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**An anterior medial cell population with an apical-organ-like  
transcriptional profile that pioneers the central nervous system in the  
centipede *Strigamia maritima***

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## Abstract

The apical plate of primary marine larvae is characterized by a common set of transcription factors comprising *six3*, *rx*, *hbn*, *nk2.1* and *FoxQ2*. It harbours the apical organ, a neural and ciliary structure with neurosecretory properties. Recent studies in lophotrochozoans have found that apical organ cells form the anterior tip of the developing central nervous system.

We identify an anterior medial tissue in the embryonic centipede head that shares the transcriptional profile of the apical plate of marine larvae, including nested domains of *FoxQ2* and *six3* expression. This domain gives rise to an anterior medial population of neural precursors distinct from those arising within the segmental neuroectoderm. These medial cells do not express *achaete scute homologue* in proneural clusters, but express *collier*, a marker for post mitotic cells committed to a neural fate, while they are still situated in the surface ectodermal layer. They then sink under the surface to form a compact cell cluster. Once internalized these cells extend axons that pioneer the primary axonal scaffold of the central nervous system. The same cells express *phc2*, a neural specific prohormone convertase, which suggests that they form an early active neurosecretory centre. Some also express markers of hypothalamic neurons, including *otp*, *vtn* and *vax1*.

These medial neurosecretory cells of the centipede are distinct from those of the *pars intercerebralis*, the anterior neurosecretory part of the insect brain. The *pars intercerebralis* derives from *vsx* positive placodal-like invagination sites. In the centipede, *vsx* expressing invaginating ectoderm is situated bilaterally adjacent to the medial pioneer cell population. Hence the *pars intercerebralis* is present in both insect and centipede brains, whereas no prominent anterior medial cluster of pioneer neurons is present in insects. These observations suggest that the arthropod brain retained ancestrally an anterior medial population of neurosecretory cells homologous to those of the apical plate in other invertebrate phyla, but that this cell population has been lost or greatly reduced in insects.

1 **Keywords:** arthropods; anterior medial region; neurogenesis; apical organ,  
2 FoxQ2, six3, neurosecretory cells, pars intercerebralis  
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## **Introduction**

There is a long history of debate as to whether the arthropod head retains structures homologous to the anterior, unsegmented tissue of annelids and other invertebrates. Morphological studies have in recent years tended to reject this idea, suggesting that the entire arthropod head is segmentally derived (Budd, 2002; Haas et al., 2001) (Note here we use the term arthropod to include hexapods, myriapods, crustaceans and chelicerates, but exclude onychophorans). New phylogenies have made any close correspondence between arthropod and annelid head organisation seem less likely (Aguinaldo et al., 1997; Dunn et al., 2008). Against this however, the conservation of transcription factor expression in the anterior regions of the most diverse animals has recently lead to the proposal that aspects of anterior patterning are conserved across, and even beyond, the bilateria (Lowe et al., 2003; Posnien et al., 2011; Sinigaglia et al., 2013; Steinmetz et al., 2010).

The morphology of the head in adult arthropods, as in other animals, shows complex adaptations to behaviour and life style. If we are to find remnants of any ancestral organisation that underlies this diversity, and is shared between widely disparate groups, it seems likely that this will be most evident during early embryogenesis, and reflected in the molecular specification of the first distinct territories and cell types to arise during head patterning. This approach has already led to a better understanding of evolutionary conserved regions in axial patterning (Lowe et al., 2003; Schilling et al., 2001; Steinmetz et al., 2010), and to the identification of evolutionarily related cell types in distant animal taxa (Arendt, 2008; Tessmar-Raible et al., 2007; Tomer et al., 2010). We have taken this approach to study the organisation of the head in a centipede, as representative of an ancient but hitherto poorly studied lineage of the arthropods.

Debate about the nature of the anterior body region of arthropods has a long history, focussing on the number of segments in the head (e.g. Rogers and

1 Kaufmann 1996), the homology of the different head segments between  
2 arthropod lineages (Damen et al., 1998; Haas et al., 2001; Scholtz and  
3 Edgecombe, 2006; Telford and Thomas, 1998) and the nature of the arthropod  
4 brain (Lichtneckert and Reichert, 2005; Urbach and Technau, 2003). Molecular  
5 markers for segment patterning, and in particular, the analysis of Hox gene  
6 expression domains (Damen et al., 1998; Hughes and Kaufman, 2002; Telford  
7 and Thomas, 1998), have largely resolved controversies about segment  
8 homologies in the post antennal region, but the structure of the most anterior  
9 part of the head and brain remains controversial.

10

11 The arthropod brain is classically divided into three units: the tritocerebrum  
12 most posteriorly, deriving from the intercalary segment in insects and  
13 myriapods, and from the homologous 2<sup>nd</sup> antennal segment of crustaceans; the  
14 deutocerebrum, deriving from the antennal segment of insects (1<sup>st</sup> antennal of  
15 crustaceans), and the protocerebrum, positioned most anteriorly. The  
16 protocerebrum comprises the ocular lobes, the mushroom bodies and the central  
17 complex, which includes the pars intercerebralis (Scholtz and Edgecombe, 2006;  
18 Strausfeld, 2012). The embryonic origin of the protocerebrum is from the pre-  
19 antennal head, but it has not been clear whether the embryonic pre-antennal  
20 region is one large territory of segmental origin (the ocular region) or might  
21 additionally comprise an anterior medial tissue. The presence of an anterior  
22 medial tissue, giving rise to parts of the central complex and the labrum, has  
23 been proposed on the basis of recent molecular work in the beetle *Tribolium*  
24 *castaneum* (Kittelmann et al., 2013; Posnien et al., 2011, 2009).

25

26 Recent support for the idea that the most anterior part of the head in arthropods  
27 and annelids may be homologous comes from studies of a homeobox  
28 transcription factor, *six3*, which is widely conserved across the animals. *Six3* is  
29 expressed in the apical plate of the annelid trochophore and the anterior medial  
30 head of several arthropods (Steinmetz et al., 2010), as well as in the anterior  
31 ectoderm of other bilaterian animals, and even in the larva of the cnidarian  
32 *Nematostella vectensis*. This suggests that a *six3* expressing anterior territory  
33 may have been inherited from the bilaterian ancestor (Sinigaglia et al., 2013;

1 Steinmetz et al., 2011). If so, parts of the central nervous system that derive from  
2 the anterior medial head, and express *six3*, are likely to have a deep evolutionary  
3 origin.

4  
5 Further studies on gene expression in free-swimming larvae of marine  
6 organisms have elucidated a conserved set of transcription factors characterising  
7 this most anterior (apical) region, comprising *six3*, *FoxQ2*, *nk2.1*, *rx* and *hbn*  
8 (Santagata et al., 2012; Sinigaglia et al., 2013; Steinmetz et al., 2010; Takacs et al.,  
9 2004; Tessmar-Raible et al., 2007; Wei et al., 2009; Yaguchi et al., 2008).

10 Orthologues of *six3*, *rx* and *nk2.1* have also been shown to be involved in  
11 development of the vertebrate forebrain and hypothalamus (Lagutin et al., 2003;  
12 Lu et al., 2013; Muranishi et al., 2012; Ohuchi et al., 1999; Oliver et al., 1995;  
13 Tessmar-Raible et al., 2007), whereas *hbn* is missing from the vertebrate  
14 genomes (Mazza et al., 2010) and *FoxQ2* is not present in mammals (Shimeld et  
15 al., 2010) and has so far not been characterised in any vertebrate.

16  
17 This apical territory of marine larvae harbours the apical organ, which is  
18 positioned centrally within nested domains of *FoxQ2* and *six3* (e.g. Santagata et  
19 al., 2012; Sinigaglia et al., 2013). Apical organs are larval sensory structures that  
20 include neurosecretory cells (Conzelmann *et al.*, 2011; 2013). A recent study  
21 argues for homology of larval apical organs among animals that develop free-  
22 swimming marine larvae, including cnidarians (Marlow et al 2014). In species  
23 that undergo a dramatic change of body plan during metamorphosis the apical  
24 organ is completely lost at the transition to the adult form (see for example  
25 Nielsen, 2005). By contrast, the polychaete annelid *Platynereis dumerilii*  
26 undergoes a gradual mode of metamorphosis and cells of the apical organ are  
27 partially maintained into late larval and adult stages. They produce pioneer  
28 neurons and are thought to form a nucleation centre for the developing nervous  
29 system of the animal (Marlow et al. 2014, Fischer et al. 2010).

