Supplementary file 1

Contents

Why do fly data lead to different conclusions with respect to the mapping of the IHB?

Three issues may explain the divergent interpretations in flies. First, Hirth et al. used a more advanced stage (stage 13/14) compared to Urbach (stage 11). Hence, the former data are based on expression within an already visible developing CNS while the latter is based on the late neuroectoderm where neuroblasts just start to delaminate. Hence, morphogenetic movements and/or secondary functions of these genes may affect expression of the later stage. Second, he used a small number of genes such that potential idiosyncrasies of the expression of single genes would not be balanced by other data. Indeed, we find that different markers hint to slightly different boundaries (red broken lines in Fig. 2A). Third, a key argument for positioning the MHB at the antennal/intercalary boundary was the expression of *shaven/Pax2* and PoxN, which were interpreted to be a Pax2/5/8 orthologs. Specifically, Hirth et al. mapped the earliest *shaven/Pax2* expression to the prospective deuto-tritocerebral boundary (i.e. boundary between antennal and intercalary segments) in stage 9 embryos (Fig 2A in that paper). Taking into account the careful re-mapping of *wg* and *hh* patterns in the procephalic ectoderm (Ntini and Wimmer, 2011) (see schematic in Fig. 1B-D and hh-patterns in Fig. 4G in that paper), the *shaven/Pax2* expression shown by Hirth et al. could be mapped to the deutocerebral-protocerebral boundary as well . However, double stainings would be required to confidently resolve that matter. For later stages, Hirth et al. use an enhancer trap line to visualize *shaven/Pax2* expression and found segmentally reiterated patterns in addition to signals in the deutocerebrum and the protocerebrum. Co-expression with PoxN (assumed to be another Pax2/5/8 ortholog) was found in the deutocerebrum but not the other domains. This was used as argument to use the Pax2 domain at the deutocerebral-tritocerebral boundary (but none of the other domains) to map the MHB-homolog (Hirth et al., 2003). In contrast, Urbach used mRNA stainings of *shaven/Pax2* and reported a more refined pattern compared to the enhancer trap. He found no *shaven/Pax2* expression in the neuroectoderm nor in any brain neuroblast. He came to the conclusion that the later segmental *shaven/Pax2* activity was too late for patterning the neuroectoderm. (Urbach, 2007).

Both studies interpreted PoxN as a second Pax2/5/8 ortholog. However, phylogenetic investigations based on the expanded genome sequences available nowadays indicate that this gene is actually not orthologous to Pax2/5/8 (Smith et al., 2018; Wollesen et al., 2015).

Ganglia are segmental structures – not parasegmental

In our scheme, the ganglia reflect the segmental frame as opposed to the embryonic parasegmental frame that was suggested by others (Deutsch, 2004). This claim is based on the following reasons: Engrailed and hedgehog mark the future posterior part of each segment but the anterior part of each parasegment (broken black lines shown only for the posterior segments in Fig. 2) (Martinez-Arias and Lawrence, 1985). Hence, engrailed expression and function is a perfect marker to distinguish between these two hypotheses. If ganglia are segmental, engrailed is expected at their posterior part while it should be found at the anterior of each ganglion if they were parasegmental structures.

In Drosophila and Schistocerca, engrailed positive neural cell bodies are located in the posterior of each neuromere (Kumar et al., 2009; Siegler and Jia, 1999; Siegler and Pankhaniya, 1997). In Drosophila, engrailed positive neurons project through the posterior commissure and in engrailed mutants, the posterior commissure is affected or even absent (Joly et al., 2007). Further, the posterior row of neuroblasts of each hemineuromere is engrailed positive in Drosophila (Urbach and Technau, 2003a) and a malocostracan crustacean (Ungerer and Scholtz, 2008). Intriguingly, a median engrailed positive neuroblast gives rise to probably homologous neurons in flies and malacostracan crustaceans, which have their cell bodies directly posterior-adjacent to the posterior commissure and project through this commissure and the posterior segmental nerve (Technau et al., 2006; Ungerer and Scholtz, 2008). Taken together, all these data are strong evidence for a contribution of engrailed positive cells to the posterior parts of the ganglia, strongly favoring the hypothesis of ganglia being segmental structures.

