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The expression pattern of the genes *engrailed*, *pax6*, *otd* and *six3* with special respect to head and eye development in *Euperipatoides kanangrensis* Reid 1996 (Onychophora: Peripatopsidae)

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Abstract

The genes *otd/otx*, *six3*, *pax6* and *engrailed* are involved in eye patterning in many animals. Here we describe the expression pattern of the homologs to *otd/otx*, *six3*, *pax6* and *engrailed* in the developing *Euperipatoides kanangrensis* embryos. Special reference is given to the expression in the protocerebral/ocular region. *E.kanangrensis otd* is expressed in the posterior part of the protocerebral/ocular segment before, during, and after eye invagination. *E.kanangrensis otd* is also expressed segmentally in the developing ventral nerve cord. The *E.kanangrensis six3* is located at the extreme anterior part of the protocerebral/ocular segment and not at the location of the developing eyes. *Pax6* is expressed in a broad zone at the posterior part of the protocerebral/ocular segment but only weak expression can be seen at early onset of eye invagination. In late stages of development, the expression in the eye is upregulated. *Pax6* is also expressed in the invaginating hypocerebral organs, thus supporting earlier suggestions that the hypocerebral organs in onychophorans are glands. *Pax6* transcripts are also present in the developing ventral nerve cord. The segment polarity gene *engrailed* is expressed at the dorsal side of the developing eye including only a subset of the cells of the invaginating eye vesicle. We show that *engrailed* is not expressed in the neuroectoderm of the protocerebral/ocular segment as in the other segments. In addition, we discuss other aspect of *otd*, *six3* and *pax6* expression that are relevant to our understanding of evolutionary changes in morphology and function in arthropods.

Keywords

Onychophora; eye; CNS; development; *pax6*-; *otd*-; *six3*-; *engrailed*-expression

Introduction

Onychophorans have a pair of small eyes with a single retina and lens situated at the base of the antenna (Dakin 1921; Storch and Ruhberg 1993). The photoreceptors have been shown to be of the rhabdomeric type although cilia are also present in each of the photoreceptor cells (Mayer 2006; Eakin and Westfall 1965). The morphological and ultrastructural aspects of eye development have been carefully described (Mayer 2006; Sedgwick 1887). However, not much is known about the molecular patterning of eye development in onychophorans.

The so-called retina determination network is a set of genes involved in eye patterning in animals such as mice and flies and usually the gene *pax6* is regarded as a key player in this network (Gehring 2004; Kumar 2009; Royet and Finkelstein 1995). However, it has been

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shown that there exists a large variation in the patterning of different eye types. Blackburn and colleagues (Blackburn et al. 2008) showed that *pax6* and *atonal* are not involved in eye patterning in the chelicerate horse shoe crab *Limulus polyphemus*. Adult eyes of the annelid worm *Platynereis dumerilii* are formed without the function of *pax6* (Arendt et al. 2002). The gene orthodenticle (*otd*) has been shown to be involved in eye formation in flies together with the segment polarity gene engrailed (*en*) (Royet and Finkelstein 1995). The vertebrate homologue to *otd*, has been shown to be important for eye development in e.g. mice (Martinez-Morales et al. 2001). The silencing of *en* expression in mice gives many defects in the CNS, including defective eye nerves (Wurst et al. 1994). In the spider *Cupiennius salei* it was demonstrated that the primary *en* head spot contributes cells to the posterior median eyes (Doeffinger et al. 2010). The gene *six3* (*optix* in *Drosophila melanogaster*) is also involved in eye formation in vertebrates (Del Bene et al. 2004; Liu et al. 2010; Seimiya and Gehring 2000).

Onychophorans are in several recent investigations placed as a sister group to euarthropods (Campbell et al. 2011; Dunn et al. 2008) and can provide important information for reconstructing the ground-pattern for eye development in euarthropods.