30  
31 Until now it has not been clear what parts of the arthropod brain derive from an  
32 anterior medial territory. One candidate is the *pars intercerebralis*, which has  
33 been shown to derive from the *six3*+ territory in the insects *Drosophila* and

1 *Tribolium*. The *pars intercerebralis* constitutes the anterior neurosecretory part  
2 of the central complex in insects (Boyan and Reichert, 2011; De Velasco et al.,  
3 2007; Posnien et al., 2011) and so might plausibly be homologous to the anterior  
4 neurosecretory brain centres in the lophotrochozoans and the hypothalamus of  
5 vertebrates (Hartenstein, 2006; Tessmar-Raible, 2007).

6 Both the annelid apical organ and the insect *pars intercerebralis* are located  
7 within the *six3+* territory at the anterior end of the the axonal scaffold. but the  
8 *pars intercerebralis* develops from bilateral ectodermal placodes at the lateral  
9 edges of the *six3+* territory (De Velasco et al., 2007; Steinmetz et al., 2010). The  
10 central anterior medial head of insects mainly gives rise to the labrum, a non-  
11 neural and probably appendicular structure (Posnien et al., 2011, 2009) (though  
12 others have interpreted the labrum as appendicular but of segmental origin [e.g.  
13 Boyan et al. 2002]). So far it is not clear whether the *pars intercerebralis* bears  
14 any further developmental or transcriptional similarity with the anterior  
15 neurosecretory cells that are part of the apical organ and connect to the anterior  
16 axonal scaffold of the marine larvae (Conzelmann et al., 2013; Fischer et al.,  
17 2010; Santagata et al., 2012; Tessmar-Raible et al., 2007).

18

19 Studies of the anterior medial head in insects are complicated by the fact that the  
20 insect head undergoes major structural rearrangements during development.  
21 This process is carried to an extreme in *Drosophila* and other *Diptera*, where the  
22 whole anterior head undergoes the process of head involution (Turner and  
23 Mahowald, 1979). In *Tribolium*, which is currently the major model for the  
24 genetic control of head development in insects (Kittelmann et al., 2013; Posnien  
25 et al., 2011, 2010; Schinko et al., 2008), the anterior medial region is a small  
26 region of tissue which comes to lie between the ocular lobes in early  
27 development, and forms the labrum Anlagen, the anterior-most portion of the  
28 medial head (Kittelmann et al., 2013; Posnien et al., 2011).

29

30 Little work has been done on head regionalization and molecular specification of  
31 anterior brain structures in non-insect arthropods. Here we study these  
32 processes in a myriapod, the centipede *Strigamia maritima*. Myriapods are now  
33 recognised as an early branch of the mandibulate arthropods, which emerged

1 basal to the pancrustacean (i.e. crustacean and insect) radiation (Regier et al.,  
2 2010; Rota-Stabelli et al., 2011) (but see (Friedrich and Tautz, 1995; Mayer and  
3 Whittington, 2009; Pisani et al., 2004) for alternative views). *Strigamia* is the first  
4 myriapod for which a sequenced genome is available  
5 (<http://www.ncbi.nlm.nih.gov/assembly/322118/>). The gene content of  
6 *Strigamia* is conservative; the genome contains a number of factors that have  
7 been lost from insect genomes. We could for example identify a clear homologue  
8 of *vax1* (Chipman et al., *under review*), a gene involved in development of  
9 anterior neurosecretory organs in vertebrates (Bertuzzi and Hindges, 1999;  
10 Bharti et al., 2011; Wataya et al., 2008), which is not present in insects (Tessmar-  
11 Raible, 2007).

12  
13 The head field of *Strigamia* condenses during early development on the egg  
14 surface (Brena and Akam, 2012), allowing gene expression to be visualised  
15 readily throughout the process of head patterning. This, together with the  
16 genome resources, makes *Strigamia* a good model for studying arthropod head  
17 development.

18  
19 We show here that gene expression in the anterior medial region of the  
20 *Strigamia* head shares striking similarities with that in the apical territories of  
21 *Platynereis* and other marine invertebrate larvae, as well as with the  
22 forebrain/hypothalamic region of vertebrates. An early specified anterior-medial  
23 neurosecretory cell population shares a transcription factor profile with the  
24 apical cells of lophotrochozoan larvae, but is distinct from the *pars*  
25 *intercerebralis* known from insects. This cell group not only forms an active  
26 neurosecretory centre but also pioneers the axonal tracts of the centipede  
27 nervous system. We discuss the implications of these observations for the origin  
28 of neurosecretory brain centres, the developmental structure of the arthropod  
29 protocerebrum and the evolution of the arthropod nervous system.

30

## 31 **Results**

32



1 **1 The anterior medial head of *Strigamia maritima* is a developmentally**  
2 **distinct territory that is demarcated by a set of conserved transcription**  
3 **factors**

4

5 In *Strigamia*, the future head becomes visible shortly after the uniform  
6 blastoderm stage, as a field of cells that condenses towards the ventral anterior  
7 part of the forming embryo (Brena and Akam, 2012). As condensation  
8 progresses, this head field becomes sharply demarcated from the surrounding  
9 single layered epithelium of the dorsal field, a presumptive extra-embryonic  
10 territory (figure 1).

11

12 We have used molecular markers to define distinct regions within this  
13 condensing head. Many genes are expressed segmentally in the more posterior  
14 part of the head field, defining the pre-antennal, antennal, intercalary and  
15 gnathal segments (e.g. *buttonhead/SP5*, figure 1AB; *sloppy paired*, *Pax6*  
16 (supplementary figure S4). (Note that *Strigamia*, like all geophilomorph  
17 centipedes, lacks eyes. We therefore refer to a pre-antennal segment rather than  
18 an ocular segment, which is the term used to describe the corresponding region  
19 in other arthropods. Despite the lack of eyes, this region in *Strigamia* is  
20 innervated by a prominent neuropil (figure 2F)).

21

22 The most anterior part of the head does not express these characteristic markers  
23 of segmented tissue (figure 1A, B) but does express the conserved anterior  
24 patterning genes *six3* (see Steinmetz et al., 2010, and figure 1J,K), *FoxQ2*, *nk2.1*  
25 and *hbn* (figure 1 C-F). We refer to the tissue defined by the expression of these  
26 genes as the anterior medial region (AMR). *Six3* is expressed throughout the  
27 anterior of the early head field, in a domain directly anterior to pre-antennal *otx*  
28 expression, (figure 1J). *FoxQ2* marks the central part of the condensing AMR  
29 (figure 1C). *nk2.1* is also expressed centrally in the AMR but more posteriorly  
30 than *FoxQ2*. The anterior part of the ring-like *nk2.1* domain overlaps with *FoxQ2*  
31 expression (figure 1D, C, L). The homeobox gene *hbn* is expressed in an arch  
32 covering the anterior rim of the AMR and extending into the dorsal field (figure  
33 1E). The posterior limit of *hbn* is directly adjacent to or slightly overlapping with

1 the anterior limit of *nk2.1* expression. The factor *rx*, part of the conserved *rx-hbn-*  
2 *otp* gene cluster (see supplementary figure S3B) is not expressed at the very  
3 early developmental stages, but slightly later, at early segmentation stages, it is  
4 expressed in a pattern similar to that of *hbn* within the anterior head (figure 1G).

