Drgx  | 292                           | KJ467077                 | 5 prime + HD        | Lymnea_Drgx (AGC24174.1) | 74% |
| Sof-Gbx   | 571                           | KJ467079                 | Entire coding       | Crassostrea_Gbx (EKC23204.1) | 89% |
| Sof-Lhx3/4| 244                           | KJ467076                 | 5 prime + HD        | Euprymna_labial (AY330184.1) | 90% |
| Sof-Hox1  | 515                           | KJ467075                 | 5 prime + HD        | Euprymna_Hox1 (AAR16188.1) | 91% |
| Sof-Hox3  | 418                           | KJ467074                 | 5 prime + HD        | Euprymna_Vsx2 (EKC18872.1) | 40% |
| Sof-xLox  | 550                           | KJ467078                 | Partial 5 prime + HD | Euprymna_xLox (ABD16192.1) | 90% (with short isoform: KJ467081) |

*derived from the NCBI “nblastn” algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). doi:10.1371/journal.pone.0109627.t001
these genes are involved in the development of neural tissues in a wide-range of animal lineages. Here we isolate a number of homeobox genes corresponding to the ANTP- PRD- and LIM-classes of HD proteins, profile their expression patterns during the latter half of cephalopod embryogenesis, and report well-defined expression territories that reflect the partitioning of the CNS into regions with distinct gene signatures (Figure 6). Temporal expression profiles of eight HD genes show variable relative expression levels within whole embryos, although analyses of spatial expression patterns of five of these genes reveal constant expression patterns from stages E24-30 – the period of embryogenesis after the establishment of the organ primordia when large-scale tissue differentiation is occurring [14]. Interestingly, we find that LIM and PRD-class genes show similar temporal expression profiles that peak within the whole embryo between stages E25–26, and have spatial expression domains restricted to the central nervous system. In contrast, ANTP-class genes show much broader temporal and spatial expression profiles. ANTP-class genes are well known for their role in early embryo patterning in other animals systems, and thus is not surprising to find these genes active in early cephalopod embryogenesis. The oscillatory nature of ANTP-class temporal gene expression between stages E15–30 suggests at least two waves of up regulation during embryogenesis. We did not examine the spatial expression patterns in embryos younger than stage E24, therefore we cannot confirm whether or not the ANTP- genes are activated in different domains corresponding to the observed early time-point peaks in our temporal analysis, however similar to the PRD- and LIM-class genes, we find ANTP-class gene expression within the CNS during the latter half of embryogenesis.

The central nervous system (CNS) of the adult cuttlefish derives from the fusion of paired ganglia that arise as placodes during the disc phase of development [24]. Neural ganglia are concentrated and fuse medially to form the lobes of the adult CNS when the embryonic disc elevates from the yolk (Stages E21–22; [15]) (see Figure 1). Although extensive comparative data is limited pertaining to PRD-class gene expression in cephalopods, available data suggests that this gene class is largely restricted to the SPM – and thus to the anterior-most portion of the CNS. Aside from transient expression of So-otx in the pedal ganglia at stages E20–22

**Figure 2. Temporal gene expression profiles of homeobox genes.** A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of ANTP-class genes (left) and PRD- and LIM-class genes (right) amplified for 30 PCR cycles. The ribosomal protein control (Sof-RPS16) shows a saturated profile after 20 cycles, whereas all developmental transcription factors show differential peaks of expression throughout embryogenesis. B) Real time quantitative reverse transcription polymerase chain reaction (qPCR) analysis of ANTP-class genes (left) and PRD- and LIM-class genes (right). Ct data for each gene was normalized to the internal control (Sof-RPS16), and represented relative to its lowest levels of gene expression present in the assay. The ANTP-class genes show oscillating expression profiles throughout embryogenesis, whereas the PRD- and LIM-class genes show very similar expression profiles with an expression peak between stages E25–26.

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Homeodomain Gene Expression in the Cuttlefish Sepia officinalis

Figure 3. Spatial gene expression profiles of PRD-class homeodomain genes. The first panel of all figures is a schematic diagram as in figure 1, summarizing gene expression domains reconstructed from analysis of serial sections of embryos from stages E24–28. The age of embryo shown is found in the upper right corner of each image. A) Sof-Arx. From left to right: Medial section through the head illustrating expression within the supraesophageal mass; Gene expression within the stellate ganglion; Medial section through the head illustrating retained expression within the supraesophageal mass. B) Sof-Vsx. From left to right: Medial section through the head illustrating expression within cells of the optic lobes, the supraesophageal mass, and the middle subesophageal mass; section through the buccal mass illustrating signal associated with the mesenchyme of the developing beak; medio-lateral section through the head demonstrating gene expression in cells scattered throughout the optic lobes, and within the supraesophageal mass. Abbreviations - asm: anterior subesophageal mass; bm: buccal mass; msm: middle suboesophageal mass; psm: posterior subesophageal mass; st: statocyst; sg: stellate ganglion; spm: supraesophageal mass.