A molecular characterisation of eye development in onychophorans is lacking and therefore we decided to investigate the expression of *en*, *otd*, *pax6* and *six3* in the embryo of the onychophoran species *Euperipatoides kanangrensis* in order to get a better understanding of the ground-pattern for the regulation of eye development in arthropods. Earlier investigations have shown that the eyes of the onychophoran species *E. kanangrensis* develop within the *otd* expressing region but out of the *six3* expressing region (Steinmetz et al. 2010), however, a detailed description of the areas where the transcripts of *six3* and *otd* occur in relation to the nervous system and eye development is lacking. In an earlier investigation in onychophorans, Eriksson and colleagues (Eriksson et al. 2009) described the expression pattern of the segment polarity gene *en* in the developing embryo and it could be shown that *en* is expressed in a patch in the most anterior segment (the protocerebral/ocular segment), however, the relation of this *en* expressing patch to the developing eye and nervous system was not addressed.

In this paper we describe in detail the expression pattern of *otd*, *six3* and *en* and their relation to eye development and protocerebral neurogenesis. We have also cloned a homologue to the eye patterning gene *pax6* and describe its expression in the developing central nervous system (CNS) and eyes.

Material and methods

Collection, animal husbandry and staging

Female *Euperipatoides kanangrensis* Reid, 1996 were collected in Kanangra Boyd National Park, NSW, Australia 33° 59'S 150° 08'E. Females were kept in containers with dampened sphagnum moss at 13°C and were fed first instar locusts or crickets once every second week. Dissected females were found to contain developing embryos for at least 12 months after collection, with individual females harbouring 20-150 embryos at various stages of development.

The development of a number of onychophoran species closely related to *E. kanangrensis* has been described and a staging system was presented (Walker and Tait 2004). The seven stages of onychophoran development are briefly: I, fertilized egg to formation of first segment; II, formation of pre-oral segment to first signs of antenna; III, mid ventral fold of embryo and start of eye formation; IV, all segments formed and eye vesicle invagination complete; V, formation of cerebral (hypo cerebral) grooves and epidermis; VI, all legs fully

extended and forming claws dermal papillae developing; VII, Completed external development but gut still undeveloped. We staged embryos according to the criteria suggested by Walker and Tait (2004).

Fixation of embryos and in situ hybridisation

Embryos were dissected from the females and, after removal of the egg membranes, fixed in 4% formaldehyde in PBS overnight at 4°C. Fixed embryos were dehydrated in a graded series of methanol (25, 50, and 75% in PBS with 0.1 % Tween-20 for 10 min each) and stored in 100 % methanol at −20° C. In situ hybridisation was done according to Eriksson and colleagues (2009).

Light microscopy

Stained embryos were mounted whole or imbedded in paraffin and sectioned. In some cases the embryos were counter stained with CYBR green (Invitrogen). Embryos were analysed and photographed on an Olympus BX51 microscope. Contrast and brightness were adjusted in Photoshop CS5.

Isolation and sequencing of *E. kanangrensis* genes

Isolation and sequencing of *E. kanangrensis* homologues to *six3* (accession EU347400), *otx* (accession EU347401) and *engrailed* (accession EU347402) were done as described before (Eriksson et al., 2009; Steinmetz et al., 2010).

Total RNA was extracted from mixed embryonic stages with the Trizol method (Invitrogen) and subsequently used to synthesise complementary DNA (cDNA) with thermoscript (Invitrogen). The obtained cDNA was used as template for polymerase chain reactions (PCR) in order to find genes homologous to *pax6* in the onychophoran genome and eventually the *E. kanangrensis* homologue to *pax6* (accession JX013948) was amplified by nested degenerate PCR using the primers and thermocycler settings as described in Arendt and colleagues (Arendt et al. 2002). The initial orthology of the *Pax6* gene was tested by searching against GenBank non-redundant protein databases using the BlastX algorithm. Orthology assignment was made based on phylogenetic analysis. The phylogenetic analyses were carried out using amino acid sequences. Bilateral *Pax6* and *Pax3/7* protein sequences were obtained from the published literature or BLAST searches of the NCBI GenBank. The abbreviation of species names, the name of the genes and their accession numbers can be found in table S1. Sequences were aligned using the program ClustalX v.2.0.10. Bayesian inference on amino acid data using MrBayes version 3.1.1 was applied for orthology analysis (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003).