5

6 *The AMR becomes positioned between the two halves of the pre-antennal region by*  
7 *a morphogenetic re-arrangement of the anterior head*

8 During the early stages of head condensation the AMR is located anterior to the  
9 pre-antennal region; only the *nk2.1* positive part of the AMR reaches between the  
10 two halves of the pre-antennal region (figure 1A, 1D). The mouth  
11 opening/stomodaeum develops in the centre of this *nk2.1* positive domain. With  
12 on-going condensation and development of the head field, the AMR converges  
13 medio-laterally and becomes enclosed laterally by the pre-antennal lobes (figure  
14 1B, M). During this process the expression domains of the anterior medial  
15 markers become more condensed (figure 1F-I). In the head of mid-segmentation  
16 and later stage embryos the pre-antennal domains reach to the anterior tip of the  
17 head, but are situated lateral to the AMR (see figure 1N). However, based on the  
18 embryonic origin of the AMR from a more anterior position, this tissue can  
19 clearly be identified as the most anterior part of the head.

20

21 In summary, we find an anterior medial tissue in the head of *Strigamia* where the  
22 factors *six3*, *FoxQ2*, *nk2.1*, *rx* and *hbn* are all expressed in partially overlapping  
23 domains (figure 1C-L). All of these factors have been shown to be expressed in  
24 the apical territory of the polychaete annelid *Platynereis dumerilii* (Marlow et al.,  
25 2014; Tessmar-Raible et al., 2007) and/or in the anterior pole ectoderm of the  
26 larvae of the brachiopod *Terebratalia transversa* (Santagata et al., 2012). A  
27 striking feature of the anterior medial head of the centipede is the *FoxQ2* domain  
28 which is entirely nested within the *six3* domain (figure 1K), an arrangement  
29 which is found in the anterior pole ectoderm of free swimming larvae of diverse  
30 marine organisms such as brachiopods (lophotrochozoa), sea urchins and  
31 cnidarians (Santagata et al., 2012; Sinigaglia et al., 2013; Yaguchi et al., 2008),  
32 but has so far not been observed in any arthropod.

33

1 Based on these gene expression patterns and the lack of segmental gene  
2 expression within the anterior medial head we propose that this developmental  
3 territory is indeed non-segmental and homologous to the apical pole ectoderm of  
4 lophotrochozoan larvae. The presence of a similar territory in deuterostome  
5 larvae and in the radially symmetric cnidarian larva indicates that this anterior  
6 developmental territory is inherited from the bilaterian ancestor (Sinigaglia et  
7 al., 2013).

8

## 9 **2 A population of neural cells originates in the anterior medial head and** 10 **pioneers the axonal tracts of the central nervous system**

11

12 Based on the deep evolutionary origin of the non-segmental anterior-medial  
13 tissue, one would expect ancient parts of the brain to derive from this region.  
14 Therefore we tested whether the anterior medial region gives rise to neuronal  
15 cells that contribute to the brain and central nervous system.

16

### 17 *The foundation of axonal pathways from an anterior medial cell population*

18 Neurogenesis in *Strigamia maritima* has been characterized by Chipman and  
19 Stollewerk (2006). Neural progenitor cell groups, expressing the pro-neural gene  
20 *achaete-scute homologue (ash)* ((Linne et al., 2012), figure 3D), invaginate in  
21 small clusters of 5-9 cells from the segmental ventral neuroectoderm. Once  
22 internalized these clusters differentiate into neural cells. In the trunk segments  
23 these pro-neural clusters show a regular bilaterally symmetric arrangement in 7  
24 rows of 3-6 invagination sites per hemisegment. Invagination sites with similar  
25 characteristics are also present in the head segments, including the pre-antennal  
26 region, but are not arranged in an obviously stereotypical pattern (Chipman and  
27 Stollewerk, 2006).

28

29 In the anterior medial region of the head no such invagination sites are visible,  
30 and *ash* is not expressed in this medial region during early development (see  
31 figure 3D). However, Chipman and Stollewerk (2006) noted that, prior to the  
32 invagination of the pro-neural cell groups, early axonal tracts were already  
33 present beneath the surface ectoderm of trunk segments. This suggested the

1 presence of pioneer axons that build up an early scaffold for the following  
2 development of the nervous system. Retrograde labelling experiments by  
3 Whittington *et al.* (1991) showed in a scolopendromorph centipede,  
4 *Ethmostigmus rubripes*, that cell bodies of longitudinal axons of the developing  
5 central nervous system are located in the brain, anterior to the stomodaeum.

6  
7 We labelled the developing nervous system of *Strigamia* with an antibody  
8 against acetylated tubulin and found that cells located in the anterior medial  
9 head are the first cells to differentiate into neurons (figure 2 A, B). These cells  
10 form a dense cluster and project axons in posterior direction (figure 2 A-C).  
11 Directly posterior-basal of the cell bodies the axons fasciculate and at least some  
12 cross-over, so that cells from the right side send their axons to the left body half  
13 and *vice versa*. This is evident from the undivided architecture of the anterior-  
14 most brain commissure, which can only be achieved by midline crossing of some  
15 axons. (figure 2B). The neurons then elongate and form the early longitudinal  
16 axonal pathways along the AP axis of the embryo (figure 2C-F). Later in  
17 development these tracts become thicker (figure 2F, G), presumably through a  
18 secondary contribution of processes from the pro-neural cell clusters of the  
19 segmental neuroectoderm. Subsequently peripheral neurons and transverse  
20 commissures that connect the left and right longitudinal strands differentiate  
21 from the segmental neuroectoderm (figure 2I).

22  
23 The first neurons to deviate from the primary axonal tracts project bilaterally  
24 into the pre-antennal region (figure 2 E-F). We call these projections 'lateral  
25 protocerebral connections'; they later connect to lobes located in the pre-  
26 antennal region that, based on marker gene expression, most likely form the  
27 mushroom bodies (see supplementary figure S4D-I). The part of the axonal  
28 scaffold lying directly basal to the anterior founder cell population is the  
29 protocerebral commissure, which connects the two longitudinal projections and  
30 crosses the anterior medial region (see figure 2F). At late developmental stages  
31 single cells are still connected via axonal projections to this anterior bridge and  
32 (figure 2F).

33

1 *The anterior pioneer axons derive from the anterior medial region and are*  
2 *characterized by expression of the neural differentiation marker collier*

3 We followed back the origin of the pioneer neurons by marker gene expression  
4 and found that they originate from the anterior medial part of the head. At stage  
5 3, (early segmentation; (Brena and Akam, 2012)) a number of cells arranged in a  
6 bow within the surface layer of the anterior medial head region start to express  
7 *collier (col)* (figure 3A, B). In other animals *col* marks cells that are postmitotic  
8 and committed to a neural fate (Baumgardt et al., 2007; Demilly et al., 2011;  
9 Garcia-Dominguez et al., 2003). With on-going condensation of the head field  
10 these *col* positive cells form a dense cluster and sink under the surface  
11 epithelium (figure 3C). At the stage of specification (first expression of *col*) and  
12 internalization of these cells the factors *six3*, *FoxQ2*, *nk2.1*, *hbn* and *rx* are  
13 expressed in the territory from which the cells derive (see figure 1 and diagram  
14 in figure 7A,B). Double labelling against *col* gene expression and acetylated  
15 tubulin shows that the *col* expressing cells are indeed identical with the axonal  
16 pioneers (figure 3D).

17

18 The early differentiation and behaviour of these anterior medial neural cells  
19 differs from that of the pro-neural cell groups in the segmental neuroectoderm.  
20 They are at first arranged in a loosely organized but coherent groups in the  
21 surface layer (figure 3B), not tight focal clusters. Unlike the segmental  
22 neuroectoderm, they do not express *ash* prior to their differentiation.  
23 Conversely, the segmental neuronal precursors do not express *col* before or  
24 during their internalization (figure 3D). These differences in development  
25 suggest that the neuronal cells of the AMR are not serially homologous to the  
26 neurons and segmental ganglia that develop from the posteriorly following  
27 segments. Another characteristic feature of the differentiation process in the  
28 anterior medial region is that, in contrast to the formation of brain parts arising  
29 from the cephalic segments and to the formation of segmental ganglia of the  
30 ventral nerve cord, it has no bilateral character; from the time of their first  
31 differentiation the cells are arranged in an undivided medial group.

