



Tracking down the “head blob”: Comparative analysis of *wingless* expression in the developing insect procephalon reveals progressive reduction of embryonic visual system patterning in higher insects

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Abstract

The evolution of larval head morphology in holometabolous insects is characterized by reduction of antennal appendages and the visual system components. Little insight has been gained into molecular developmental changes underlying this morphological diversification. Here we compare the expression of the segment polarity gene *wingless* (*wg*) in the pregnathal head of fruit fly, flour beetle and grasshopper embryos. We provide evidence that *wg* activity contributes to segment border formation, and, subsequently, the separation of the visual system and protocerebrum anlagen in the anterior procephalon. In directly developing insects like grasshopper, seven expression domains are formed during this process. The activation of four of these, which correspond to polar expression pairs in the optic lobe anlagen and the protocerebral ectoderm, has shifted to postembryonic stages in flour beetle and *Drosophila*. The remaining three domains map to the protocerebral neuroectoderm, and form by disintegration of a large precursor domain in flour beetle and grasshopper. In *Drosophila*, the precursor domain remains intact, constituting the previously described “head blob”. These data document major changes in the expression of an early patterning gene correlated with the dramatic evolution of embryonic visual system development in the Holometabola.

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1. Introduction

The evolution of complete metamorphosis in the Holometabola facilitated an increasing divergence of larval and adult body plan resulting in a wide range of juvenile head morphologies among extant species (Fig. 1). In directly developing primitive insects, the head of first instar juvenile or nymph differs little from adult morphology and functionality besides

being of smaller size. The major peripheral sense organs, antennae and compound eyes, are *fully* differentiated and innervate neuropils of adult-like neuroanatomy. Various stages of departure from this ancestral state exist in the larval forms of holometabolous insects. A general trend in the evolution of the holometabolous insect larval head is the reduction of sensory organs. The most primitive forms are represented by eucephalic larvae, which carry a solid head capsule equipped with gnathal appendages of adult-like proportions but extremely reduced antennae (Fig. 1). Even more dramatic reduction is characteristic for the larval visual system. Small clusters of ocelli-like lateral eyes, called stemmata, serve as main visual organs in place of the nymphal compound eyes of primitive insects. The stemmatal photoreceptors project

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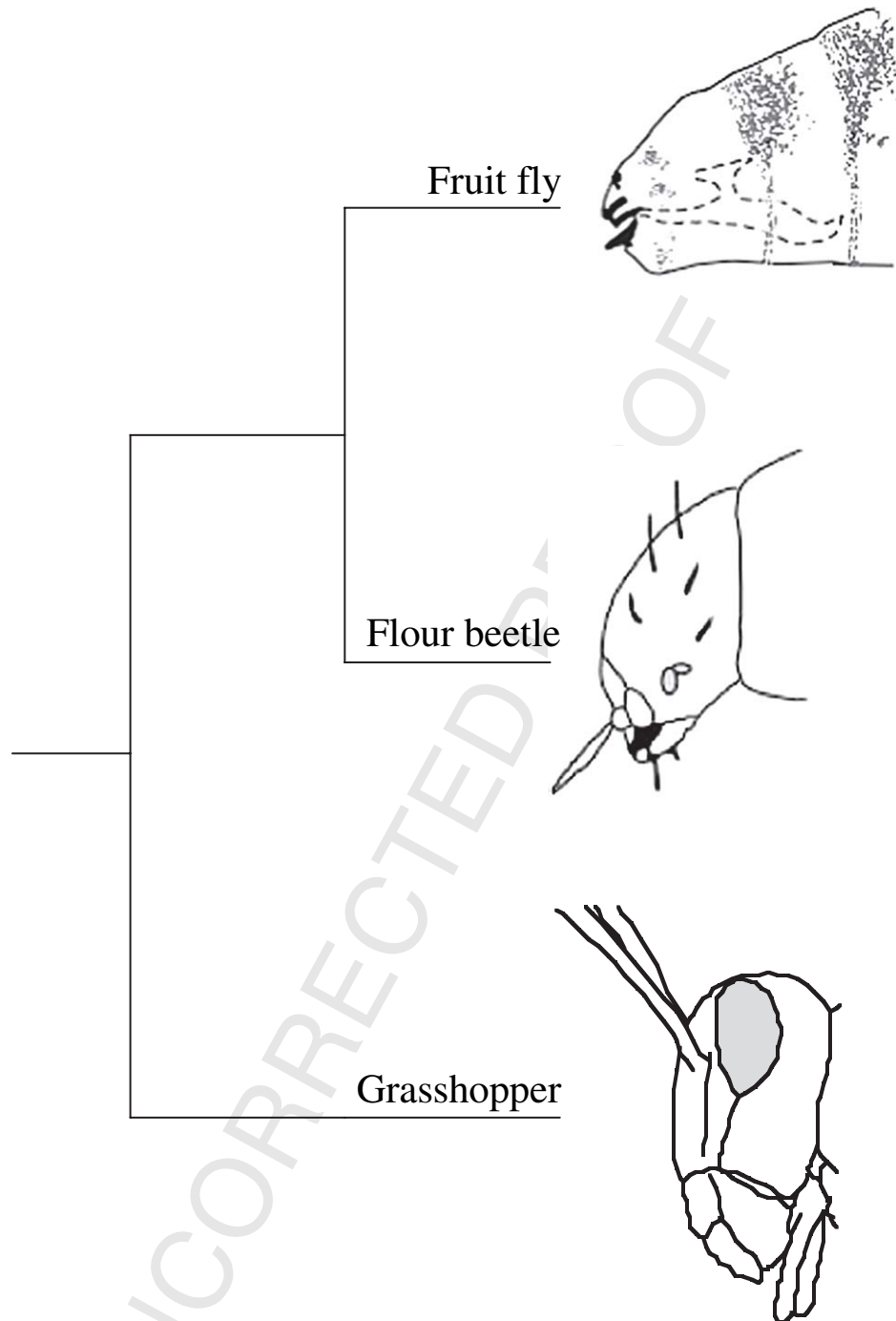


Fig. 1. Evolution of insect juvenile head morphology. Maximal divergence of juvenile head morphology is represented by the adult body plan like nymphal head of directly developing insects like grasshopper (*Schistocerca americana*), the reduced head capsule of primitive holometabolous species such as the flour beetle *Tribolium castaneum*, and the extreme degree of reduction in the inverted and extremely reduced head skeleton of *Drosophila*. Most pronounced is the trend towards reduction of the major peripheral sense organs in the larval forms of holometabolous species. The nymphal antenna of grasshopper counts 13 segments. The larval antenna of *Tribolium* is reduced to five segments. In the *Drosophila* maggot, the antenna is replaced by the small dorsomedial papilla of the maxillary sense organ and the likewise minute antennal sense organs. In grasshopper nymphs, vision is facilitated by fully developed compound eyes. These are replaced by highly reduced larval eyes, the so-called stemmata (indicated by grey shading), in the flour beetle larva. Further reduction is seen in *Drosophila*, which is equipped with internalized larval eyes, the Bolwig organs.

into specific larval optic neuropils, which remain distinct from the anlagen of the later developing adult optic lobes (Heming, 1982).

In diverse lineages of the Holometabola, prominently in Diptera and Hymenoptera, further evolutionary transformation

of the larval head resulted in the emergence of acephalic larval body plans (Fig. 1). In this case, head segments of the larva have acquired trunk-like quality due to conversion of peripheral head capsule structures into interior structures. Larval acephaly is widely known by virtue of being one of the defining

characters of the Cyclorrhapha, which includes *Drosophila* (Fig. 1) (Yeates and Wiegmann, 1999). In the latter, a soft cuticle furnished pseudocephalon, which lacks external appendages, builds the anterior tip of the larva. The internal cephalopharyngeal head skeleton includes a feeding apparatus strongly modified for detritus uptake. Structural stability is provided by hydrostatic turgor.

Correlated with the extreme overall reduction of ancestral head morphology, the procephalic sense organs of higher Diptera belong to the most radically miniaturized such structures in insects. Two small sensory elevations in the dorsal anterior larval head ectoderm, the olfactory organs of the *Drosophila* larva, represent the remnants of the ancestral antennae (Jurgens et al., 1986). The visual sense organs have shifted into the interior of the larva. A bilateral pair of 12 cell photoreceptor clusters, which are attached to the outer surface of cephalopharyngeal head skeleton, constitutes the *Drosophila* larval eyes commonly known as Bolwig organ (Bolwig, 1946; Green et al., 1993). In the absence of separate larval optic lobe neuropils, the Bolwig organ photoreceptors contact guidepost cells in the early adult optic lobe anlage and project directly into target cells of the central brain (Campos-Ortega and Hartenstein, 1997; Meinertzhagen and Hanson, 1993; Schmucker et al., 1997).

The molecular developmental basis of antennal and visual system reduction in the larvae of higher insects has not been addressed yet. Catalyzed by the availability of a cross-reactive antibody against the segment polarity gene *engrailed* (*en*), much effort has been targeted towards molecular comparative studies of embryonic insect head segmentation (for review, see (Patel et al., 1989a,b; Urbach and Technau, 2003a)). *En* expression has been interpreted to indicate the existence of three postoral or gnathal segments in the posterior head and four segments in the preoral head, i.e. procephalon *sensu* Snodgrass (Schmidt-Ott et al., 1994a; Schmidt-Ott and Technau, 1992). The morphologically unambiguous postoral head segments are characterized by each bearing a specific pair of gnathal appendages: mandibles, maxillae and labium. Likewise generally accepted procephalic head regions are the antennal and intercalary segments. Conflicting views exist regarding the organization of the procephalon anterior to these segments, which encompasses the eyes and the labrum (for recent review see Urbach and Technau, 2003a). Assuming homology to the terminal acron of segmented Annelida, the anterior procephalon has traditionally been considered of non-segmental nature (Jurgens and Hartenstein, 1993). More recently however, phylogenetic, paleontological and *en* expression data has been interpreted to suggest that the anterior procephalon of modern arthropods represents a true segmental unit: the ocular segment (Schmidt-Ott and Technau, 1992). Second, neuroanatomical, genetic and gene expression data have been interpreted to indicate an origin of the labrum from appendages (Boyan et al., 2002; Budd, 2002; Haas et al., 2001). These may have either been associated with the intercalary segment or a fourth procephalic segment (Boyan et al., 2002; Haas et al., 2001; Schmidt-Ott and Technau, 1992; Urbach and Technau, 2003a). In light of this unresolved situation and to emphasize

uniqueness of developmental and morphological organization, the head region anterior to the intercalary segment will here be referred to as anterior procephalon.