Results

Early expression of *otd* and *six3*

The first observed expression of the head and eye patterning gene *otd* is during stage I on both sides of the blastopore slit (Figs. 1 a-b). *Otd* is expressed diffusely in the blastodisc centered around the blastopore slit. The expression is lacking in the posterior area around the posterior part of the blastopore.

The first sign of *six3* expression coincides in developmental time (stage I) with the early expression of *otd*. The expression is restricted to a thin band around the most anterior part of the slit-like blastopore (Figs. 1c-d).

Expression of *otd* and *six3* during germ band extension

At stage II, both *otd* and *six3* are expressed in the developing protocerebral/ocular segment. *Otd* is initially restricted to the posterior margin, extending dorso-ventrally (Fig. 2) and *six3* at the anterior margin of the brain anlage (Fig. 3). It appears as if the two genes to a large extent are expressed in mutually exclusive zones but we cannot rule out some limited overlap. *Otd* is then expressed in a field perpendicular to the original marginal expression, extending towards the anterior part but not reaching the most anterior quarter of the anterior-posterior distance (Fig. 2). *Otd* is also expressed around the stomodeum when it has been separated from the posterior blastopore-slit. In the stage IV embryo *otd* is expressed in a relatively larger area of the brain anlage but expression is lacking in the anterior-most part and in a wedge shaped field pointing towards the eye (Fig. 4). In addition, *Otd* expression is observed in different neuronal precursor layers but not in the underlying mesoderm (Fig. 5). The area of eye invagination is expressing *otd* and the invaginated ectoderm continues to express *otd* (Fig. 5). There is no expression of *otd* in the antenna. Segmental expression in the future trunk nervous system is also appearing during the stage IV embryo (Fig. 4a-b).

The expression of *six3* during the stages III and IV continues to be in the anterior part of the protocerebral/ocular segment and is including the anterior part of the antenna (Figs. 3c-d, 5). The *six3* expressing zone is confined to the outermost (youngest) neuronal precursors (Fig. 5c-d). The *six3* expression can also be seen in the ectoderm and mesoderm of the antenna (Fig. 5c). There is no expression of *six3* in the developing eye.

Engrailed expression in the head

We made a detailed analysis of the dorso-posterior *en* expression in relation to the eye and nervous system in stage III and IV embryos and found that the *en* domain is outside of the neuroectoderm just abutting the invaginating eye on the non-neuroectodermal side (Figs. 6a-d). It appears as if the *en* expressing domain is only including a few cells of the invaginated eye vesicle (Fig. 6d).

Characterisation and analysis of *pax6* expression

We cloned a 586 bp fragment of a *pax6* homologue by degenerate and nested PCR. This fragment was characterised as a *pax6/toy* homologue (supplementary data S. 1 and S. 2). Sequence alignment of *pax6* domains within bilaterians is demonstrated in Fig S1. Pax6 is strongly conserved at the primary sequence level, particularly in the area of the DNA-binding paired and homeodomains (Fig. S1 a and b). A less degree of sequence conservation can be observed in the linker region between the two DNA-binding domains (Fig. S1 residues 130 to 155). The phylogenetic analysis places the *E. kanangrensis* *pax6* clearly with the arthropod *pax6/toy* homologues. (Fig. S2). *Pax6* is expressed in the lateral parts of the protocerebral/ocular segment at stage II (Figs. 7a-b), the expression includes the area, as judged by distances to segment border, where the eye and antenna are formed in a later stage embryo. There is also expression in the developing ventral nerve cord (Figs. 7a-b). During stage III expression can be seen weakly in the invaginating eye and stronger in the antenna and in a wedge shaped area in the ventral and posterior corner of the neuroectoderm of the protocerebral/ocular segment (Figs. 7c-d). During stage V expression is stronger in the eye vesicle and in the antennal tract and in the invaginating hypocerebral organ (Figs. 7e-f). The expression of *pax6* in the ventral nerve cord continues during stage V (Fig. 7e).