The diverging location of eye anlagen in flies versus beetles

The co-expression of the transcription factors *sine oculis* and *eyes absent* in Drosophila embryos marks the anlagen of the eye field (including the anlagen of the compound eyes) (Chang et al., 2001; Friedrich, 2013). For this discussion we use *sine oculis* as a very faithful marker for the eye field because a respective imaging line in flies marked eye neuroblasts, eye target neurons of the larval eye and the some optic lobe pioneers among other cells (Chang et al., 2003). In fly embryos, *sine oculis* starts expression in a stripe across the dorsal midline of the embryonic head before it splits into the two lateral eye anlagen. Hence, the early eye anlagen were mapped to a continuous dorsal eye field, which later separates into two lateral eye anlagen, which migrate ventrally (Chang et al., 2001; Friedrich, 2013). This seems not unlike the situation in vertebrates where the two eye anlagen are located in close proximity at the midline.

The situation in beetles is different. Here, *sine oculis* starts being expressed in two separate domains, which are very actually very far apart from each other (Fig. 1 F) (Posnien et al., 2011a). In line with this finding, the eye anlagen of beetles and grasshoppers have been mapped to these separate regions based on wingless and eyeless expression (Dong and Friedrich, 2005; Liu et al., 2006; Yang et al., 2009a; Yang et al., 2009b).

Which of these insect species should be used for the comparison with vertebrates? Within insects, the head anlagen typically emerge at a *ventral median* or even posterior position in the blastoderm and they start out in an essentially two dimensional setting. Indeed, the expression of all genes shown in Fig. 1 start out in this 2D setting, where the eye anlagen emerge on opposite sides of the head lobes (see eye anlagen marked in Fig. 1F). Only later, the 2D anlagen undergo morphogenesis to fold into a 3 dimensional head according to the “bend and zipper” model of head formation (Posnien and Bucher, 2010; Posnien et al., 2010). In flies, in contrast, the head anlagen are located at an *anterior dorsal* position (Posnien et al., 2010). When considering the different morphogenetic movements of the head anlagen, the later aspects of expression accord quite well between these insects. Actually, when dissecting the fly germband from the egg and flattening it like Urbach et al. has been doing (e.g. Technau et al., 2006; Urbach and Technau, 2003b), the two species’s early head anlagen look quite similar.

In summary, only the situation at early embryonic stages strongly diverges such that early patterning events are likely to be under the control of different signaling cues (e.g. the dorsal versus ventral signals) and it is likely that the early head patterning system of Drosophila was modified to this new environment. For example, mutating dpp as the key dorsal morphogen leads to an enlargement of the head anlagen in beetles (both morphologically and in terms of otd expression) (van der Zee et al., 2006) similar to the effect in vertebrates (Reversade et al., 2005). However, at least on the level of otd expression no effect was not found in fly dpp mutants (van der Zee et al., 2006).

Hence, we assign the differences of the early eye field specification observed between flies and beetles to the different morphological settings at early stages and we have argued previously that the beetle situation is probably more typical for insects (Posnien et al., 2010). Importantly, the Drosophila situation resembles the beetle situation at later stages of embryogenesis and the conclusions drawn from marker gene expression in beetles is mostly reflected by fly data as well (e.g. compare the data shown in Urbach 2007 with our data).

Notes on expression patterns shown in Fig. 1

The expression patterns for the vertebrate genes were extracted from late gastrulation and early neurulation stages of different vertebrates: Embryonic day 7-8.5 (mouse), 8-12 hours post fertilization (zebrafish), stage 11-14 (Xenopus laevis) and HH4-7.5 (chick) (see Table S1 in Posnien 2011 for references). They were plotted onto the early 4-6 somite stage mouse neural plate (Rubenstein, 1997).