Each head segment is linked to a specific neuromere of the central nervous system. This relationship is expressed by the segmental origin of the neuromere forming neuroblasts and by segment specific projection patterns of the peripheral sensory neurons. The sense organs of the antennal and intercalary segments project into the deutocerebral and tritocerebral ganglia respectively (Schmidt-Ott et al., 1994a; Urbach and Technau, 2003a). Each of these procephalic neuromeres develops from neuroblasts that originate from within neuroectoderm of the later innervating segments. One exception to this rule has been documented in the embryonic head of grasshopper, where *en* expressing neuroblasts from the protocerebrum in addition to the deutocerebrum contribute to the antennal lobe (Boyan and Williams, 2000). The neuroectoderm of the anterior procephalon gives rise to the mushroom bodies, protocerebrum and the visual system.

Comparison of *en* expression between *Drosophila* and lesser derived eucephalic species reveals a high degree of conservation in the segmental organization of embryonic head despite the divergence of juvenile head morphology, and substantial differences in the upstream regulation of segmentation gene expression (Schroder, 2003; Stauber et al., 1999). Preliminary observations indicate that this may not be the case for the signaling factor encoding gene *wingless* (*wg*), a second segment polarity gene (Rijsewijk et al., 1987). In the trunk segments, *wg* is expressed in 3–4 cell-wide domains immediately anterior to *en* segmentation stripes. *wg* signaling is essential for maintaining *en* expression in this context (Martinez Arias, 1993). It has previously been shown that *wg* is expressed in a conserved manner in the embryonic head of species with eucephalic juvenile forms (Dong and Friedrich, 2005; Liu and Friedrich, 2004). Published *wg* expression patterns in the *Drosophila* head are difficult to relate to lesser-derived insects (Baker, 1988; Schmidt-Ott and Technau, 1992; van den Heuvel et al., 1989). Most striking is a large protocerebral neuroectoderm expression domain in the anterior procephalon, dubbed “head blob”, which exhibits little similarity to the complex and dynamic expression of *wg* in the anterior procephalon of primitive insects (Schmidt-Ott and Technau, 1992). To explore the possibility of modified spatial regulation of *wg* in the *Drosophila* embryonic head, we investigated the homology relationships of procephalic *wg* expression domains in *Drosophila*, the red flour beetle *Tribolium castaneum*, and the American desert locust *Schistocerca americana*. Using embryological landmarks we identified conserved neuroblast contributing *wg* expression domains in the intercalary and antennal segments. Conserved expression domains also exist in labrum and stomodeum. Major expression pattern divergence is, however, confirmed in the anterior procephalon. Specifically, the data suggests that the evolution of embryonic head development in the Holometabola involved reduction of *wg* patterning functions in the embryonic visual system concurrent with the reduction of photoreceptive organs of the larval stages.

2. Materials and methods

2.1. Animal culture

Embryos were obtained from cultures of the American desert locust *Schistocerca americana*, wild type red flour beetle *Tribolium castaneum*, and Canton S wild type *Drosophila melanogaster*. Cultures of all three species were maintained in in-house facilities as described (Dong et al., 2003; Dong and Friedrich, 2003; Liu and Friedrich, 2004).

2.2. Molecular biology

The ortholog of the *Tribolium hh* gene was cloned by guessmere PCR. Based on protein sequence alignment of *hh* entries from *Drosophila* (acc# L02793), *Anopheles* (acc# XM_321721), *Junonia* (acc# AF117742), cricket (acc# AB044709) and *Gallus* (acc# NM_204821), two pairs of degenerate primers were designed for nested PCR. The forward outer primer corresponded to amino acid sequence DEEGTGA (GA(T/C) GA(A/G) GA(A/G) GGI ACI GGI GC), the forward inner primer to amino acid sequence EGTGAD (GA(A/G)GA(A/G)GGIACGGIGCIGA), the backward outer primer to amino acid sequence HWYANA (gc(c/t)ttigc(g/a)tacct(g/a)tg), and the inner backward primer corresponded to amino acid sequence DFGAE with an added Cla I restriction site (CCATCGATGGACCCA(A/G)TC(A/G)ACICICG).

Pupal stage total RNA was extracted with the RNAqueous kit (Ambion, Inc.), and cDNA prepared with RETROscript kit (Ambion) using random decamers. One microliter of cDNA product was used as template for the first round PCR. First and second round PCR reactions were carried out with following touchdown cycle conditions: denaturation for 60 s at 94 °C, annealing for 1 min in all cycles starting at 55 °C but dropping 2 °C with each of the first five cycles to hold at 45 °C in the remaining 30 cycles, elongation was held for 50 s for the first five cycles, 2 min for the following 15 cycles, and 4 min for the remaining 15 cycles. After isolation of an RT-PCR fragment, 3'RACE was carried out using the First-Choice RLM-RACE kit (Ambion) and the following forward primers: 5'-GGGATGAAGAAGGGTATCATAC-3' (3'RACE outer primer) and 5'-ACGAAGGACGTGCTGTTGAT-3' (3'RACE inner primer) resulting additional about 900 bp downstream *hh* cDNA sequence. RT-PCR and RACE fragments were cloned into pGEM-T vector (Promega), sequenced with the BigDye Terminator sequencing kit (Applied Biosystems) and forwarded for electrophoretic separation to the Applied Genomics Technology Center of Wayne State University. Sequence analysis and alignments were carried out with MacVector 6.0.1 (Oxford Molecular Group) and Clustal W (Thompson et al., 1994).

2.3. Whole-mount in situ hybridization

Single and double labeling whole mount in situ hybridization experiments with digoxigenin or biotin labeled RNA probes were carried out as previously described (Friedrich

and Benzer, 2000; Liu and Friedrich, 2004). Probes utilized in this study included the *wg* ortholog from *Schistocerca americana* (Friedrich and Benzer, 2000), *Drosophila melanogaster* (Baker, 1987), and *Tribolium castaneum* (Nagy and Carroll, 1994), the *glass (gl)* gene ortholog from *Tribolium castaneum* (Liu and Friedrich, 2004), and *Drosophila melanogaster* (Moses et al., 1989), and the *Tribolium hh* ortholog described in the present study (acc# DQ493452).

2.4. Expression pattern analysis and documentation

Grasshopper embryos were staged following the morphological criteria introduced by (Bentley et al., 1979). Grasshopper neuroblast cells were identified based on their enlarged size compared to epithelial cells and their position basal of the ectoderm cell layer in the neurogenic region (Zacharias et al., 1993). Flat mount preparations of labeled *Drosophila* embryonic heads were prepared as described by Urbach et al., 2003. Differential interference contrast (DIC) images were taken with a Zeiss Axioscope coupled to a SPOT RT digital camera (Diagnostic Instruments, Inc.). Brightness and contrast were adjusted with Photoshop 6.0 (Adobe).

3. Results

3.1. Conserved segmental distribution of *wg* expressing neuroblasts in the grasshopper embryonic procephalon

Although the early expression of *wg* has been analyzed in a large range of arthropods, the conservation of its role in neurogenesis is little documented (Damen, 2002; Duman-Scheel and Patel, 1999; Duman-Scheel et al., 2002; Hughes and Kaufman, 2002). This aspect is well investigated in *Drosophila*, where *wg* is expressed in delaminating neuroblasts of the central nervous system of the trunk and head (Chu-LaGraff and Doe, 1993; Patel et al., 1989c). In the trunk, *wg* expressing neuroblasts delaminate from the median neuroectoderm partition of the segmental *wg* expression domains. In the head, *wg* expressing neuroblasts have been detected in all three procephalic neuromeres, i.e. proto-, deutero- and tritocerebrum (Richter et al., 1998; Urbach and Technau, 2003b).

To further validate homology of the *Drosophila* procephalic *wg* expression domains to those in primitive species, we investigated the presence of *wg* expressing neuroblasts in the grasshopper. The early expression pattern of *wg* in the developing grasshopper procephalon has been described previously (Dear den and Akam, 2001; Dong and Friedrich, 2005). *wg* expression domains form in all pregnathal segments but the presence of *wg* expressing neuroblasts has only been analyzed in the optic lobe and the protocerebrum (Dong and Friedrich, 2005). Taking advantage of the distinct neuroblast cells size in grasshopper, we investigated potential *wg* expressing neuroblast contributions in the entire developing procephalon.

3.1.1. Intercalary segment

Weak expression of *wg* can be detected starting from 25% of embryonic development in the intercalary segment (ic) (not

shown). At 28% of development, *wg* expression is clearly visible (Fig. 2a,c). Unlike in the more posterior gnathal and trunk segment, *wg* expression is confined to neurogenic ectoderm close to the midline. Expression levels appear slightly lower compared to other expression domains. Two to three *wg* positive neuroblast cells can be detected per segment hemisphere expression domain (Fig. 2c'). All *wg* positive neuroblasts are located immediately underneath the neuroectodermal layer and thus in direct contact with it. After stage 40% of development, *wg* expressing neuroblasts fade away in the intercalary segment. These data suggest that transiently *wg* expressing neuroblasts segregate or contribute ganglion mother cells to the tritocerebrum.