Discussion

Otd, *six3*, *en* and *pax6* are expressed in non-overlapping domains in the brain

During early segmentation the expression of *otd* in *E. kanangrensis* is initially restricted to the protocerebral/ocular segment. In euarthropods the early expression varies with some

examples of expression in more posterior structures but it appear as if the early expression in arthropods is restricted to the protocerebrum (Browne et al. 2006; Finkelstein et al. 1990; Hirth et al. 2003; Li et al. 1996; Steinmetz et al. 2011; Telford and Thomas 1998), which is the first neuromere of euarthropods. From cross sections of the *otd* expressing areas, expression can be seen in neuronal precursors of all ages indicating a function during the entire process of neurogenesis. *Six3* on the other hand is restricted to the neuroectodermal layer, suggesting a function in early neurogenesis only. The neuroectoderm of the protocerebral/ocular segment is completely devoid of *en* expression, which contrasts with the other head segments as well as the trunk segments where segmental *en* expression can be seen in the neuroectoderm as well as in deeper layers with more mature neuronal precursors (Eriksson et al. 2009). However, the antenna that is situated on the protocerebral/ocular segment express *en* in its posterior half just as in the rest of the appendages of the head and trunk. The eye invaginates at the border between the neuroectoderm and dorsal non-neuronal areas and *en* expression is located on the non-neuronal side of the eye vesicle. Also the gene *wingless* is lacking in the developing neuroectoderm of the protocerebral/ocular segment but is present in the neuroectoderm of all posterior head and trunk segments (Eriksson et al. 2009).

***Otd*, *en* and *pax6* but not *six3* are involved in onychophoran eye development**

Otx and *pax6* play key roles in the so-called retina determining network that control eye development in *D. melanogaster* (Friedrich 2006) and they appear to be important for eye development in many animals (Martinez-Morales et al. 2001; Royet and Finkelstein 1995; Hanson and Van Heyningen 1995). However, in the chelicerate *Limulus*, *pax 6* is not expressed in the developing eyes (Blackburn et al. 2008) indicating that *pax6* might not be universally present in eye development. In this study we have shown that the onychophoran homologues *otd* and *pax6* are expressed in the developing eye of *E. kanangrensis*, and hence, at least two of the genes involved in the retina determining network are present in onychophorans. The analysis of additional chelicerates will provide an answer to the question if *pax6* is not necessary for eye development in all chelicerates or only in *Limulus*. *Optix* or *six3* as it is called in most other animals than *D. melanogaster* is a target of *eyeless* (*pax6*) (Ostrin et al. 2006) but is also capable of inducing ectopic eyes in *D. melanogaster* independent of *eyless* (Seimiya and Gehring 2000). Perhaps *optix* represents a parallel to the early retina network in *D. melanogaster* (Friedrich 2006; Ostrin et al. 2006; Seimiya and Gehring 2000). It appears as if *six3* is not involved in eye formation in the onychophoran *E. kanangrensis* since it is not expressed in the developing eye. The segment polarity gene *en* is a target of *otx* in development of ocelli in *D. melanogaster* (Royet and Finkelstein 1995). Earlier reports have suggested, based on anatomy and innervation pattern, that the onychophoran eye is homologous to the insect ocelli (Mayer 2006) and the ocelli of insects are regarded as homologous to the anterior median eyes of spiders (Paulus 1979). The *en* function in *D. melanogaster* in median ocelli formation and the expression of *en* at the site of eye formation in *E. kanangrensis* would also support a homology between median ocelli and onychophoran eyes. However, *en* is expressed at the site of the posterior median eye in the spider *Cupiennius salei* (Doeffinger et al. 2010). Despite their name, posterior median eyes (PM eyes or secondary eyes), the PM eyes are actually considered as belonging, in an evolutionary sence, to the lateral eyes of Arachnida and only the anterior median eyes (AM eyes) are true median eyes and as such homologous to the median eyes or ocelli of other arthropods. The *en* expression at the site of PM eye formation thus present a puzzle, either the PM eyes belong to the median eye type (not at all supported by structure) or more likely *en* has changed its function in respect to eye formation in the spider.