32

### 3 The late expression domains of *six3*, *irq-A*, *FoxQ2*, *hbn* and *rx* substructure the anterior medial head around the group of pioneer neurons

By Stage 4.3 (Brena and Akam, 2012), when the anterior medial neuronal cells are specified and lie at the anterior tip of the axonal scaffold, the AMR is substructured by a specific expression profile of the regional patterning genes. *Six3* continues to be expressed throughout the whole anterior medial head, but its expression is significantly reduced in the group of medial neuronal cells. Cells lying ventral and dorsal to this cell cluster still show strong expression of *six3* (figure 4A, B). *FoxQ2* expression is nested within the *six3* expression domain, and by contrast to *six3*, it is expressed strongly within the medial neuronal cells (figure 4 D, E). In addition *FoxQ2* is expressed in two lobes lying lateral to this central cell population (which will give rise to the *pars intercerebralis*, see below) and its expression reaches posteriorly into the anterior lip of the stomodaeum (figure 4 D, E). A marker that is not expressed during early development of the AMR but is found in this tissue at this later stage (4.3) is *iroquois-A* (*irq-A*). The *Strigamia* genome contains three *iroquois* genes of which two, *irqB* and *irqC* are expressed in the anterior medial region but also within the entire segmental neuroectoderm (data not shown). *Irq-A* (most closely related to *Drosophila caupolican* and *araucan*) is expressed within the AMR but only dorsally in the cells overlying the medial neuronal cell cluster (figure 4F, G). Both *rx* and *hbn* are expressed only ventrally of the neurogenic cells, mutually exclusive with *irq-A* (figure 4G-J). Neither *rx/hbn* nor *irq-A* is expressed in the medial neurogenic cells themselves. With proceeding development *rx* and *hbn* retreat from the anterior-most part of the AMR.

### 4 Development of the anterior neurosecretory protocerebrum

#### *Neurosecretory activity of the neuronal founder cells*

In the polychaete *Platynereis* the anterior *nk2.1/rx* positive region (which is also *six3* positive (Steinmetz et al., 2010)), gives rise to a neurosecretory fibre plexus that is located at the anterior tip of the axonal scaffold. Hence we suspected that the neurogenic cells that derive from a territory with similar molecular

1 characteristics might form a neuroendocrine nucleus. We therefore tested  
2 whether they express *pro-hormone convertase 2 (phc2)*, an enzyme involved in  
3 neurohormone processing, which in *Platynereis* marks the anterior  
4 neurosecretory fibre plexus (Tessmar-Raible, 2007; Tessmar-Raible et al., 2007).  
5 The centipede *phc2* gene is expressed in exactly the same cells that express *col*  
6 (compare figures 3 E,F; 5 A, B) and pioneer the early axonal scaffold (see figure  
7 2). Hence the pioneering neuronal cell population that derives from the  
8 centipede AMR is an early active neurosecretory centre that shares molecular  
9 and positional similarities with the neurosecretory fibre plexus in the polychaete  
10 (Tessmar-Raible et al., 2007).

11

### 12 *Development of the centipede pars intercerebralis from bilateral invaginating head* 13 *placodes*

14 We wondered whether the neurosecretory cells that are specified in the anterior  
15 medial head give rise to the centipede *pars intercerebralis*, which in insects is the  
16 anterior neurosecretory centre of the central complex (De Velasco et al., 2007)  
17 and develops from the *six3* positive territory in *Drosophila* and in the beetle  
18 *Tribolium* (Posnien et al., 2011; Steinmetz et al., 2010). Characteristic of the  
19 developing insect *pars intercerebralis* is that it develops from head ectodermal  
20 placodes that invaginate from the surface and form ectodermal compartments  
21 inside the embryo (De Velasco et al., 2007).

22 Two bilateral pairs of placodal invagination sites are found in the anterior head  
23 of the centipede. The central ones, which we term the main head placodes,  
24 develop at the border between the anterior medial region and the pre-antennal  
25 region (figure 5E). A pair of smaller invagination sites of unknown fate forms  
26 more laterally in the anterior head (the lateral head placodes). The ectoderm of  
27 both pairs expresses *six3* (see figure 5F).

28 In *Drosophila* cells that form the *pars intercerebralis* express *Dchx*, the orthologue  
29 of vertebrate *vsx*, throughout development (De Velasco et al., 2007). We tested  
30 expression of the centipede *vsx* gene and found that it is expressed in cells that  
31 lie immediately lateral to the medial neurosecretory cells and marks the medial  
32 part of the ectodermal compartments that derive from the main head placodes  
33 (figure 5C, D, G). (The more lateral part of these placodes express mushroom

1 body markers, see supplementary figure S4E, F, I). Based on *vsx* expression and  
2 the mode of development from invaginating ectoderm we conclude that the  
3 medial parts of the main head placodes give rise to the centipede *pars*  
4 *intercerebralis*. The *vsx* expression domains are within the nested *six3* and *FoxQ2*  
5 expression domains (see figure 5A, D), but notably *hbn/rx* and *nk2.1* expression  
6 is absent from the invagination sites and ectodermal compartment of the *pars*  
7 *intercerebralis* (figure 1G, H, L, 4G-J). The *pars intercerebralis* compartments are  
8 also devoid of *col* and *phc2* expression that mark only the central neurosecretory  
9 cells. Hence there are two distinct anterior structures that develop from the  
10 *six3/FoxQ2* domain, the unpaired median population of neurosecretory pioneer  
11 neurons and the bilateral *pars intercerebralis*.

#### 12 13 *Hypothalamus-like cell types in the central pioneer-neuronal/neurosecretory cell* 14 *cluster and in the pars intercerebralis*

15 The apical plate derived neurosecretory region of the polychaete shares  
16 molecular similarities with the neuroendocrine brain centre of vertebrates, the  
17 hypothalamus. Cells that give rise to the zebrafish hypothalamus derive from a  
18 *six3*, *nk2.1* and *rx* positive region at the anterior end of the neural plate, which is  
19 reminiscent of the neurosecretory plexus formation in the polychaete (Steinmetz  
20 et al., 2010; Tessmar-Raible et al., 2007). Based on this Tessmar-Raible and  
21 others have proposed that the anterior neurosecretory cells in both these  
22 territories likely share a common evolutionary origin (Marlow et al., 2014;  
23 Steinmetz et al., 2010; Tessmar-Raible, 2007; Tessmar-Raible et al., 2007).  
24 To test for similarities in the molecular identity of vertebrate hypothalamus  
25 neurons and the anterior neurosecretory cells of *Strigamia* we examined some of  
26 the factors that are known to mark hypothalamic neurons. The gene *ventral*  
27 *anterior homeobox 1* (*vax1*) is involved in formation of the pituitary gland and  
28 also defines rostral hypothalamic progenitors in the forebrain (Bertuzzi and  
29 Hindges, 1999; Bharti et al., 2011; Wataya et al., 2008; Bharti et al., 2011). *Vax1*  
30 genes are absent from sequenced insect genomes and so far no *vax1* gene has  
31 been characterized in any arthropod. We found a clear *vax1* orthologue in the  
32 centipede genome. Embryonic expression of *vax1* in the centipede is restricted to  
33 the anterior-most part of the head (figure 6A, B). In the medial domain the



1 pattern is punctate and marks single cells, which are located within the medial  
2 neurosecretory cell cluster (figure 4B). The lateral expression of *vax1* is within  
3 the compartmentalized ectoderm that derives from the head placodes. It is  
4 however absent from the medial part of the main head placode that expresses  
5 *vsx* and produces the *pars intercerebralis* (figure 5H).

6

7 A second factor that is required for the specification of neurosecretory  
8 hypothalamic neurons (Wang and Lufkin, 2000) is *orthopedia (otp)*. We find  
9 expression of the centipede *otp* gene in a punctate pattern concentrated within  
10 the anterior medial region. *Otp* positive cells are found in both the medial  
11 neurosecretory cell population and the *pars intercerebralis*, and also more  
12 laterally (figure 6C, D).