3.1.2. Antennal segment

In the antennal segment (an), *wg* expression starts at about 17% of development (Dearden and Akam, 2001) (not shown). An initially segmentation stripe-like-domain transforms into the ventral ectoderm expression domain of the antennal appendage, which starts to elongate by 28% of embryonic development (Fig. 2a). *wg* positive neuroblast cells are less prominent in the antennal segment than in the intercalary segment. At 28% of development, one to two *wg* expressing neuroblasts can be detected at the ventral base of the antenna (Fig. 2 b,b'). As in the case of the intercalary segment, the antennal segment neuroblasts are closely associated with the ectodermal *wg* expressing cells. The *wg* expressing neuroblasts most likely contribute to the deutocerebrum.

3.1.3. Anterior procephalon

Major aspects of the complex and dynamic expression of *wg* in the anterior procephalon have been described (Dong and Friedrich, 2005). Expression starts out as a large neuroectodermal patch, the protocerebral neuroectoderm domain (pne), which resolves into three discrete domains by 28% of embryonic development (Fig. 2a). The median protocerebral neuroectoderm domain (mpn) is located in the protocerebral compartment of the procephalon. The substantially larger dorsal and ventral protocerebral neuroectoderm domains (dpn + vpn) mark the dorsoventral poles of the border between protocerebrum and the emerging anlage of the visual system (vs) (Fig. 2a,d,h). By 30% of embryonic development, large *wg* expressing neuroblast cells can be identified immediately underneath all three protocerebral neuroectoderm domains (Fig. 2d–g'). The *wg* positive neuroblasts associated with these three domains become more pronounced with progressing development. At about 35% of embryonic development, the neuroblast cells that segregate from the dorsal and ventral protocerebral neuroectoderm domains can be divided into two groups: those which extend into the protocerebral side, and a second population located on the side of the visual anlage, which most likely contributes to the proximal part of the outer optic lobe anlage (oa), the medulla (Fig. 2h–j').

With further development, the protocerebral neuroectoderm domains are supplemented by a dorsoventral pair of protocerebral ectoderm domains (dpe + vpe) that do not contribute neuroblasts (Fig. 3). In addition, the visual anlage harbors

a second pair of *wg* expressing cell clusters, which are positioned at the dorsoventral poles of the outer optic lobe anlage (doa + voa) (Fig. 3). This domain pair originates separately from the neuroectodermal domains (Dong and Friedrich, 2005). By 35% of embryonic development, *wg* positive cells of the protocerebral neuroectoderm domains reach the optic lobe neuroblasts in both hemispheres of the visual anlage (Fig. 3).

In the developing labrum (lr), *wg* expression starts relatively late at the beginning of about 30% of embryonic development (Fig. 2d). The expression domain appears to remain ectodermal. Consistent with the lack of *wg* expressing neuroblast cells in the developing clypeolabrum of *Drosophila* (Richter et al., 1998), at no stage were *wg* positive neuroblasts detected in the grasshopper embryonic labrum.

Based on the histological examination four *wg* expression domains contribute neuroblasts in the procephalon: the intercalary domain, the antennal domain, the protocerebral neuroectoderm domain, which separates into subdomains, and the outer optic lobe anlagen domain pair. With exception of the outer optic lobe anlagen domains, the situation is consistent with the localization of *wg* expressing neuroblasts in all three procephalic neuromeres of *Drosophila* (Richter et al., 1998; Urbach and Technau, 2003b). It is therefore reasonable to assume that the situation described in *Drosophila* represents ancestral aspects of embryonic head patterning in insects.

3.2. *wg* separates visual system from protocerebrum compartment within the anterior procephalon

The protocerebral neuroectoderm expression domains of *wg* in the grasshopper are unique by contributing neuroblasts to two different neuronal compartments: medially to the protocerebrum, and peripherally to the outer optic lobe anlagen in the visual system. This raises the question if these domains are located between two potential segments or represent a border within the anterior procephalon. Previously published expression of *wg* in relation to a second segmentation gene, the signaling factor *hedgehog* (*hh*), in the two spotted cricket *Gryllus bimaculatus* suggests that *wg* acts first as segmentation gene and subsequently acquires patterning functions within the anterior procephalon (Miyawaki et al., 2004). To examine if these indicative expression aspects of *wg* and *hh* in the anterior procephalon are conserved, we investigated the expression of these genes in *Tribolium* as representative of lesser derived holometabolous insects (Fig. 4).

At the time of stomodeum formation, two procephalic *hh* expression domains can be detected in the early *Tribolium* germband. These mark the posterior borders of the prospective antennal segment and anterior procephalon (ap) (Fig. 4a,b). At this point, the protocerebral neuroectoderm precursor domain of *wg* outlines the anterior margin of the *hh* domain in the anterior procephalon (Fig. 4b). The same close association of *hh* and *wg* segmentation domains is observed in the trunk segments (Fig. 4a,c,e). Thus, even though the developing stomodeum interrupts the anterior procephalon expression domains of *hh* and *wg* at the midline (Fig. 4b,d), the expression patterns

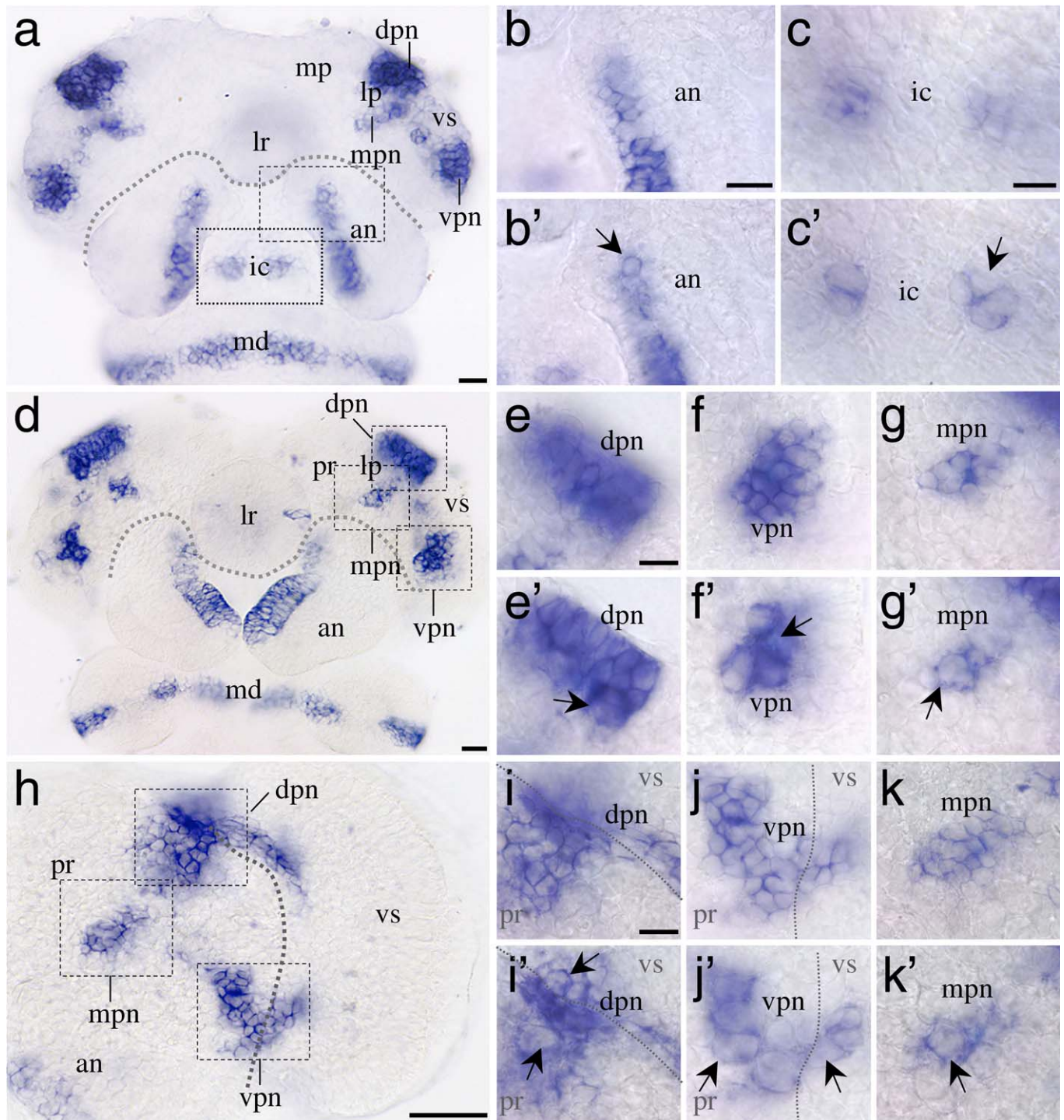


Fig. 2. *wg* expression in the embryonic grasshopper procephalon. (a) Frontal view of grasshopper head at 28% of embryonic development. *wg* expression domains have been established in the head lobes, the antennal appendages, and intercalary and mandibular segments. Approximate border between anterior procephalon and antennal segment indicated by dotted line. (b) High magnification view of epithelial *wg* expression at the base of the antennal segment corresponding to the area in the hatched window of panel (a). (b') Deeper level optical section revealing *wg* positive neuroblasts (arrow) underneath the *wg* expressing epidermal cell layer. (c) High magnification view of epithelial *wg* expression in the intercalary segment corresponding to the area in the dotted window of panel (a). (c') Deeper level optical section revealing *wg* positive neuroblasts of the intercalary segment. (d) Frontal view of grasshopper head at 30% of embryonic development. Approximate border between anterior procephalon and antennal segment indicated by dotted line. *wg* expressing neuroblasts can now be identified, which are associated with the dorsal (e and e'), ventral (f and f') and median (g and g') protocerebral neuroectoderm expression domains. (h) Frontolateral view of right grasshopper head hemisphere at 33% of embryonic development. The embryonic eye lobes have become prominent. The dorsal (i and i') and ventral (j and j') protocerebral neuroectoderm expression domains contribute *wg* positive neuroblasts to both the visual anlage and the adjacent protocerebral compartment. The median protocerebral neuroectoderm domain (k and k') is linked to *wg* positive neuroblasts in the protocerebral compartment. Median border of visual system compartment indicated by dotted line. An, antenna; dpn, dorsal protocerebral neuroectoderm expression domain; in, intercalary segment; lp, lateral protocerebrum; lr, labrum; md, mandible; mpn, median protocerebral neuroectoderm expression domain; pr, protocerebrum; vpn, ventral protocerebral neuroectoderm expression domain; vs, visual anlage. Scale bars correspond to 100 μ m in (h), 50 μ m in (a) and (d), and 25 μ m in (b), (c), (e) and (i).