Other aspects of *otd*, *six3* and *pax6* expression

Onychophoran *otd* expression combines patterns that are divided between two homologues in other arthropods—In some animals two paralogues of *otd/otx* have been described, for instance, in the crustacean *Parhyale hawaiiensis* there are two paralogues of *otd* with differing expression patterns and difference in onset of expression. *Otd2* is distinguished by a special motif consisting of the amino acid residues tryptophan, serine and proline (WSP). *Otd2* is expressed later and in a different pattern to that of *otd1* (Browne et al. 2006). In *E. kanangrensis* we found one gene homologous to *otd* and it contained the WSP motif that is included in some *otd* homologues. The single *otd* gene found in *E. kanangrensis* is both expressed early in development and later continues to be expressed in the protocerebral/ocular segment as well as in a cluster of central cells in the neuroectoderm in each segment in the ventral nerve cord. Thus it is possible that the functions of a single *otd* homologue in onychophorans have been divided between the two homologues that appear to be present in some euarthropods. In the early *E. kanangrensis* embryo (stage I), before segmentation, the *otd* gene is expressed in a broad area in the blastodisc surrounding the slit-like blastopore, it is missing from the most posterior part around the posterior blastopore. This expression pattern is similar to the expression pattern seen in *Drosophila otd* and for the *otd2* expression in *Tribolium* and *Parhyale* (Browne et al. 2006; Li et al. 1996) in that the expression is in the anterior of the extending germ band. However, the early expression of onychophoran *otd* is also extended in a region far anterior to the emerging germ band along the blastopore. The ectoderm and mesoderm of the embryo is traditionally believed to migrate anteriorly from the location of the posterior part of the blastopore (Anderson 1966; Eriksson and Tait 2012; Manton 1949) and, hence, the area outside of the germ band is regarded as extra embryonic ectoderm that do not contribute to the embryo. Perhaps the early *otd* expression in the so-called extra embryonic ectoderm indicates that it is not really extra embryonic but do contribute with embryonic ectoderm. Another factor that early *otd* expression may be connected to germ band extension is that the expression of *otd* is sometimes, asymmetrical around the blastopore (Figs. 1a-b). This asymmetry might reflect the fact that the early development of the germ band is also sometimes developing asymmetrical, i.e. the germ band extends with different speed, on each side of the blastopore (Eriksson and Tait 2012; Manton 1949).

Early *six3* expression correlates with axis formation—The early expression of *six3* takes place around the anterior margin of the slit-like blastopore. This looks very similar to what has been described in the Medaka fish, where the early *six3* expression is seen around the anterior margin of the embryonic shield (Loosli et al. 1998). This, together with the general resemblance between onychophoran and vertebrate segmentation tempts to do further investigation on the molecular aspects of onychophoran axis formation and segmentation, e.g. does the onychophoran slit-like blastopore have an organising function like the fish embryonic shield or amphibian organizer? Given the fact that both onychophorans and fish are bilaterian animals, and the fact that bilaterians are regarded as a monophyletic group (Nielsen 2001) one could expect to find similarities in the formation of the body axis. The *Drosophila six3* gene is expressed in the anterior part of the stage 5, or blastoderm stage, but is lacking in the extreme terminal areas (Seo et al. 1999). However, from a *Drosophila* fate map (Campos-Ortega and Hartenstein 1985) one can see that the area of *six3* expression corresponds to the prospective areas of the most anterior neurogenic area, which would correspond to the early expression pattern seen in *E. kanangrensis*.