13

14 We identified within the centipede genome a single *vasotocin*  
15 (*vasopressin/oxytocin*) -*neurophysin (vtn)* orthologue. The vertebrate  
16 orthologues encode neuropeptides secreted by the periventricular  
17 hypothalamus (Pearson and Placzek, 2013). This gene has been lost from the  
18 *Drosophila* genome but is present in other insects, including at least some  
19 orthopterans and the beetle *Tribolium* (named *inotocin* in insects; Stafflinger et  
20 al., 2008). The expression data for *vtn* in the centipede is not as clear as for  
21 transcription factors (perhaps because the levels of expression are low at these  
22 embryonic stages), and long staining times led to increased background.  
23 Nevertheless we detected transcripts of the centipede *vtn* gene in a punctate  
24 pattern throughout the ventral neuroectoderm and ventral midline, with more  
25 concentrated expression within the anterior medial neurosecretory cell  
26 population, and also within cells of the *pars intercerebralis* (figure 6E, F). This is  
27 similar to the *otp* expression pattern, which is consistent with findings in other  
28 animals that both factors are co-expressed and that *otp* is involved in the  
29 regulation of vasopressin/oxytocin expression in the mouse hypothalamus  
30 (Acampora et al., 1999; Tessmar-Raible, 2007).

31

32 Thus the medial neurosecretory cells of the centipede share similarities with  
33 both the polychaete neurosecretory plexus and the vertebrate hypothalamus.

1 These medial cells occupy an anterior position within the nervous system and  
2 originate from an *nk2.1+ / rx+ / six3+* territory. They contain cells that express the  
3 transcription factors *vax1* and *otp*, which specify hypothalamic neurons (Wang  
4 and Lufkin, 2000; Wataya et al., 2008) and cells that express the hypothalamic  
5 neuropeptide *vtn*. The *pars intercerebralis* is developmentally distinct from the  
6 medial cells and does not have a pioneering function in nervous system  
7 development. It also derives from a territory that expresses *six3* (and FoxQ2),  
8 but is devoid of *vax1*, *nk2.1* and *rx*. The latter two factors are characteristic of the  
9 hypothalamus progenitor tissue (Pearson and Placzek, 2013; Tessmar-Raible et  
10 al., 2007). However, cells expressing markers of hypothalamic neurons, *vtn* and  
11 *otp*, are also found in the developing *pars intercerebralis*.

12

### 13 **Discussion**

14

15 We have identified an anterior cell population in centipedes that lies medial to  
16 the primordia of the *pars intercerebralis*, and which appears to be without a  
17 recognised counterpart in insects (but see below). The fate of these cells in the  
18 adult brain is unclear, but given that they establish the anterior commissure,  
19 they are likely to contribute to the centipede central complex.

20

21 The central complex is well defined in insects, where it consists of different  
22 elements including the central body, ellipsoid body and a protocerebral bridge  
23 (Boyan and Reichert, 2011; Strausfeld, 2012). In millipedes the midline neuropil  
24 of the central complex is greatly reduced, but a distinct midline neuropil  
25 corresponding to the insect central body has been found in several centipede  
26 species (Loesel et al., 2002; Strausfeld, 2012). It is situated between the  
27 mushroom bodies and is innervated by allatostatin-like immunoreactive  
28 peptidergic cells (Loesel et al., 2002). The anterior neurosecretory pioneer cells  
29 that we identify in *Strigamia* show several similarities to this central body. They  
30 lie between the protocerebral lobes anterior to the first developing brain  
31 commissure and the initial fibres originating from their cell bodies project  
32 longitudinally in a parallel array, before joining the anterior commissure.

33

1 In insects it has been shown that most elements of the central complex, including  
2 the central body, are produced by neuroblasts located within the *pars*  
3 *intercerebralis* (see Boyan and Reichert, 2011). Knockdown of *six3* function in  
4 the beetle *Tribolium* disrupts central body formation (Posnien et al., 2011),  
5 supporting the idea that the central body derives from the *six3* positive territory.  
6 In the centipede both embryonic structures, *pars intercerebralis* and medial cell  
7 cluster, are in close proximity within the *six3* (and *FoxQ2*) expressing territory.  
8 Therefore it seems likely that both cell populations produce the central body  
9 neuropil of *Strigamia*.

10 Anterior midline neuropils are found in most arthropods and in onychophorans  
11 (Strausfeld, 2012), but little is known about their embryonic development  
12 outside the insects. In the spider *Cupiennus salei* the arcuate body, which is a  
13 possible homologue to the insect central body (Loesel et al., 2011; Strausfeld,  
14 2012), is formed by bilateral invaginations of the protocerebral ectoderm and a  
15 subsequent fusion at the midline. In addition many postmitotic neural precursor  
16 cells located in the medial pre-cheliceral domain contribute to to the central  
17 protocerebrum (Doeffinger et al., 2010). The lateral invaginations bear  
18 similarities to the invaginating 'head-placodes' of the centipede. It is however not  
19 clear whether the medial neural precursors are similar to the anterior medial  
20 cells of *Strigamia*. More comparative developmental work is required to  
21 elucidate the evolutionary relationships of embryonic cell populations across the  
22 arthropods, and of the adult structures that they give rise to.

23

24 *The anterior pole of the head axis and the anterior-posterior organisation of the*  
25 *protocerebrum*

26 The interpretation of the anterior neuro-axis in arthropods is still subject to  
27 dispute. Some authors have interpreted structures that derive from the pre-  
28 antennal (ocular) region, in particular the eyes, as the anterior-most tip of the  
29 neural axis of arthropods (Haas et al., 2001; Rempel, 1975; Siewing, 1963), but  
30 molecular work in insects hints at the central complex as being the anterior-most  
31 brain structure (Posnien et al., 2011; Urbach and Technau, 2003). In 1963  
32 Siewing proposed a subdivision of the protocerebrum into archicerebrum  
33 comprising the ocular lobes and the mushroom bodies, and prosocerebrum,

1 comprising the central complex. He interpreted the archicerebrum as the  
2 anterior-most part of the brain (Siewing, 1963; discussed in Scholtz and  
3 Edgecombe, (2006)). Urbach and Technau (2003) also suggested a subdivision of  
4 the protocerebrum into archi- and prosocerebrum, but see the *pars*  
5 *intercerebralis* and the central complex, which is at least partially formed by  
6 progenitors located in the developing *pars intercerebralis* (Boyan and Williams,  
7 2011; De Velasco et al., 2007; Williams and Boyan, 2008) as the anterior-most  
8 brain structures. This is based on a map of neuroblasts in the head, where cells  
9 that give rise to the *pars intercerebralis* are located in the anterior-most, medial  
10 part of the insect head lobes (Urbach and Technau, 2003). Similarly Strausfeld  
11 2012 argues that the *pars intercerebralis* is part of an ancestral, rostral and a-  
12 segmental brain (Strausfeld, 2012).

13

14 Our work in the centipede now clearly supports an embryonic origin of the  
15 protocerebrum from two developmentally distinct regions, an  
16 ocular/preantennal region, and the anterior medial region. These regions are  
17 characterised by the expression of largely non-overlapping sets of transcription  
18 factors (see figure 7H). Brain structures that derive from the AMR include the  
19 *pars intercerebralis*, well documented in insects and other arthropods, and the  
20 anterior *col+* medial/neurosecretory cell cluster, which has not previously been  
21 described in insects or any other arthropod. Together these structures  
22 represent the anterior tip of the neural axis, as Urbach and Technau, and  
23 Strausfeld, proposed. Brain parts deriving from the ocular/pre-antennal region  
24 are more posterior structures.

25

26 *The evolutionary origin of the anterior-most part of the centipede protocerebrum*  
27 *and origin of the axonal scaffold from an apical-organ like neurosecretory cell*  
28 *population*

29 The anterior-medial neurosecretory cell cluster derives from a region expressing  
30 a broadly conserved suite of transcription factors. Central to this system is a  
31 domain of *FoxQ2* expression nested within *six3* expression. In many marine  
32 larvae, the cells of the apical organ are specified centrally within this *FoxQ2*  
33 domain, a pattern that has been found in organisms as diverse as brachiopods,

1 polychaete annelids, cnidarians, echinoderms and hemichordates (Darras et al.,  
2 2011; Lowe et al., 2003; Marlow et al., 2014; Santagata et al., 2012; Sinigaglia et  
3 al., 2013; Wei et al., 2009; Yaguchi et al., 2008; Yankura et al., 2010). This  
4 molecular topography has been particularly well characterised in the anterior  
5 pole ectoderm of the Brachiopod *Terebratalia transversa* (Santagata et al., 2012).  
6 This reveals parallels with *Strigamia* also in dorso/ventral organisation:  
7 Expression of *hbn* in *Terebratalia* is restricted to the ventral side of the animal, as  
8 in *Strigamia*.