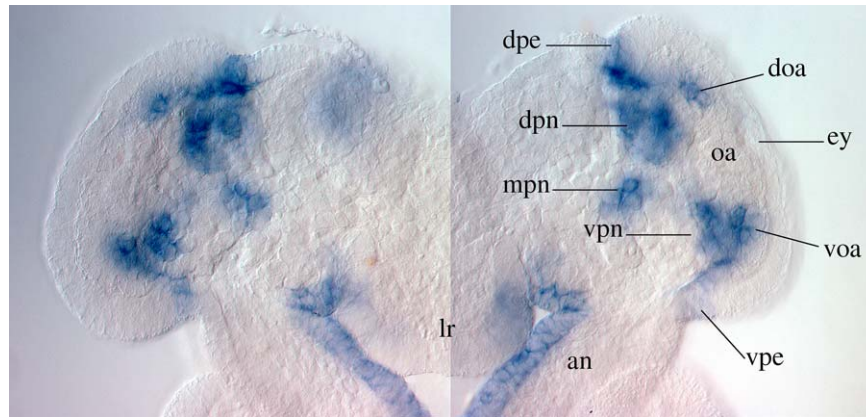


Fig. 3. Complexity of *wg* expression domains in the anterior procephalon of the grasshopper *Schistocerca americana*. Frontal view of embryonic head at 35% of development. A total of seven *wg* expression domains are detected in association with the eye lobes. In addition to the protocerebral neuroectoderm domains (dpn, mpn, vpn) shown in Fig. 2, separate dorsoventral domain pairs are initiated in the eye lobe ectoderm (dpe and vpe) and the outer optic lobe anlage (doa and voa). An, antenna; doa, dorsal optic lobe anlagen domain; dpe, dorsal protocerebral ectoderm domain; dpn, dorsal protocerebral neuroectoderm domain; ey, eye lobe ectoderm; lr, labrum; mpn, median protocerebral neuroectoderm domain; oa, outer optic lobe anlagen; voa, ventral optic lobe anlagen domain; vpe, ventral protocerebral ectoderm domain; vpn, ventral protocerebral neuroectoderm domain.

of both genes indicate involvement in segmentation defining the posterior border of the anterior procephalon.

In the antennal segment, the onset of *wg* expression is slightly delayed in comparison to *hh*. Once the third thoracic *hh* segmentation stripe has fully formed, weak *wg* expression joins at the anterior margin of the antennal *hh* segmentation stripe (Fig. 4c,d). At the same time, the antennal and anterior procephalon *hh* domains become separated by a wider field of cells. The anterior procephalon domain of *hh* expands along its peripheral edge. The protocerebral neuroectoderm domain of *wg* continues to outline the anterior margin of the *hh* domain (Fig. 4d). At a more advanced germband elongation stage, when the second abdominal segmentation stripe is formed, the expression domains of *wg* and *hh* in the anterior procephalon have separated (Fig. 4e–g). The protocerebral neuroectoderm domain of *wg* has shifted more anteriorly and begins to partition into the three daughter domains of the protocerebral neuroectoderm domain (Fig. 4g). Similar to the situation in grasshopper, the dorsal and ventral protocerebral neuroectoderm domains are positioned at the border between protocerebrum and the visual anlage (compare Fig. 4g with Fig. 2a). Once separation of the protocerebral neuroectoderm subdomains is completed, the gap between the protocerebral *wg* and the anterior procephalon *hh* domain has widened further (Fig. 2h–j).

The dissociation of *wg* and *hh* segmentation domains is unique for the anterior procephalon. *wg* and *hh* domains remain adjacent in all other segments (Fig. 4e,h). The dynamics of *wg* expression in the *Tribolium* anterior procephalon suggests that the *wg* signaling domain transforms from a segmental to an intrasegmental patterning center.

3.3. *wg* expression dynamics in the anterior procephalon of *Tribolium*

At the completion of germband extension, the *Tribolium* *wg* protocerebral neuroectoderm expression domains have acquired

a very similar distribution compared to that in the grasshopper (compare Fig. 2a,d with Fig. 5a,b). The visual primordium at the lateral head lobe margins is fronted with a pair of *wg* domains (Fig. 5c), which correspond to the dorsal and ventral protocerebral neuroectoderm domains based on similarity in position and ontogenetic origin compared to grasshopper. More medially, a third domain corresponding to the median protocerebral neuroectoderm domain has formed (Fig. 5b,c). However, unlike in the grasshopper, no further distinct expression domains can be detected. This is true with regards to potential equivalents of the grasshopper optic lobe anlagen domain pair, as well as with regards to distinct ectodermal *wg* regions in the anterior eye field poles (compare Fig. 3 with Fig. 5b,c). Also in subsequent stages of embryonic development, no further diversification of *wg* expression domains seems to take place in the anterior procephalon. Although increasing in size, the dorsal protocerebral neuroectoderm domain remains contiguous throughout the first half of germband retraction (Fig. 5d,e,f,g,h,k). The expression of *wg* in this domain appears to extend along the dorsal median head ectoderm. *wg* signal is also evident in cells of the developing protocerebrum immediately underlying the expression domain (Fig. 5f,k). Due to the small size of *Tribolium* embryonic cells and the compounded nature of the expression domain, potential neuroblasts are difficult to distinguish with certainty at this point. At later stages, scattered *wg* expressing cell clusters are detected in the dorsal compartment of the protocerebrum (Fig. 5n,q). Although it cannot be excluded that expression of *wg* is activated independently in these cells, the expression data convey the impression that the dorsal protocerebral neuroectoderm domain functions as a source of *wg* expressing neuroblasts as in grasshopper. It is noteworthy, however, that the domain remains compact for a longer time compared to grasshopper.

A second notable aspect is the fact that the ventral protocerebral neuroectoderm domain reduces in size during germband retraction. This contrasts with the massive size increase of the dorsal domain (Fig. 5e,h,m,p). At the end of germband