Whereas in vertebrates Vsx expression in the visual system has been well documented, expression data for visual system homeobox (Vsx) orthologs from non-vertebrate lineages is limited to three model systems: the fruitfly Drosophila melanogaster expresses Vsx in the optic-lobe progenitor cells [34], the nematode roundworm Caenorhabditis elegans expresses Vxs within a small number of sensory interneurons neurons [35], and the ascidian Ciona intestinalis where expression in the tadpole is limited to four cells within the visceral ganglion [36]. Expression throughout the optic lobes of the Sof-Vsx is unsurprising as it is assumed that this lobe is used for processing of visual information. These lobes also express Sof-Lhx3/4 (this paper) and the PDR-class gene so-pax6 [15], and all three of these genes have been proposed as a common molecular signature for a homologous cell type involved in visual processing [37]. We do not comment on possible expression in the retina of any of the genes examined here due to high levels of non-specific background staining regularly found associated with the eye (data not shown). Vxs are also known to be expressed in populations of interneurons in both deuterostomes (Ciona and vertebrates including the hind brain and spinal cord in zebrafish: [38]), and protostomes (C. elegans [35]), and have been proposed to play an ancestral role in integration of sensory information in general [35]. In the cuttlefish, the dorsal basal lobe innervates the optic glands, and lesions in this area of the adult brain show no detectable motor effects [39]. The presence of a Sof-Vxs expression domain in this lobe suggests involvement in the specification of a neuronal sub-type involved in the integration of visual cues. Expression of Sof-Vsx in cells of the buccal mass and mSM is more intriguing, and may indicate the presence of sensory tissues associated with the buccal mass, whereas expression within the mSM in similar domains as described for Sof-Lhx3/4 could indicate a concentration of interneurons in this lobe, as both genes are known to be involved in interneuron development. [33,38,40].
Whereas the lobes of the SPM are predominantly cognitive centers, the SM is largely composed of motor centers [39]. Lhx3/4 forms part of a motor neuron molecular signature (together with Mnx) [26]. The combination of Lhx3/4 and islet, another LIM-class homeodomain protein, is necessary for motor neuron specification in Drosophila [11] and vertebrates (chick: [10]; zebrafish: [8]). No data is currently available regarding islet expression in cephalopods, however analysis of chromatophore motor neuron distribution reveals the SM, in particular the pSM, as a site of concentrated motor neurons [41] [42], confirming earlier reports of motor activity in response to electrical stimulation in these lobes [39]. Here we find that Sof-Lhx3-4 is broadly expressed throughout the nervous system of the cuttlefish, including the anterior-most portion of the mSM and aSM, potentially overlapping with so-Mnx expression domains (Figure 6, [26]) and thus suggesting the presence of a conserved motor neuron molecular signature in cephalopods. The expression profile of one other LIM-class gene, an ortholog of the Apterous/Lhx2/9 paralogy group, has also been reported from E. scolopes (Es-ap: [43]). Es-ap is expressed within the neural tracts of the arms and the optic lobes, similar to Sof-Lhx3/4 reported here, as well as within the SPM, in broader domains with respect to Sof-Lhx3/4 in

Figure 4. Spatial gene expression profiles of ANTP-class homeodomain genes. Figure layout as in Figure 3. A) Sof-Gbx. Clockwise from upper left: Medial section through the head showing signal within the buccal mass, oesophagus, and posterior and middle subesophageal masses; Higher magnification from the same embryos showing gene signal within the posterior subesophageal mass and statocyst; Media-lateral section through the mantle cavity highlighting expression in the gills and posterior subesophageal mass; Expression is detected in the arm epithelium; Medial section of the subesophageal mass, signal is evident in the ventral-most regions; Staining of the stellate ganglion; Medial section through the mantle cavity highlighting gene expression in the intestinal apparatus. B) Sof-Hox3. Clockwise from upper left panel; Medial section through the head showing extensive staining within the posterior and middle subesophageal masses, highlighting a domain of concentrated gene expression posteriorly. Signal is also present at the level of the brachial ganglia (arrows); Section showing medial staining within the funnel; Section shows staining in the head epithelium in the buccal mass and a small population of 2-4 cells (arrow) in the anterior-most portion of the supraesophageal mass; Buccal mass staining is no longer present by stage E26, whereas the supraesophageal mass cells retain expression (arrows); Section through the base of the arms in a stage E25 embryo shows staining within the arm epithelium and the nerve cord surrounding the neuropil of the arms (np); Similar staining within the arms is not present in earlier stage E24 embryos, while expression in the brachial ganglia primordial is present; Abdominal section showing staining in the stellate ganglia; Lower magnification image showing staining in gills, stellate ganglia, subesophageal mass, and ventral-most portion of the funnel tube. Abbreviations - br.g: brachial ganglia; g: gills; hp: hepatopancreas; int: intestine; iym: inner yolk mass; m: mantle; np: neuropil; oe: oesophagus; all others as in Figure 3.

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the cuttlefish. Together, these data suggest conservation of a LIM code [9] for neuronal sub-type cell specification within cephalopods.