9

10 Despite the apparently ancient origin and conserved molecular fingerprint of  
11 this anterior territory (the apical plate), we did not anticipate finding a cell  
12 population homologous to the apical-organ itself in the centipede, because  
13 neither primary free-swimming larvae nor ciliated epithelia are present in  
14 ecdysozoans, and nothing recognisable as an apical organ has been reported in  
15 any extant arthropod (Telford et al., 2008). However, although the larval apical  
16 organ degenerates completely during metamorphosis in those groups such as  
17 sea urchins and cnidarians, that undergo a complete reorganisation of the body  
18 plan at metamorphosis (Nielsen, 2005, and literature cited therein), there are  
19 other groups in which the apical organ, and other structures derived from the  
20 apical plate, integrate into the axonal scaffold of later larval and adult stages  
21 (Fischer et al., 2010; Santagata et al., 2012). This would be typical, for example,  
22 of annelids, which show a continuity of function from trochophore to larva to  
23 adult. It is now clear that in at least some Spiralia, cells of the apical organ form a  
24 neuropil that contributes to the larval and sometimes even to the adult central  
25 nervous system (Fischer et al., 2010; Santagata et al., 2012; Tessmar-Raible et al.,  
26 2007) and execute important neuroendocrine functions (Conzelmann et al.,  
27 2013, 2011; Tessmar-Raible et al., 2007). This makes the finding of a similar cell  
28 population persisting within some arthropods less surprising.

29

30 Whether or not ecdysozoans evolved from an ancestor with a free-swimming  
31 larval stage, our results suggest that the common ancestor of ecdysozoans and  
32 lophotrochozoans possessed an anterior domain characterised by a conserved  
33 regulatory signature that gave this anterior tissue the competence to form

1 neural/neurosecretory cells. Either arthropods lost the ability to form a ciliated  
2 apical tuft from cells within this territory (as they lost ciliation in general), or the  
3 ciliated tuft might have been acquired independently during the evolution of  
4 larval forms.

5

#### 6 *Anterior pioneer neurons in centipedes, insects and crustaceans*

7 Our results suggest that the centipede anterior medial cell population, which  
8 expresses the apical organ markers *col*, *phc2* and *otp* (Conzelmann et al., 2013;  
9 Jackson et al., 2010; Marlow et al., 2014; Pang et al., 2004; Santagata et al., 2012;  
10 Tessmar-Raible et al., 2007) serves an important function in erecting the  
11 primary scaffold of at least the anterior central nervous system. Similar long  
12 range pioneer neurons originating from the anterior pole have not so far been  
13 characterized in any other arthropod. In most insects for instance the  
14 neuroblasts that pioneer the axon tracts of the anterior nervous system are  
15 located in the bilateral head neuroectoderm (Posnien et al., 2011; Urbach and  
16 Technau, 2003; Younossi-Hartenstein et al., 1996).

17

18 Interestingly two short range pioneer neurons differentiate within the anterior  
19 medial domain of the head of the grasshopper *Schistocerca gregaria* (Boyan and  
20 Williams, 2008; Ludwig et al., 1999), an insect that forms most of its nervous  
21 system through stem-cell like progenitor cells (Shepherd and Bate, 1990). These  
22 two cells originate directly from the epithelium and not from intermediate  
23 progenitors. They are the pioneers of the primary brain commissure of the  
24 grasshopper (Boyan and Williams, 2008; Ludwig et al., 1999). Although this  
25 alternative mode of formation of the brain commissure is restricted to single  
26 pioneer cells, it shows intriguing similarity to the development of the pioneer  
27 neurons that are directly specified within the surface epithelium of the *Strigamia*  
28 anterior medial head, and are quite distinct from the invaginating pro-neural cell  
29 clusters of the ventral neuroectoderm, which are the myriapod equivalent of the  
30 insect neuroblasts (Chipman and Stollewerk, 2006; Dove and Stollewerk, 2003;  
31 Stollewerk and Simpson, 2005). It would be exciting to see whether these cells  
32 arise from a territory in the grasshopper that expresses similar molecular  
33 markers as the *Strigamia* AMR.

1

2 Anterior medial cells with a neurogenic character have also been reported in  
3 some but not all crustaceans. In the amphipod *Orchestia cavimana* one of the first  
4 signs of axogenesis is that about six cells in a medial domain arrange in a row  
5 and contribute to the anterior protocerebral commissure (Ungerer et al., 2011).  
6 Pioneering neurons that have their origin in the brain have also been described  
7 in the crayfish *Cherax destructor* whereas in another malacostracan crustacean,  
8 the woodlouse *Porcellio scaber*, the first neurons have a segmental origin  
9 (Whittington et al., 1993). These authors do however comment that the  
10 pioneering axons from the brain in the crayfish are in their morphology not  
11 similar to the centipede pioneer axons (Whittington et al., 1993, 1991).

12

13 None the less, based on the conserved molecular characteristics of the anterior  
14 medial pioneer neurons in centipedes, we believe that this cell population  
15 probably does go back to the arthropod ancestor. It is possible that this ancestor  
16 possessed long range anterior axonal pioneers like the centipede, which during  
17 evolution have gradually been replaced by neurons from the segmental  
18 neuroectoderm. On the other hand it is also possible that ancestrally the axons  
19 from the medial domain only contributed to the anterior part of the axonal  
20 scaffold, as do the medial cells in the grasshopper (Boyan and Williams, 2008;  
21 Ludwig et al., 1999) and that they have been modified to long range pioneers in  
22 the myriapod lineage.

23

#### 24 *Conservation of anterior neurosecretory brain centres*

25 There is some disagreement surrounding the structure of the vertebrate anterior  
26 neural plate, but the rostral hypothalamus probably marks its anterior-most tip  
27 (Puelles and Rubenstein, 2003; Rubenstein and Shimamura, 1998). Similarities  
28 in the markers expressed in the rostral hypothalamus and in the apical  
29 plate/apical organ of polychaete annelids have led Tessmar Raible et al to  
30 propose that these structures share a common evolutionary origin (Tessmar-  
31 Raible et al., 2007). Our results argue that the centipede retains a derivative of  
32 the same ancestral structure.

33

1 Three of the regional transcription factors that characterize the AMR of the  
2 centipede, *six3*, *rx* and *nk2.1* are expressed in the medial forebrain region of  
3 vertebrates that produces the hypothalamus (Lagutin et al., 2003; Muranishi et  
4 al., 2012; Tessmar-Raible et al., 2007). In addition, several genes that mark  
5 neurosecretory cell populations in the hypothalamus are also expressed within  
6 the centipede anterior medial neurosecretory cells. For example, *otp* is required  
7 for the correct development of the hypothalamus from the rostral neural plate  
8 and for secretion of the neuropeptides arginine-vasotocin, oxytocin (both  
9 orthologous to *Strigamia vtn*), somatostatin and corticotropin releasing hormone  
10 (Wang and Lufkin, 2000). We found scattered cells in the medial population  
11 expressing *otp*, and a concentration of *vtn* expressing cells within the anterior  
12 medial population and the *pars intercerebralis* of *Strigamia*. *PC2 (phc2)*, which  
13 distinctively marks the anterior medial cells that pioneer the axonal scaffold of  
14 *Strigamia*, is expressed in the paraventricular and arcuate nuclei of the  
15 hypothalamus (Nillni, 2007). In addition one of the three mouse orthologues of  
16 *col*, *Olf-1/EBF-like 3*, is expressed in some cells of the hypothalamus (Wang et al.,  
17 1997). *Vax1*, which is expressed in a subset of the anterior medial pioneer  
18 neurons in the centipede, is required for axonal tract formation in the ventral  
19 forebrain and is prominently expressed in the presumptive hypothalamus  
20 (Bertuzzi and Hindges, 1999). In mouse embryos *vax1* is also involved in  
21 formation of the pituitary gland. Its absence in the anterior-most ectoderm  
22 seems to be required for the invagination of pituitary gland progenitor tissue, as  
23 the complete lack of *vax1* in the anterior ectoderm leads to a second invagination  
24 further posterior (Bharti et al. 2011). This bears some similarity to the absence  
25 of *vax1* in the *pars intercerebralis* tissue, and its expression in surrounding areas.