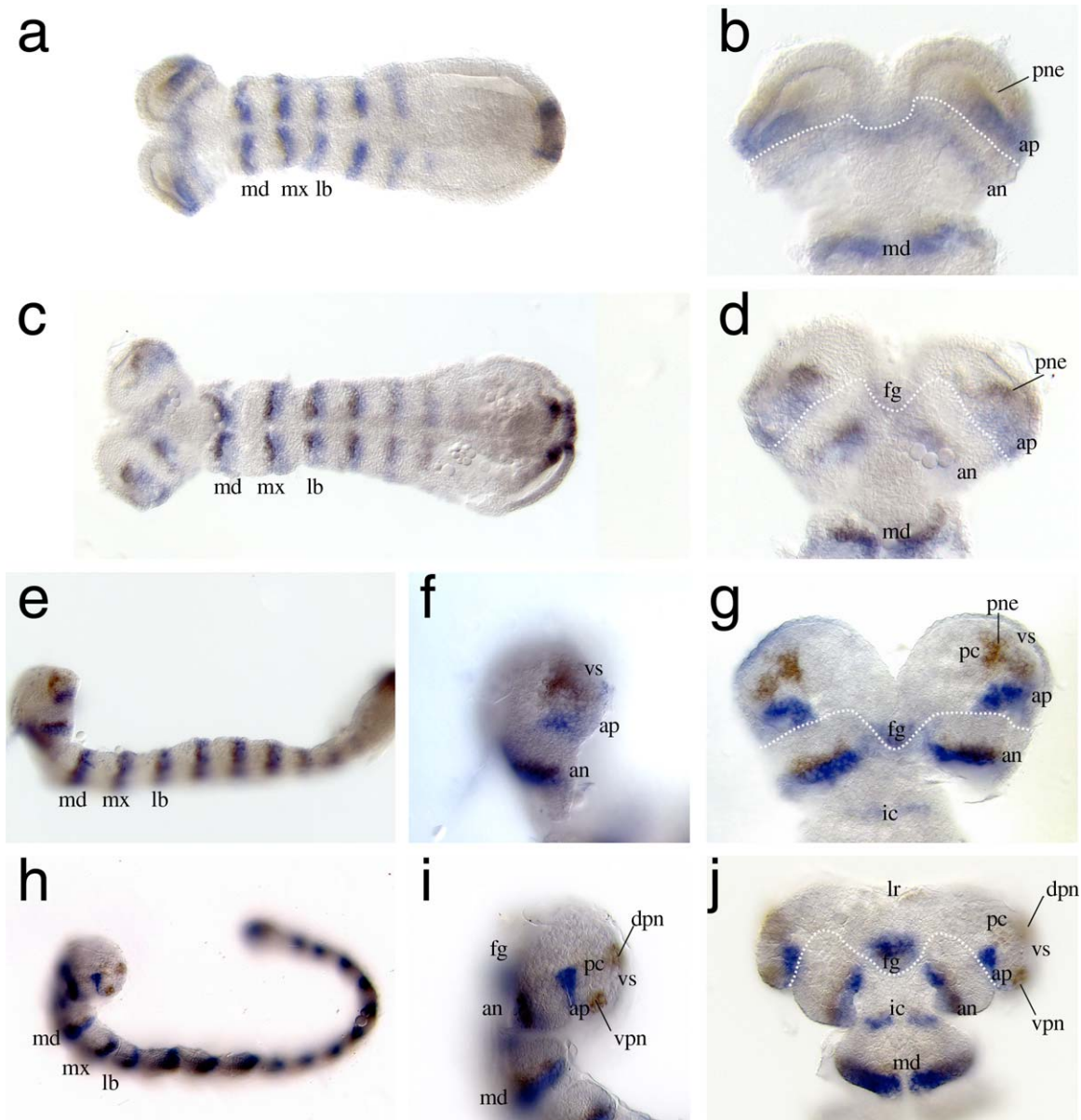


Fig. 4. *wg* separates protocerebral and visual compartments in the anterior procephalon. (a)–(j) Double labeling of *Tribolium* embryos for expression of *hh* (blue) and *wg* (brown). (a)–(c) Late germband elongation stage. (a) Ventral view of early germband elongation stage embryo. (b) Frontal view of the same stage as in (a). Two *hh* stripes are detected in the embryonic procephalon. The antennal *hh* stripe extends across the germband. *wg* is not yet expressed in the antennal segment. The most anterior expression domain of *hh* defines the posterior margin of the anterior procephalon. It is interrupted at the stomodeum and associated with anteriorly adjacent *wg* expression. (c) Ventral view of early germband elongation stage slightly more advanced compared to (d). The *hh* stripe of the anterior procephalon has widened. The associated protocerebral ectoderm domain of *wg* has become more prominent. *wg* expression also initiated in the antennal segment. (d) Front view of the same stage as in (c). (e) Lateral overview of advanced germband elongation embryo. (f) High magnification view of head region in (e). *hh* and *wg* are expressed in discrete and non-overlapping domains of the anterior procephalon. (g) Frontal view of embryonic head. The anterior procephalon domains of *wg* and *hh* have separated. In the antennal, gnathal and trunk segments, *wg* and *hh* remain expressed in immediately adjacent segmentation stripes. (h)–(j) Early germband extension stage. Antennal segment acquires appendicular character. (h) Lateral overview of embryo. (i) High magnification view of head region in (h). The *wg* protocerebral neuroectoderm domain has split up into subdomains. (j) Frontal view of embryonic head. *wg* and *hh* continue to be expressed in non-overlapping patterns at this stage. The anterior procephalon segmentation stripe of *hh* has retracted to the border between the antennal appendage and the anterior procephalon. The latter includes separate protocerebral and visual anlagen compartments divided by a pair of *wg* protocerebral neuroectoderm domains. An, antenna; ap, anterior procephalon; dpn, dorsal protocerebral neuroectoderm domain; fg, foregut; lb, labial segment; lr, labrum; md, mandible; mx, maxillary segment; pc, protocerebrum; vpn, ventral protocerebral neuroectoderm domain; vs, visual anlage. (b), (d), (g), (j) White dotted line indicates posterior border of anterior procephalon.

retraction, the ventral protocerebral neuroectoderm domain has become restricted to a comparatively weak spot in the head ectoderm, ventral of the cleft generated by the invaginating optic lobe anlagen (Fig. 5m). At this point, the ventral protocerebral neuroectoderm appears at distance from the developing protocerebrum. Moreover, expression appears to be exclusively ectodermal. The ventral protocerebral neuroectoderm therefore seems a less prolific source of *wg* expressing neuroblasts, which may, however, be generated during earlier stages of development.

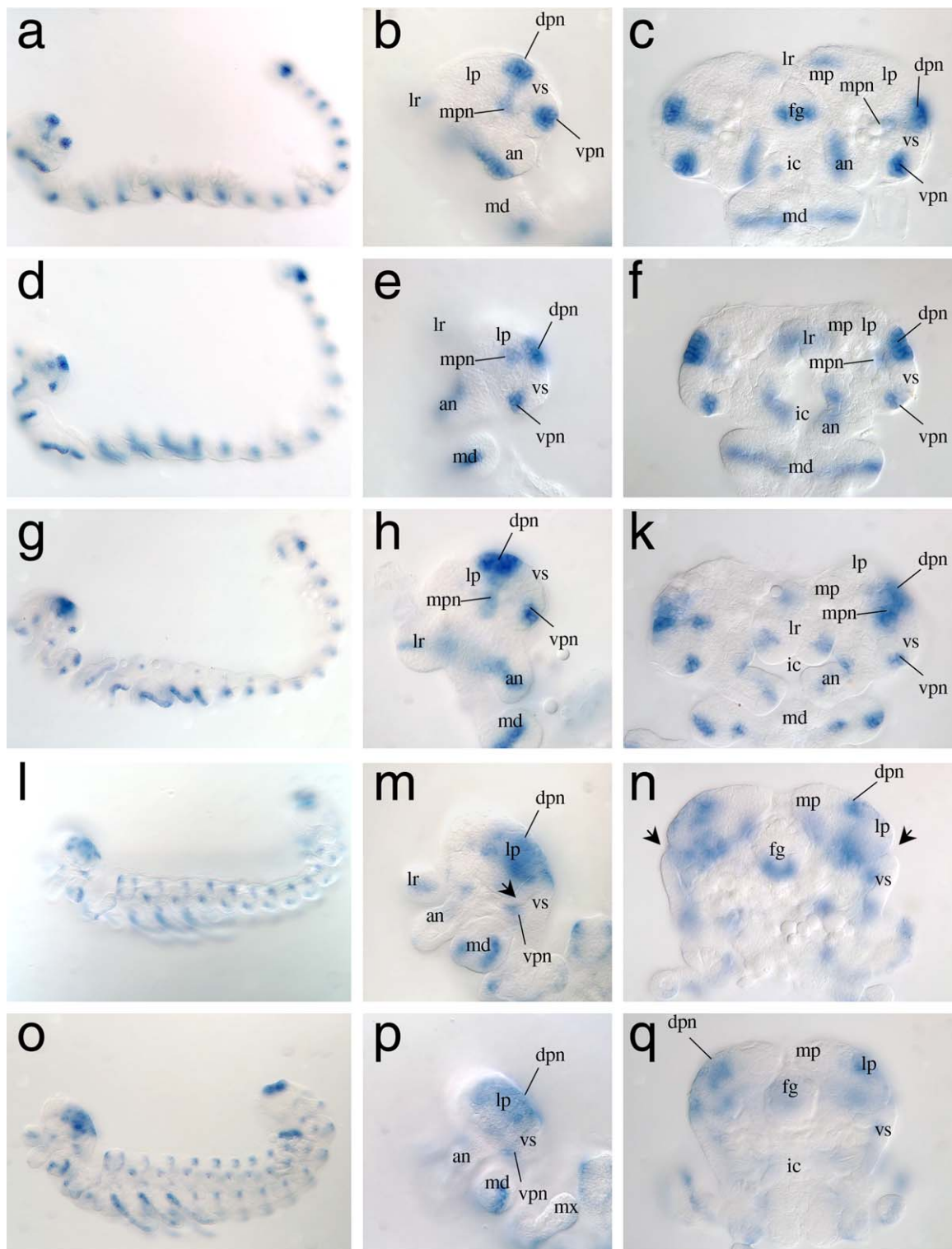


Fig. 5. *wg* expression in the *Tribolium* procephalon. (a), (d), (g), (l), (o) Lateral view of embryo. (b), (e), (h), (m), (p) High magnification of lateral view of embryonic head. (c), (f), (k), (n), (q) High magnification views of frontal embryonic head. (a)–(c) Early germband retraction stage. *wg* expression domains are detected in mandible and intercalary segments, labrum, and antennae. Dorsal, median and ventral protocerebral neuroectoderm expression domains can be discriminated in the anterior procephalon. The dorsal and ventral protocerebral neuroectoderm domains are of equivalent size. (d)–(f) Progressed germband retraction stage. The dorsal protocerebral neuroectoderm domain has gained in size while the ventral protocerebral neuroectoderm domain appears reduced. (g), (h), (k) Late germband retraction stage. The dorsal protocerebral neuroectoderm domain has further widened while the ventral protocerebral neuroectoderm domain continues to decrease. (l)–(n) Beginning of dorsal closure stage. The dorsal protocerebral neuroectoderm domain transformed into widely scattered domains in the dorsal and posterior protocerebrum. The ventral protocerebral neuroectoderm domain has become reduced to detectable spot underneath the optic lobe invagination fold (arrow). (o)–(q) Early progressed dorsal closure stage. Optic lobe invagination is completed. Extent and distribution of the protocerebral *wg* expression domains has remained largely unchanged. An, antenna; dpn, dorsal protocerebral neuroectoderm expression domain; fg, foregut; ic, intercalary segment; lp, lateral protocerebrum; lr, labrum; md, mandible; mp, median protocerebrum; mpn, median protocerebral neuroectoderm domain; mx, maxillary segment; vpn, ventral protocerebral neuroectoderm domain; vs, visual anlage.