The Tribolium fgf8 and Dll expression is based on Beermann and Schröder, 2008 and Beerman et al. 2001, respectively. Engrailed expression is based on Brown et al., 1994. Pictures of stained embryos of the respective stages were manually transformed to fit a standard embryo using Tc-wg co-expression and morphology of the head lobes as criteria (Adobe Illustrator CS4 software). The outline of the expression domains were redrawn (Adobe Illustrator CS4 software). This allowed mapping the relative position of all combinations of expression patterns in “virtual double stainings” but the exact patterns of co-expression remain to be elucidated by direct double-stainings.

Notes on expression patterns shown in Fig. 2

FGF-8 is expressed in the antennal segment of Tribolium but not Drosophila (Beermann and Schroder, 2008; Posnien et al., 2011b; Urbach, 2007). Its posterior boundary coincides with the one from wingless (Posnien et al., 2011b) while the anterior boundary is less well mapped but might be at some distance to the ocular hh-stripe. Other aspects of FGF-8 expression are omitted. Otd has a very narrow overlap with ems expression (not shown) (Schinko et al., 2008), while the anterior boundary of ems is exactly abutting the ocular wg stripe (unpublished data). Therefore, we show otd expression extending a bit into the hh-domain but not into the antennal segment. Note that in flies, otd seems to mark parts of the deutocerebrum/antennal segment (Hirth et al., 2003; Urbach, 2007). No ungp/gbx data was available for Tribolium but based on data from flies and other animals it is likely to abut the otd expression (not depicted in Fig. 2). Labial/Hox1 is the anterior-most expressed Hox gene in insects and many other animals but in the vertebrate CNS, it is expressed subterminally in rhombomere 4 (taken from Urbach 2007). Hence, the anterior boundary of HOX marked tissue is defined by non-orthologous Hox genes. Engrailed forms a stripe at the vertebrate MHB but in all insect segment boundaries except for the ocular/antennal boundary. Here, engrailed expression is reduced to a domain called the “head spot”, which we assume to be a deviation from the ancestral stripe like pattern. In line with this, hh is expressed in a stripe like pattern at the ocular/antennal parasegment boundary and the respective engrailed domain is indeed a stripe in spiders and a crustacean (Damen, 2002; Patel et al., 1989). The btd domains anterior to the antennal segment emerge later in development and do not have the stripe-like appearance of the posterior segments (Schinko et al., 2008). One pre-antennal domain seems to be located in the six3 positive tissue and one behind it. However, their positions have not been mapped precisely.

Notes on expression patterns and fate maps shown in Fig. 3

The mouse fate map and expression of otx, emx and pax6 is based on (Inoue et al., 2000; Shimamura et al., 1995). Six3 expression was added based on the description in (Oliver et al., 1995) considering co-expression with both otx and pax6 and its expression in optic vesicle anlagen. The anlagen of the olfactory bulb was added based on a chick fatemap shown in (Cobos et al., 2001) and considering that six3 expression in olfactory bulb anlagen was not stated in mouse (Oliver et al., 1995) and seems to be median to those anlagen in chick as well (Puelles et al., 2005). The expression of the panplacodal marker six4 and the location of the placodes is based on *Xenopus* data summarized in (Schlosser, 2014). The expression patterns of the insect fate-maps are based on Figure 1 and data from (Posnien et al., 2011c). The location of the larval/compound eye anlagen betweenm the two lateral wingless domains is based on (Luan et al., 2014; Yang et al., 2009a). The ocelli anlagen are placed within the six4/eya marked tissue, because they have been mapped to a respective eya expression in grasshopper. Further, we tentatively placed the anterior ocelli within six3 and otd positive regions while the posterior ocelli were postulated to be six3 negative and maybe rx positive.

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