26  
27 In conclusion, there is substantial overlap in the sets of markers that  
28 characterize the anterior medial neurosecretory cells of *Strigamia* (and other  
29 invertebrates) and the developing hypothalamus of vertebrates.

30  
31 These results together suggest that the anterior neurosecretory brain centre of  
32 the bilaterian ancestor already possessed a relatively high degree of complexity  
33 and cell type diversification, a principle that also emerges from recent studies in



1 cnidarian and lophotrochozoan larvae (Conzelmann et al., 2013, 2011; Marlow et  
2 al., 2014, 2009; Tessmar-Raible et al., 2007).

3

4 The development and structure of the anterior neurosecretory system seems to  
5 be less conserved in the insects used as major experimental models. The *pars*  
6 *intercerebralis* is conserved and developmentally well characterized in insects  
7 (De Velasco et al., 2007), but no cell population corresponding to the *collier*-  
8 expressing pioneer cells has so far been characterized in *Drosophila* or in  
9 *Tribolium*. In these insects, the cells of the *pars intercerebralis* merge to form the  
10 most medial neurosecretory structure (De Velasco et al., 2007), whereas in  
11 *Strigamia*, the bilateral parts of the *pars intercerebralis* remain separated by the  
12 *collier* expressing population. Both the medial pioneer cells and the *pars*  
13 *intercerebralis* derive from the *FoxQ2* and *six3* expression domain, and both  
14 express some of the hypothalamic cell type specific marker genes (*otp*, *vtn*).  
15 Hence both might originate from an ancient anterior neuroendocrine system that  
16 has diversified during evolution of the arthropod brain.

17

## 18 **Experimental procedures**

19

### 20 *Embryo collection and fixation*

21 Embryos were collected from a wild population near Brora, Scotland (Chipman  
22 et al., 2004a). The material was fixed for several days in 4% Formaldehyde/0.5 x  
23 PBS (details can be found in (Brena and Akam, 2012)). Embryos were stages  
24 according to morphological features as described in (Brena and Akam, 2012).

25

### 26 *Gene identification and cloning*

27 Genomic resources for *Strigamia maritima* are available at  
28 <http://www.ncbi.nlm.nih.gov/assembly/322118/>. Gene orthologues were  
29 identified by *Blast* searches against the genomic and transcriptomic sequence.  
30 Gene identities were validated by reciprocal searches of the sequences against  
31 generic databases. Models of all identified head patterning genes were annotated  
32 on the genome and can be found at

1 [http://metazoa.ensembl.org/Strigamia\\_maritima/](http://metazoa.ensembl.org/Strigamia_maritima/). Ensembl gene IDs are listed  
2 in the supplementary material (suppl. table S1).

3 In addition, for the characterization of *FoxQ2* and *irq-A* genes, phylogenetic trees  
4 (supplemental figures S2 and S3) were created using Phylemon2 (Sánchez et al.,  
5 2011). Multiple sequence alignments were performed on protein sequences  
6 using MUSCLE (Edgar, 2004) and gene trees were built by maximum likelihood  
7 analysis in PhyML (Guindon and Gascuel, 2003). Tree calculation parameters are  
8 given in the accompanying material (Suppl fig. S2 and S3). A classification of the  
9 centipede neuropeptides and homeobox genes can also be found in the *Strigamia*  
10 genome publication (Chipman et al., *under review*).

11 Specific primers were designed against the identified gene sequences and  
12 products were amplified by standard PCR reaction and subsequently cloned into  
13 the pGEM-T-Easy vector system (Promega). Inserts were verified by sequencing  
14 and then used as templates for *in situ* probe synthesis.

15

16 *In situ staining of gene expression and antibody stain of the central nervous system*

17 Single and double colorimetric *in situs* were performed as described in (Chipman  
18 and Stollewerk, 2006; Chipman et al., 2004b). For *in situ* stains in conjunction  
19 with antibody stain against acetylated tubulin, embryos were pre-treated in a  
20 buffer containing 5% mercaptoethanol and 0.3 % Triton (Yoshida-noro et al.,  
21 2000) to increase tissue permeability and allow increased penetration of the  
22 antibody. Permeabilised embryos were first taken through the probe incubation  
23 steps of the *in situ* hybridisation protocol and then incubated in the primary  
24 antibody (from mouse, clone 6-11-B1, Sigma) (1:250 v/v) at 4° over night.  
25 Embryos were washed several times in PBT (PBS+0.1% Tween-20) and then  
26 incubated with the secondary antibody (A488 goat anti mouse IgG, Molecular  
27 Probes) (1:500 v/v) at 4° over night. After several washes in PBT embryos were  
28 post-fixed for 10 minutes in 4% Formaldehyde. Finally embryos were incubated  
29 in the anti-DIG-AP antibody (1:3000 v/v) and a Fast Red (Roche) staining  
30 reaction was carried out. All embryos were counterstained with the nuclear dye  
31 Hoechst (H33342; used at 1/1000 v/v). A detailed protocol is available on  
32 request from the authors.

33

## 1 *Image acquisition*

2 Specimens were mounted in 90% Glycerol and analysed using a Leica SP5  
3 upright confocal laser scanning microscope. The fluorescing properties of Fast  
4 Red (Murdoch et al., 1990) were used for laser scanning detection of the *in situ*  
5 stain using a 543nm He-Ne laser. The A488 labelled acetylated tubulin was  
6 visualized using an Ar laser at 488nm and for Hoechst detection we used a  
7 405nm diode. For analysis and reconstruction of the image stacks we used the  
8 free software bundle FIJI (Schindelin et al., 2012). Brightness and contrast of  
9 images were adjusted using Photoshop CS5 (Adobe).

10

## 11 **Acknowledgements**

12

13 This work was made possible by collaborations initiated within the Marie Curie  
14 training network “Evonet”, which funded VSH. We thank members of the Akam  
15 group for assistance with embryo collection and the *Strigamia* Genome consortium  
16 for sharing data prior to publication. We thank particularly Heather Marlow, Maria  
17 Tosches and Detlev Arendt from the Evonet group at EMBL for discussions  
18 concerning apical patterning. Maria Tosches, Angelika Stollewerk and Nicholas  
19 Strausfeld provided valuable comments on the manuscript prior to publication.

20

21

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35

36 **Figure legends**

37

1 **Figure 1: Morphogenesis of the head field and early gene expression within**  
2 **the anterior medial tissue. A-L** *In situ* hybridisation, probes as indicated in the  
3 pictures. **A** (head condensation, stage 2.3), **B** (late head condensation/early  
4 segmentation, stage 3.1): Expression of *SP5* marks the segmental territories of  
5 the early head. White arrow points at expression in the pre-antennal region.  
6 Double headed arrow points to the anterior medial tissue. Dashed line in **A**  
7 marks the anterior margin of the condensed head field. **C-E**: Expression of *FoxQ2*,  
8 *nk2.1* and *hbn* during early head condensation. **F-I**: Expression domains of  
9 anterior medial markers (now including *rx*) during early segmentation stage  
10 (Stage 4.1-4.2). **C'**, **D'**, **E'**, **F'**, **G'**, **H'**, **I'** show nuclear stain of the specimens. **J**: *six3*  
11 expression in AMR is directly anterior to pre-antennal *otx* expression. **K**: Nested  
12 domains of *FoxQ2* and *six3* in AMR. **L**: *FoxQ2* overlaps with the anterior portion of  
13 *nk2.1* expression (dashed line, compare to **I**). **M**, **N**: Schematic drawings of the  
14 anterior medial domain, and its inclusion between the two halves of the pre-  
15 antennal region. **M** - head condensation stage; **N** -segmented germ band; **N** only  
16 includes *six3* and *FoxQ2* expression. pa=pre-antennal region, ant=antennal  
17 segment, AMR=anterior medial region. Bracket in **A**, **B**, **E** and **E'** marks the 'dorsal  
18 field' (**df**), the thin epithelium covering the anterior hemisphere which may be  
19 extra-embryonic. A-I = whole embryo diameter is between 1.1-1.2 mm; scale  
20 bars J-L = 100  $\mu$  m.