3.4. *wg* expression in the *Drosophila* procephalon

Due to the dramatic differences in head morphogenesis, the expression of *wg* in the *Drosophila* embryo is quite different

from that in grasshopper and flour beetle when viewed in whole mount preparations (Fig. 6). Previous work described five expression domains in procephalic region of the *Drosophila* embryo: the conspicuous anterior procephalic domain or

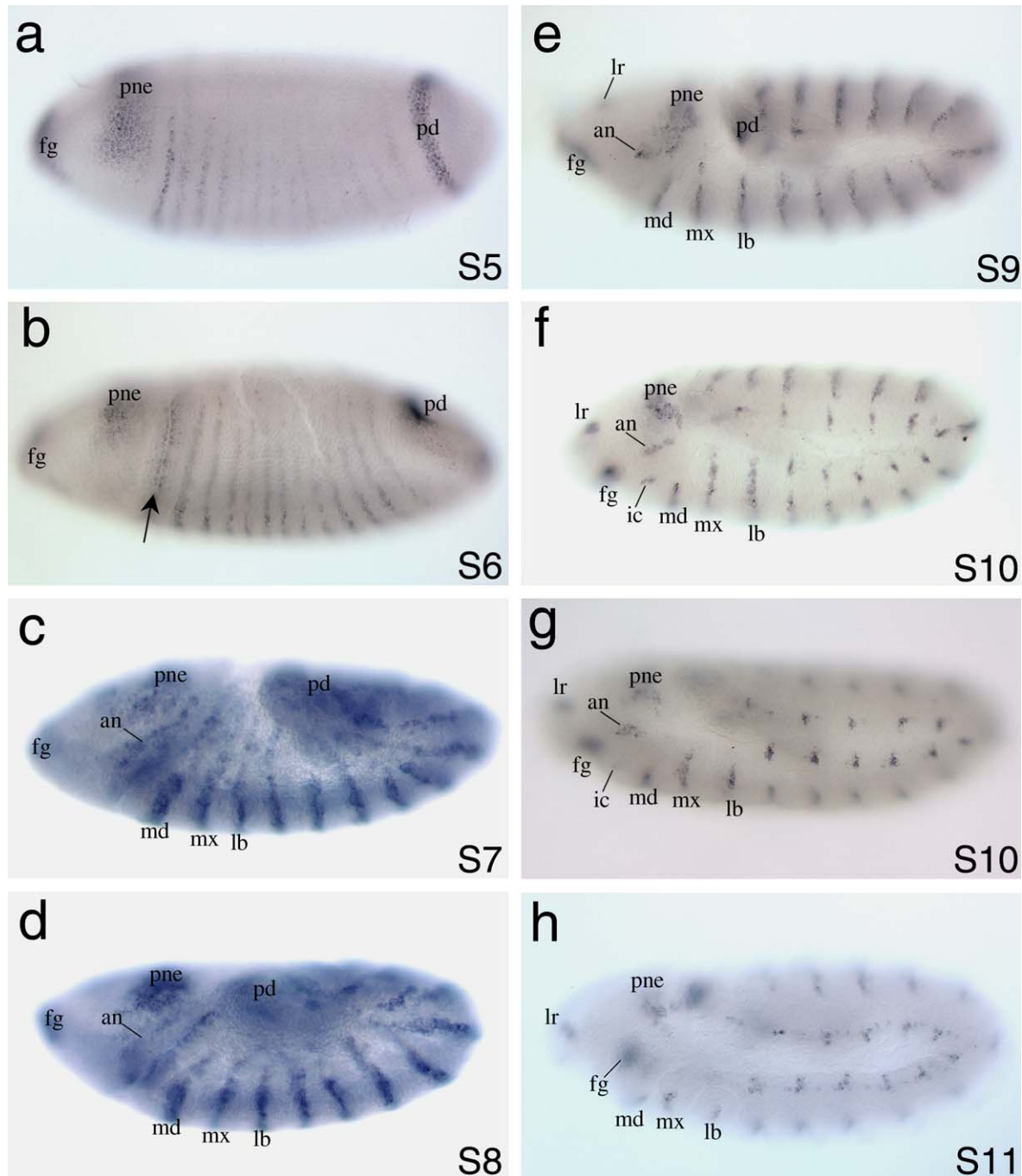


Fig. 6. *wg* expression in the *Drosophila* procephalon. (a) Stage 5 late blastoderm embryo. *wg* expression is visible in the prospective foregut, proctodeum, and the protocerebral neuroectoderm domain at the posterior margin of the anterior procephalon. (b) Stage 6 embryo at onset of head fold formation (arrow). The protocerebral neuroectoderm expression domain has dorsally concentrated. (c) Stage 7 embryo and progressed germband extension stage. The protocerebral neuroectoderm expression domain is joined by the more ventrally positioned antennal expression domain. (d) Stage 8 embryo and extended germband stage. The antennal domain can be detected as concise stripe. The protocerebral neuroectoderm expression domain is dorsally further compressed. (e) Stage 9 embryo. *wg* is now also detected in the labral anlage. The protocerebral neuroectoderm expression domain is the widest expression domain in the embryo. (f) Early stage 10 embryo. Strong expression detected in antennal and intercalary segments. (g) Late stage 10 and early germband retraction stage. The protocerebral neuroectodermal expression domain has become weaker. The antennal and intercalary expression domains begin to fade. (h) Stage 11 embryo. The antennal and intercalary expression domains have disappeared. *wg* continues to be expressed in the protocerebral neuroectoderm domain and labrum. An, antenna; fg, foregut; ic, intercalary segment; lb, labial segment; lr, labrum; md, mandibular segment; mx, maxillary segment; pne, protocerebral neuroectoderm expression domain; pd, proctodeum.

“head blob”, an antennal segment domain, an intercalary segment domain, and expression domains in the invaginating foregut and the developing labrum (Schmidt-Ott and Technau, 1992). The anterior procephalon and foregut domains trace back to the early blastoderm (Fig. 6a). A cap of *wg* expression at the anterior tip of the embryo represents the cell population of the future foregut opening. The anterior procephalon domain is the widest expression domain at this stage, filling a large field in the posterior dorsal hemisphere of the embryonic head region. Its position and later development suggest that it corresponds to the early undivided protocerebral neuroectoderm domain in grasshopper and flour beetle. During germband extension, the anterior procephalon domain becomes somewhat more compressed but remains contiguous (Fig. 6c,d). Likewise, little change can be noted in the foregut domain. By the end of germband extension, the antennal expression domain emerges in form of a slender oblique stripe in the lateral head ectoderm (Fig. 6d). In stage 9 embryos, the number of procephalic *wg* expression domains increases further by onset of expression in precursor cells of the labrum (Fig. 6e). The initiating labral expression domain is located in the dorsal anterior head region. During head involution, this domain shifts in anterior direction, eventually coming to rest at the tip of the embryo (Fig. 6h). Correlated with this, the foregut expression domain shifts ventrally. Throughout these morphogenetic events, expression in the anterior procephalon remains unmodified and contiguous. This is still true at stage 11 of embryogenesis, immediately before the beginning of germband retraction. (Fig. 6e–h). Two additional events in the regulation of procephalic *wg* expression fall into this time window. During stage 10, expression is transiently activated in the intercalary segment (Fig. 6f,g), while the antennal expression domain fades at the end of stage 10 to completely disappear by stage 11 (Fig. 6g,h).

In summary, most aspects of procephalic *wg* expression in *Drosophila* are very similar to those in the eucephalic grasshopper and flour beetle except for the fact that the expression in the presumptive protocerebral neuroectoderm domain is not split into daughter domains. This observation raises questions regarding the homology of the *Drosophila* protocerebral neuroectoderm domain at later stages of development.

3.5. Comparative mapping of the *Drosophila wg* head blob

Seeking additional information to clarify the relationship of *wg* expression in the anterior embryonic procephalon of *Drosophila* to that in more primitive species, we studied the expression of *wg* in flat mount preparations (Fig. 7). In frontal overview perspective, the correspondence of cephalic *wg* expression domains in *Drosophila* and *Tribolium* is striking. Most domains in the head region of a stage 10 embryo of *Drosophila* align in similar register with that in the head of a late germband extension embryo of *Tribolium* (Fig. 7a,b). The most anterior *wg* expression domain is located in the developing labrum. A pair of widely separated punctate expression domains in *Drosophila* labrum compares with closely adjacent horizontal stripe like domains in the flour beetle

labrum. Also the foregut expression domain appears virtually identical between beetle and fruit fly. Remarkably, even though no appendages are formed in the antennal segment of *Drosophila*, the expression of *wg* occurs in the form of oblique stripes with respect to the longitudinal body axis, with the high tip pointing towards the midline just like in *Tribolium* (Fig. 7b). High similarity is also observed in the intercalary segment, where *wg* is expressed in a transient manner in both *Tribolium* and fruit fly. The only non-matching domain is the protocerebral neuroectoderm domain in the anterior procephalon, which straddles as a broad stripe from the anterolateral margin towards the antennal stripe without indication of subdomains (Fig. 7b).

Origin and position of the *Drosophila wg* domain in the anterior procephalon suggest that it is equivalent to the early contiguous protocerebral neuroectoderm domain but does not resolve into subdomains. To test this hypothesis further and obtain a better understanding of its relevance for the patterning of visual system, we compared flat preparations of *Tribolium* and *Drosophila* embryonic heads double labeled for *wg* and the transcription factor *glass* (*gl*), which marks the differentiating larval photoreceptors in the embryonic visual system of both species (Liu and Friedrich, 2004; Moses et al., 1989). In *Tribolium*, *gl* is expressed at the posterior margin of the embryonic eye field embedded between the dorsal and ventral neuroectodermal protocerebrum domains of *wg* (Fig. 7c). In *Drosophila*, *gl* expression can be detected in stage 12 embryos in the differentiating Bolwig organs (Fig. 7d). Similar to the median and dorsal protocerebral neuroectoderm domains in *Tribolium*, the *Drosophila* protocerebral neuroectoderm domain is positioned anterior of the Bolwig organ photoreceptors along the longitudinal body axis (Fig. 7c,d). Unlike in *Tribolium*, no discrete ventral *wg* expression domain can be detected in association with the Bolwig organ photoreceptors. The closest *wg* expression domain in the ventral embryonic head is in the maxillary segment (Fig. 7d). The antennal and intercalary domains have been reduced at this stage. In combination with the distribution of *wg* expression domains in the single label preparations, these data suggest specifically a lack of the ventral protocerebral neuroectodermal domain in *Drosophila*. The *wg* domain in the anterior procephalon of *Drosophila* may correspond to either the median or dorsal protocerebral *wg* expression domains of primitive insects. Alternatively it may be equivalent to the primordial non-dissociated protocerebral ectoderm domain in primitive insects.