Onychophoran hypocerebral organs are probably glands—Pflugfelder (1948) suggested that the hypocerebral organs of onychophorans are analogous structures to the *corpora allata* of insects. Earlier ultrastructural investigations have also suggested that the onychophoran hypocerebral organs are probably including glandular cell types (Eriksson et

al. 2005). The glandular function of the hypocerebral organ in onychophora is thus supported given the involvement of *pax6* in the development of the pituitary gland in vertebrates and in insulin producing neurons in the fly *D. melanogaster* (Clements et al. 2008; Kioussi et al. 1999). *Pax6* is also expressed in other tissues of animals like the olfactory epithelium and olfactory bulb of vertebrates (Callaerts et al. 1997; Griffin et al. 2002). However, since the hypocerebral organ is not connected to the brain with any nerve fibres a sensory function is probably not the case.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Expression of *otd* (a-b) and *six3* (c-d) in *Euperipatoides kanangrensis* embryos. (a-b) Stage I embryo with elongated blastopore (Bp), but before segment formation. *Otd* is expressed diffusely in the area around the slit-like blastopore with the exception of the posterior part. (c-d) Stage I embryo with elongated blastopore (between arrowheads) and with a developing germ band (arrow) but before segment formation. Expression of *six3* is restricted to the area bordering the anterior blastopore (upper arrowhead). Gb = germ band, Pbp = posterior part of blastopore, Vee = ventral extraembryonic ectoderm, scale bars (a-d) = 500µm.

Fig. 2.

Expression of *otd* in *Euperipatoides kanangrensis* embryos of stage II. **(a-b)** Expression is in the dorso-ventral area of the posterior part of the protocerebral/ocular segment (Pc), ventral view anterior is up. The anterior (A), dorsal (D), posterior (P) and ventral (V) parts of the protocerebral segment is marked. **(c-d)** Stage II embryo slightly later than the one shown in a-b. Expression is now also detected in a zone perpendicular to the expression domain of the previous embryo as well as around the stomodeum (arrowhead). A = antenna, J = jaw, W = walking leg, scale bars **(a-b)** = 500 μ m, **(c-d)** = 300 μ m.

Fig. 3.

Expression of *six3* in *Euperipatoides kanangrensis* embryos. **(a-b)** A stage II embryo with a segmenting germ band (Gb). The slit-like blastopore has been divided up into three parts; proctodeum (black arrowhead), a middle part and the stomodeum (star), areas that separate these blastopore regions are marked with white arrowheads. The expression of *six3* is restricted to the anterior part of the protocerebral/ocular segment (Pc) and extend ventrally and dorsally to the edge of the ventral and dorsal extra embryonic ectoderm (Vee and Dee respectively). **(c-d)** Late stage II embryo with developing antenna (A). The expression of *six3* can now be seen in the antenna. J = jaw, Dee = dorsal extra-embryonic ectoderm, Sp = slime papilla, Vee = ventral extra-embryonic ectoderm, W = walking leg, scale bars **(a-d)** = 500 μ m.

Fig. 4.

Expression of *otd* in *Euperipatoides kanangrensis* embryos of stage IV. **(a-b)** Expression is in the dorsal and ventral area of the posterior part of the protocerebral/ocular segment (Pc), ventral view anterior is up. **(b)** A different embryo of the same stage to that in **(a)**. **(c-d)** The areas of ventral and dorsal expression meet in the dorsal area where the eye rudiment is situated (arrow), lateral view anterior is up. A = antenna, J = jaw, W = walking leg, scale bars **(a-b)** = 400 μ m, **(c-d)** = 300 μ m.