In contrast to LIM and PRD-class genes, ANTP-genes show broader temporal expression profiles (see Figure 2b), and are largely restricted to the subesophageal mass (the posterior CNS); Sof-Hox3 is expressed within the pSM (this paper), Sof-Gbx is expressed within the posterior regions of the pSM and mSM (this paper), and Sof-Msx is expressed within the anterior mSM and aSM [26]. Hox gene expression has been previously reported in whole mount in situ preparations for the Hawaiian bobtail squid Euprymna scolopes, another Cephalopod Mollusk [44]. The authors demonstrate dynamic Hox gene expression patterns, and suggest conservation of expression collinearity within the CNS along the anterior-posterior axis as well as novel non-collinear expression domains within the peripheral nervous system. Hox3 orthologs are commonly expressed within the derivatives of the pedal ganglion (the pSM) in both cephalopod species, as well as in the abalone Haliotis asinina [45]. Interestingly, Sof-Hox3 is also expressed within a small number of cells in the inferior frontal lobe of the SPM (derivative of the cerebral ganglion) in the cuttlefish, whereas the pSM is included within the SPM in E. scolopes [44]. The more diffuse expression of Sof-Hox3 within the pSM and mSM reported here may reflect a similar role in axonal patterning of the CNS as described for E. scolopes, whereas the well-defined expression domain within the dorsal-posterior pSM and the few cells of the SPM likely reflects the use of Sof-Hox3 as part of the molecular signature of specific cell types involved in the functional partitioning of this lobe as opposed to conferring positional axonal information.

The expression territory of the ANTP-class gastrulation brain homeobox (gtx) homolog is consistent with a role in axial patterning of the nervous system. In vertebrates the mid-brain/ hind-brain boundary is specified by two HD transcription factors, gtx and the PRD-class gene orthodenticle (otx) [46]. Platynereis dumerilii (polychaete) orthologs are used in a similar manner along the anterior-posterior axis [47]. The cuttlefish orthodenticle gene (So-otx) is expressed within the anterior portion of the CNS (SPM and OL) from stages E20 through hatching [25], whereas we show here that Sof-Gbx is expressed more posteriorly in the pSM and mSM. Taken together, these data demonstrate that otx/gbx patterning of the A-P axis of the nervous system may be conserved in the cuttlefish. Restriction of expression to the ventral-most regions of the SM however, suggests a role in dorsal/ventral patterning, and nonetheless helps define a specific gene signature for neurons of the magnocellular lobe (ventral-most portion of the SM).

Hirth and Reichert [48] have proposed a general scheme wherein general neural patterning with regards to the body axis can be defined by HD gene expression: ANTP-class genes defining the posterior, and PRD-class genes of the orthodenticle group defining the anterior portion of metazoan central nervous systems. Our data certainly conforms to this hypothesis: ANT-class HD gene expression is largely restricted to the posterior derivatives of the CNS, the subesophageal mass, conferring spatial information in the form of broad expression domains along the A/P axis; PRD-class HD gene expression is more prevalent in the anterior CNS, the supraesophageal mass. On the other hand, studies of HD gene expression in the ctenophore Hydra have lead to the proposal that neural specification may precede axial patterning in terms of ancestral HD gene function [49], and thus axial patterning and cell type specification may be considered distinct genetic modules during development. Evidence is accumulating that strongly suggests a role for ANTP-class genes in specifying post-mitotic neuron sub-types, in both vertebrate and invertebrate lineages (reviewed in [50]). The restricted, mostly non-overlapping, expression territories of each individual HD gene described here reflects the functional partitioning of the adult CNS, and illustrates
that the individual lobes of the brain possess a unique molecular signature in terms of HD gene expression. Although cephalopods are not widely used as experimental genetic models, they have a large, well characterized, behavioral repertoire (reviewed in [51]). As our understanding of the genetic architecture underlying the functional differentiation of their nervous systems is unraveled, the cephalopod research community is well placed to make rapid progress in connecting behavioral circuitry with the underlying genetic components.

Supporting Information

Figure S1  Phylogenetic analysis of recovered homeodomain gene fragments. *Sepia* sequences recovered in this study are indicated in boldface type. All major gene clades are indicated, and the *Sepia* sequences resolve together with other mollusk sequences where available. (*) indicates elongation of already known gene sequences. (**) indicates new sequence data. Genes for which expression data is presented here are highlighted. Sequences without a GenBank accession number listed were
Figure S2 Effect of Adobe Photoshop image manipulation used in this work. On the left is an original image taken at the microscope showing the background coloration on a scale from pink to green; on the right is the adjusted image after HIGHPASS Filter with a 250 pixel radius and adjustment of brightness and contrast. The background colouration results in an even grey scale after this procedure. Circles represent the areas where background colour was sampled from both images.

Supporting Information S1 Homeodomain multiple sequence alignment. a) CLUSTAL O (1.2.0) multiple sequence alignment used for phylogenetic analysis. Sequences recovered in a) CLUSTAL O (1.2.0) multiple sequence alignment.

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