4. Discussion

4.1. Conserved *wg* expressing neuroblast contributions to the insect procephalon

To determine the evolutionary conservation of procephalic *wg* expression, we compared expression in the directly developing grasshopper with that in the flour beetle *Tribolium castaneum*, a primitive holometabolous species that develops through a eucephalic larval form, and *Drosophila melanogaster*, the well studied paradigm of acephalic head

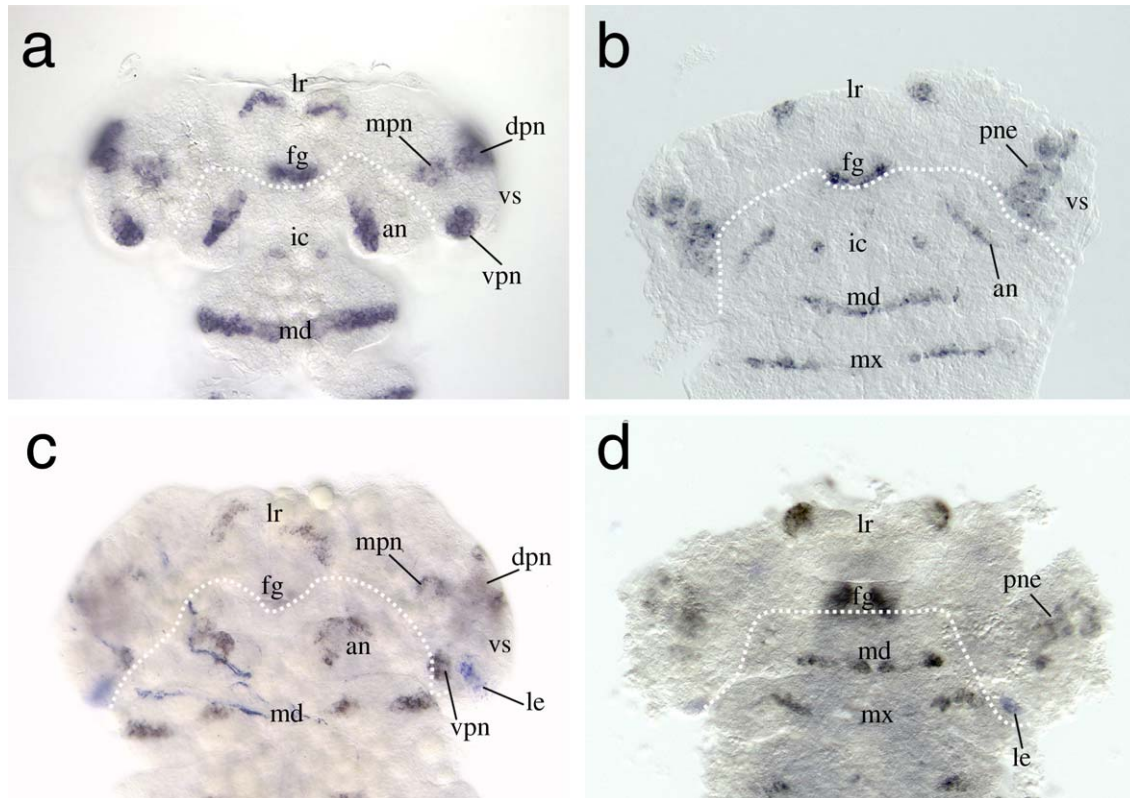


Fig. 7. Comparative mapping of the procephalic *wg* expression domains in *Tribolium* and *Drosophila*. (a) Frontal view of *Tribolium* embryonic head at early germ-band retraction stage (compare Fig. 5c). (b) Frontal view of flat preparation of stage 10 *Drosophila* embryonic head at (compare Fig. 6f,g). Similar *wg* expression domains are detected in all procephalic segments except for the anterior procephalon. The protocephalic neuroectoderm domain, however, has remained compact in *Drosophila*, while being subdivided into three ancestral procephalic neuroectoderm domains in *Tribolium*. (c) Frontal view of *Tribolium* embryonic head at late germ-band retraction stage (compare Fig. 5k) double labeled for *wg* (brown) and the photoreceptor specific gene *gl* (blue). The larval eyes begin to differentiate at the head lobe margins wedged between the dorsal and ventral protocerebral neuroectoderm expression domains of *wg*. (d) Frontal view of flat preparation of stage 11 *Drosophila* embryonic head at (compare Fig. 6h) double labeled for *wg* (brown) and *gl* (blue). In comparison to panel (b), the mandibular segment has shifted forward, *wg* expression in the intercalary and antennal segments has faded. The procephalic neuroectoderm domain has remained compact. The larval eyes are positioned at the lateral margins of the head lobes similar to *Tribolium*, but wedged between the procephalic neuroectoderm and maxillary *wg* domains. An, antenna; dpn, dorsal protocerebral neuroectoderm domain; fg, foregut; ic, intercalary segment; le, larval eye; lr, labrum; md, mandibular segment; mpn, median protocerebral neuroectoderm domain; mx, maxillary segment; pne, protocerebral neuroectoderm expression domain; vpn, ventral protocerebral neuroectoderm domain; vs, visual anlage. White dotted line indicates posterior border of anterior procephalon.

development. Considering the massive morphological divergence between the strongly derived larval head region of *Drosophila* and that of two more primitive representatives, the pattern of *wg* expression in the anterior head of *Drosophila* is remarkably conserved. Several similarities can be listed:

- (I) Specific expression domains form in the intercalary and antennal segments, the anterior procephalon, the labrum and the foregut.
- (II) Expression in the intercalary segment is transient. In *Drosophila*, the antennal segment also expresses *wg* only transiently, which is not the case in *Tribolium* and *Schistocerca*. This difference is explained by the complete reduction of antennal appendage development in the *Drosophila* embryo. In *Tribolium* and *Schistocerca*, the segmental antennal expression domain persists as the ventral patterning domain of the extending antennal appendage.
- (III) *wg* expressing neuroblasts are formed in the intercalary and antennal segments as well as in the anterior

procephalon, but not in the developing labrum and foregut. This conclusion emerges from the consistency between the nature of *wg* expressing neuroblast segments in the present analysis of *wg* expressing neuroblasts in the embryonic grasshopper head and that previously reported in *Drosophila* (Reichert and Boyan, 1997; Urbach and Technau, 2003b; Younossi-Hartenstein et al., 1996).

- (IV) The contribution of *wg* positive neuroblasts in the anterior procephalon of grasshopper and *Tribolium* is substantially larger compared to the antennal and intercalary segments. This difference fits well with the relative numbers of *wg* positive cells reported in the procephalon of *Drosophila*. Urbach and Technau (2003) identified 16–20 *wg* expressing cells in the *Drosophila* anterior procephalon compared to three in the antennal segment and one in the intercalary segment. Although different in absolute numbers, similar ratios have been reported in earlier studies (Chun-LaGraff and Doe, 1993; Reichert and Boyan, 1997;

Richter et al., 1998; Younossi-Hartenstein et al., 1996). According to Urbach and Technau (2003), *wg* positive neuroblasts represent up to 20% of all protocerebral neuroblasts. In line with this, the effect of genetically eliminating *wg* function is most severe on the protocerebrum in the embryonic head region. About 50% of the protocerebrum has been found to degenerate in *Drosophila* *wg* null mutant embryos, whereas the deutero- and tritocerebral neuromeres do not show detectable phenotypes (Chu-LaGriffa and Doe, 1993; Richter et al., 1998). Although we have not investigated the presence of *wg* expressing neuroblasts in *Tribolium* in detail, the widespread expression in the dorsal protocerebrum in this species is consistent with the results in grasshopper and *Drosophila*.

These data indicate that the majority of early head segmentation and neurogenesis patterning steps are highly conserved in insects, consistent with previous conclusions from comparative work on *en* (Schmidt-Ott and Technau, 1992; Schmidt-Ott et al., 1994b) (Rogers and Kaufman, 1996).

4.2. Developmental organization of the insect anterior procephalon

The segmental organization of the most anterior areas of the insect head has remained a controversial issue (Urbach and Technau, 2003a). The interpretation of segmentation gene expression domains as indicators of segmental organization has a strong rationale. It is based on the serial homology of segmentation gene expression and function along the longitudinal body axis, which is unambiguous in the trunk region. Due to their strong conservation, segmentation gene expression domains have also proven extremely useful for comparisons across arthropod orders despite differences in early embryonic development and final phenotype (Damen et al., 1998; Duman-Scheel and Patel, 1999; Duman-Scheel et al., 2002). Unfortunately, the serial homology of segmentation gene expression domains tends to be less pronounced in the head tagma. The gnathal segment polarity expression domains of *hh*, *en* and *wg* are still shaped and regulated like those in the trunk. The procephalic segments, however, deviate from the trunk segmentation paradigm. In *Drosophila*, segmentation in the procephalic region and the mandibular segment is unique in being established largely without pair rule gene input (Finkelstein and Perrimon, 1991). In addition, the regulatory relationships between the segment polarity genes differ from those in the trunk and between procephalic segments (Gallitano-Mendel and Finkelstein, 1997b). It has been suggested that the divergence of procephalic segment polarity gene regulation may reflect older evolutionary age or their pronounced morphological diversity (Gallitano-Mendel and Finkelstein, 1997b). Regardless the cause, due to obvious departures from the trunk segmentation paradigm, it is not trivial to differentiate between segmentation related and non-segmentation related expression domains in the procephalon. The controversy regarding the segmental nature of *en*

expression domains in the *Drosophila* labrum and visual system is paradigmatic of this problem. In the gastrulating embryo, we find that *wg* is expressed in immediate association with *hh*, and thus in perfect correspondence with the trunk segmentation paradigm. It seems reasonable to conclude that the *wg* and *hh* implement a segmental boundary at this stage. This observation supports the concept of an ocular segment containing the visual anlage and protocerebrum. No further insights, however, are added by the data on the anterior organization of the ocular segment. It may be followed by a labral segment or by a terminal non-segmental region.