Fig. 5.

Expression of *otd* and *six3* in *Euperipatoides kanangrensis* embryos. **(a)** Cross section of a late stage III to early stage IV embryo showing *otd* expression in the developing protocerebral part of the brain (Pc). The expression can be seen in both proliferating neuroectoderm (below the arrowhead) as well as in developmentally older neuronal precursors towards the mesodermal cavity (M). There is no expression in the antenna (An). **(b)** Cross section of the same stage as in (a) at the level of the invaginating eye vesicle. Expression of *otd* can be seen in the ectoderm in and around the invaginating eye. The slight blue colour in the yolk (Y) is due to unspecific staining in yolk particles. **(c)** Cross section of a stage IV embryo showing *six3* expression in the anterior part of the brain (arrowheads) as well as in the mesoderm (M) of the protocerebral/ocular segment (right arrow) and antenna (left antenna). **(d)** Cross section of a stage IV embryo showing expression of *six3* in the apical layer of the neuroectoderm (arrowhead). Ec = ectoderm, G = gut, scale bars **(a, c-d)** = 200 μ m, **(b)** = 100 μ m.

Fig. 6.

Expression of *engrailed* in *Euperipatoides kanangrensis* embryos. **(a)** The anterior end of and stage II embryo with segmental *eng* expression in each segment the patch of *eng* expression in the protocerebral/ocular segment is abutting the invaginating eye (arrowhead) just posterior of the antenna (A). **(b)** A close up of a stage IV embryo showing the head patch of *eng* expression just abutting (arrow) the invaginated eye vesicle. **(c-d)** Cross section of dorsal brain region including eye of a stage IV embryo. Expression of *eng* in the ectoderm (Ec) and dorsal part of the invaginated eye vesicle. The expression is on the dorsal side facing away from the brain process contacting the eye vesicle (Bp), the border between the eye and brain process is marked with an arrow. J = jaw segment, Sp = slime papilla segment, W = walking leg segment, Scale bars **(a)** = 300 μm , **(b-d)** = 50 μm .

Fig. 7.

Expression of *pax6* in *Euperipatoides kanangrensis* embryos. **(a-b)** Expression of *pax6* in the protocerebral/ocular segment (Pc) and the neuroectoderm (Jn) of the jaw (J) segment in a stage I embryo. In the protocerebral/ocular segment expression can be seen in parts of the neuroectoderm as well as in the area of the future antenna (A) in the jaw segment there is only expression in the neuroectoderm. **(c-d)** A stage II embryo showing expression of *pax6* in the developing antenna and areas lateral, dorsal and medial to it, expression is also seen in a crescent shaped area of the ventral and posterior neuroectoderm of the protocerebral/ocular segment. There is also expression in the neuroectoderm of the jaw (Jn) and slime papilla (Spn) segments as well as in the trunk (not shown on the image). Note the weak expression

in the invaginating eye (E). **(e)** A stage IV embryo from the ventral side showing expression of *pax6* in the central and lateral parts of the neuroectoderm of the developing brain, including the invaginating hypocerebral organs (upper arrows), the lower arrow show the developing “tounge”. There is also expression in the ventral nerve cord (star). In the area medial to the developing ventral nerve cords the ventral closure has not completed, therefore the yolk is shining through the thin extra embryonic ectoderm and the area appear dark (arrowhead). **(f)** A stage IV embryo from the dorsal side showing expression of *pax6* in the eye vesicles (arrow). There is also expression in the area of the developing antennal (At) tracts as well as medially corresponding to the protocerebral commissure (C). Inset shows the staining of the eye vesicle in bright field illumination. L = lips, Fp = frontal processes, St stomodeum, Scale bars **(a-b)** = 100 μm , **(c-f)** = 200 μm , **(f inset)** = 50 μm .