21

22 **Figure 2: Axons originating from an anterior medial cell population erect**  
23 **the primary scaffold of the central nervous system.** Labelling of the nervous  
24 system with an anti-acetylated tubulin antibody, developmental series.  
25 Reconstructions of confocal microscope image stacks. **A**: Early segmentation  
26 (stage 4) embryo. White arrow points at neurogenic cell population in AMR; the  
27 ventral neuroectoderm with invaginating cell clusters lies in the Y-shaped area  
28 between the dashed lines. A white asterisk marks the stomodaeum **B**: White  
29 arrow points at cell bodies, red arrows at elongating ends of the axonal bundles  
30 **C-E**: Elongation of axonal bundles (red arrows) during stages 4.3-5; cell bodies  
31 remain in anterior medial position. **F**: Elaboration of the anterior nervous  
32 system, brain development. **G** Addition of commissures and peripheral neurons  
33 to the longitudinal axonal tracts. AMR=anterior medial region, lb=labrum,

1 pa=pre-antennal segment, ant=antennal segment, **prn**=pre-antennal neuropil,  
2 **pc**=protocerebral commissure, **an**=antennal nerve, **pn**=peripheral neurons,  
3 **co**=commissure. Scale bars: A, C, D-F = 100  $\mu$  m, G= 50  $\mu$  m.

4

5 **Figure 3: The neural cells originating in the AMR express *collier*.** A-D *In situ*  
6 stains of gene expression, developing nervous system. Reconstructions of  
7 confocal microscope image stacks. **A-B** (early segmentation, stage 3.2): *Col+* cells  
8 appear in a loosely arranged arch in the AMR. *Col+* cells are in the surface cell  
9 layer (see B'). **C** (stage 4.1): *Col+* cells have sunk beneath the surface (see C') and  
10 form a dense cell cluster. Yellow lines in B and C indicate planes of orthogonal  
11 section in B' and C'. **D** (stage 3.2): The pro-neural gene *ash* is expressed in cells of  
12 the lateral neuroectoderm. The central AMR where the *col+* cells are situated  
13 (marked by white asterisks) is mostly free of *ash* expression. At this stage  
14 expression is only seen in few cells at the border of the AMR (marked by white  
15 arrows). **E, F** (stage 4.3): *Col* expression in the cell bodies of the pioneering  
16 axons. White arrows in F point at axonal connections to the central neuropil.  
17 Scale bars: F = 300  $\mu$  m; C-F=100  $\mu$  m.

18

19 **Figure 4: Regional patterning of the AMR.** *In situ* stains of gene expression,  
20 developing nervous system. Reconstructions of confocal microscope image  
21 stacks. All stage 4.3 embryos. **A, B, C:** Regional expression of *six3* in the entire  
22 AMR tissue, down-regulation of expression within the pioneer neuronal cell  
23 population (encircled in B, C). **D, E:** *FoxQ2* expression in a compact domain  
24 surrounding and including the anterior medial cells and the *pars intercerebralis*  
25 compartments (white arrows). **F, G:** Expression of *irq-A* in the anterior medial  
26 tissue is only dorsally (internally) in the embryo above the medial neuronal cells  
27 (see G', G''). **H, I:** Expression of *rx* in the AMR, ventral to the pioneer neuronal  
28 cells (see I', I''). **J:** *Hbn* is expressed in a pattern similar to that of *rx*; both are  
29 absent from the placodal invagination sites of the *pars intercerebralis* (white  
30 arrows); expression retracts from the AMR as development proceeds. Encircled  
31 area in B, C, F, G and J marks the position of the medial neural cell cluster.  
32 **AMR**=anterior medial region, **pa**=pre-antennal segment, **ant**=antennal segment,  
33 **ic**=intercalary segment **lb**=labrum. Scale bars: A, D-J = 100  $\mu$  m; B, C= 50  $\mu$  m.

1

2 **Figure 5: Neurosecretory activity of the axonal pioneers and development**  
3 **of the pars intercerebralis.** *In situ* stains of gene expression, developing

4 nervous system. Reconstructions of confocal microscope image stacks. All stage  
5 4.3 embryos. **A, B:** Expression of *phc2* within the medial neurogenic cells. **C, D:**  
6 Expression of the pars-intercerebralis marker *vsx* in developing brain structures  
7 situated directly lateral to the medial neural cells, and around the stomodaeum  
8 (white asterisk in D). **E:** Surface rendering using the stain of cytoskeletal  
9 acetylated-tubulin. Invagination sites of the main head placodes are visible  
10 (encircled areas). The medial part of the invagination site co-localizes with *vsx*  
11 stain shown in C and D. **F:** *Six3* is expressed in the sub-surface ectoderm that  
12 derives from the main head placodes (encircled areas) and the lateral head  
13 placodes (white arrows). **G** *Vsx* is expressed only in the medial part of the main  
14 head placode-derived ectoderm (encircled). *Vax1* is expressed only in the lateral  
15 part of the main head placode-derived ectoderm (encircled), mutually exclusive  
16 with *vsx*. **AMR**=anterior medial region, **pa**=pre-antennal segment, **ant**=antennal  
17 segment, **ic**=intercalary segment, **lb**=labrum. Scale bars A, C, E, F = 100  $\mu$  m.

18

19 **Figure 6: Expression of the hypothalamic marker genes *vax1*, *otp* and *vtn* in**  
20 **the anterior medial region.** *In situ* stains of gene expression, developing

21 nervous system. Reconstructions of confocal microscope image stacks. A-D stage  
22 4.3, E-F stage 5 embryos. **A, B:** Expression of *vax1* in some of the medial neural  
23 cells and laterally in the placodal derived ectoderm, but not in the *pars*  
24 *intercerebralis* (compare 5D). **C:** Expression of *otp* in a punctate pattern within  
25 the anterior head. **D:** *Otp* is expressed in some of the cells belonging to the  
26 medial neural cell cluster. **E, F:** Expression of the RNA encoding the neuropeptide  
27 *vtn* is detected at higher levels within cells of the medial neural cell cluster and  
28 *pars intercerebralis* than in the remaining ectoderm. **AMR**=anterior medial  
29 region, **oc**=ocular segment, **ant**=antennal segment, **ic**=intercalary segment,  
30 **lb**=labrum. Scale bars: A-C, F = 100  $\mu$  m; D = 50  $\mu$  m.

31

32 **Figure 7: Summary of structure and gene expression characterizing**  
33 **subdomains of the AMR tissue. Schematic drawings. A, B:** Early gene

1 expression domains (at late head condensation/early segmentation stage)  
2 anterior to the mouth field (mf). *Col* expressing neural cells are specified within a  
3 domain of nested *six3* and *FoxQ2* expression and adjacent *rx/nk2.1* expression.  
4 Black lines indicate invagination sites of the *pars intercerebralis* (pi) **C-F**: Gene  
5 expression domains in relation to the developing nervous system. *Col+/phc+*  
6 cells are located at the anterior end of the primary axonal scaffold, laterally  
7 bordered by the *vsx*-expressing *pars intercerebralis* and surrounded by *six3*,  
8 *FoxQ2* and *irq-A* expression. Black arrows in C indicate the extension of the  
9 primary axonal scaffold in posterior direction, **G**: Architecture of the developing  
10 protocerebrum and anterior axonal scaffold. **pi**=pars intercerebralis placodal  
11 invagination site, **mf**=mouth field, **lhp**=lateral head placode, **mhp**=main head  
12 placode, **cpnc**=central pioneer neuronal cells, **mo**=mouth, **pas**=primary axonal  
13 scaffold.  
14  
15  
16