The early segment border formation phase is followed by an event unique for the anterior procephalon. The *wg* domain dissociates from that of *hh* by moving anteriorly, thereby generating a median compartment in the anterior procephalon encompassing the protocerebrum, and a peripheral compartment containing the visual anlage. It remains to be tested if the spatial separation of *hh* and *wg* expressing cells results from formation of new cells in this area, which seems most likely, or from translocation of the expression domains across existing cells. Regardless of the displacement mechanism, the relocation of the *wg* domain to a position within the anterior procephalon suggests that this domain acquires a secondary intrasegmental patterning function once the posterior border of the anterior procephalon has been consolidated. The subsequent function consists of the elaboration of the visual and protocerebral compartments within the anterior procephalon. In the course of this process, *wg* expression in the anterior procephalon loses its stripe-like shape, which is characteristic of segmentation function, and dissociates into the three protocerebral neuroectoderm domains.

The changing role of *wg* in the anterior procephalon highlights two points. First, the function of initial segmentation gene domains change during the course of embryogenesis. Caution must therefore be taken in interpreting expression domains of segmentation genes by default to reflect involvement in segmental patterning. Second, protocerebrum and visual system derive from the same germ band region, which appears to be of segmental character. This is consistent with the interpretation of visual system patterning based on neuronal structure and head gap gene phenotypes, which affected elements of the visual system (Schmidt-Ott et al., 1995). The boundary between visual system and protocerebrum separates two compartments within one segment rather than two independently patterned early segments.

The relation of *wg* and *hh* expression domains in the anterior procephalon described here for *Tribolium* exhibits precisely the same dynamics in the cricket *Gryllus bimaculatus* (Miyawaki et al., 2004). Hence, compartmentalization of the anterior procephalon by *wg* is an ancestral aspect of insect head development. The question if this patterning aspect is also conserved in *Drosophila* is more difficult to interpret as the anterior procephalon *wg* domain does not undergo secondary compartmentalization (see below). *hh* is expressed in the anterior procephalon ventral to *wg*, corresponding to a posterior position along the longitudinal body axis consistent with the situation in *Tribolium* (Chang et al., 2001). The relative

positions are therefore conserved. However, unlike in the trunk segments, the activation and maintenance of *wg* in the anterior procephalon is independent of *hh* (Gallitano-Mendel and Finkelstein, 1997a). This regulatory relationship is compatible with the fact that the anterior procephalon *wg* domain can separate from that of *hh* in primitive species. It, however, also implies that the initiation of *wg* is independent of *hh* despite their initial proximity of expression domains arguing against a canonical segmentation gene character of its early expression phase. It would be very desirable to revisit this issue in primitive insects with functional assays.

4.3. *wg* expression pattern changes correlate with reduction of the larval visual system in higher insects

Previous investigations in the grasshopper embryonic visual system, a paradigm for embryonic visual system patterning in directly developing insects, revealed a highly dynamic and complex spatiotemporal control of *wg* expression associated with a multitude of patterning events (Dong and Friedrich, 2005). The pair of protocerebral neuroectoderm domains located in regions that correspond to the dorsal and ventral poles of the adult visual system are likely involved in at least four aspects: (I) contributing neuroblasts to the protocerebrum and the outer optic lobe anlage, (II) specifying the border between visual system anlage and adjacent head compartments, (III) stimulating cell division in the visual system anlage, and (IV) coordinating the spatiotemporal onset of retina differentiation by repression from a distance (Dong and Friedrich, 2005). The somewhat later emerging dorsoventral ectodermal expression domains cooperate in functions II–IV. Independent growth activation of the outer optic lobe anlage is likely exerted by a pair of conserved polar expression domains in the developing medulla neuropil (Dong and Friedrich, 2005). The pleiotropic involvement of *wg* signaling in the grasshopper visual system is indicative of the complexity of integrated embryonic visual system development in directly developing insects, which produces optic neuropils and retina of adult like functionality.

Some of the ancestral *wg* expression domains of the embryonic grasshopper visual system are not initiated in the embryo of beetle or fruit fly but during later stages. This is consistent with the spatial and temporal decoupling of the development of various components of the larval and adult visual system in holometabolous insects. No larval or adult optic lobe anlagen expression domains of *wg* could be detected in the *Tribolium* embryo. Despite the difficulty of identifying the smaller sized neuroblasts in the intercalary or antennal segments of *Tribolium*, potential optic lobe anlagen neuroblast cells should be detectable considering their separation from other *wg* expression domains in grasshopper. In *Drosophila*, *wg* expression in the homologs of the *wg* expressing embryonic grasshopper optic lobe anlagen neuroblasts are initiated during larval development (Kaphingst and Kunes, 1994). This may also be the case in *Tribolium*. The delay of growth-intensive adult optic lobe development into postembryogenesis provides a likely explanation for the lack of these expression domains in the

relatively small embryonic visual system anlage. The postembryonic initiation of these domains may thus represent a change that occurred in the ancestral lineage of the Holometabola in conjunction with the reduction of the larval optic neuropils.

The same may hold for the delay of distinct ectodermal *wg* expression domains in the visual system. The examination of *wg* expression in the *Tribolium* embryo yielded no indication of independent ectodermal expression domains in addition to the three neuroectodermal domains. Considering the small size of the embryonic visual system and the fact that the development of the larger adult eye field is postembryonic, it seems conceivable that the polar neuroectodermal domains are sufficient to instruct dorsoventral polarity in the developing visual anlage. Conserved ectodermal expression domains in front of the developing adult eye field begin being expressed in the last larval instar of *Tribolium*. These may be initiated *de novo* during postembryogenesis like in *Drosophila* or represent reinitiated derivatives of the neuroectodermal expression domains in the embryo (Friedrich and Benzer, 2000).

As most obvious in the *Drosophila* embryonic visual system, also the regulation of the protocerebral neuroectoderm expression domains in the anterior procephalon underwent significant changes during holometabolous insect evolution. These domains are still present in *Tribolium*, where they emerge from the primordial protocerebral neuroectoderm domain in a manner very similar as in grasshopper. However, during subsequent stages of development, the ventral protocerebral neuroectoderm domain appears to become smaller, while the dorsal domain expands. This is different from the expression dynamics in the grasshopper, where also the ventral neuroectodermal domain, although being smaller than the dorsal domain, continues to expand throughout embryogenesis. These observations in *Tribolium* indicate an early trend of ventral protocerebral neuroectoderm domain reduction during the evolution of holometabolous insects.

A yet more extreme modification of protocerebral neuroectoderm expression must have occurred during the evolution of the lineage leading to *Drosophila*. In this case, *wg* remains expressed in a single domain throughout development. The respective domain, known as the “head blob”, is homologous to the initial contiguous protocerebral neuroectoderm domain in directly developing species like grasshopper. The data further suggests that the *Drosophila* head blob domain evolved by elimination of the step that leads to the dissociation of the protocerebral neuroectoderm domain in lesser derived species. The comparison with *Tribolium* suggests that this “frozen” protocerebral neuroectoderm domain most likely fulfills patterning functions of the ancestral dorsal and median protocerebral neuroectoderm domains. *wg* expression seems to be completely missing in the strategic area of the ventral domain in the *Drosophila* procephalon. As noted, reduction of the ventral protocerebral domain is already apparent in the *Tribolium* embryonic visual system suggesting that its beginnings reach far back in the evolution of the Holometabola.

The extreme state of reduction in *Drosophila* is most likely mechanistically linked to the dramatic reduction of both the larval eyes and outer optic lobe anlagen. The larger larval

eyes of *Tribolium* are formed from a field of five photoreceptor preclusters in the visual anlagen ectoderm (Liu and Friedrich, 2004). In the ancestrally organized *Tribolium* embryonic head, larval eye differentiation still proceeds in an anlagen field that requires *wg* signaling mediated dorsoventral polarity input. The *Drosophila* Bolwig organ involves formation of a single photoreceptor cluster. Although a quantitative comparison has not been carried out yet, it also seems that the embryonic optic lobe anlage is substantially larger in *Tribolium* than in *Drosophila*. There may thus still be a higher need for activation of cell proliferation in the *Tribolium* visual anlage. Stimulation of cell division is an ancestral patterning function of *wg* in the visual system (Dong and Friedrich, 2005). Thus, given the extreme reduction of optic lobe anlagen and larval eyes, evolution left the undivided protocerebral neuroectoderm domain in *Drosophila* mainly as neuroblast generator in the development of the protocerebrum. The spatial control of Bolwig organ differentiation may still be under the repressive control by the protocerebral neuroectoderm domain, perhaps in cooperation with the medially situated antennal or maxillary domains of *wg*. This can be tested by investigating the effect of manipulating *wg* expression levels on Bolwig organ development, as has been carried out for *Decapentaplegic* and *hh* signaling (Chang et al., 2001). Studying the expression of *wg* in Dipteran species that represent intermediate forms of head reduction, the most primitive forms of which are expected to still possess the ventral protocerebral neuroectoderm domain, will equally be informative (Melzer and Paulus, 1989).

Despite its highly derived organisation, the *Drosophila* system has proven extremely informative concerning ancestral mechanisms of animal head patterning (Chang et al., 2001). The data presented here give reason to think that the study of lesser derived insect species holds the key to discovering an even greater degree of conservation of cephalic patterning in animals.

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