Differences in size and number of embryonic type-II neuroblast lineages are associated with divergent timing of central complex development between beetle and fly

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Abstract

Background: Despite its conserved basic structure, the morphology of the insect brain and the timing of its development underwent evolutionary adaptions. However, little is known on the developmental processes that create this diversity. The central complex is a brain centre required for multimodal information processing and an excellent model to understand neural development and divergence. It is produced in large parts by type-II neuroblasts, which produce intermediate progenitors, another type of cycling precursor, to increase their neural progeny. These neural stem cells are believed to be conserved among insects, but their molecular characteristics and their role in brain development in other insect neurogenetics models, such as the beetle *Tribolium castaneum* have so far not been studied.

Results: Using CRISPR-Cas9 we created a fluorescent enhancer trap marking expression of *Tribolium fez/earmuff*, a key marker for type-II neuroblast derived intermediate progenitors. Using combinatorial labelling of further markers including *Tc-pointed*, *Tc-deadpan*, *Tc-asense* and *Tc-prospero* we characterized the type-II neuroblast lineages present in the *Tribolium* embryo and their sub-cell-types. Intriguingly, we found 9 type-II neuroblast lineages in the *Tribolium* embryo while *Drosophila* produces only 8 per brain hemisphere. In addition, these lineages are significantly larger at the embryonic stage than they are in *Drosophila* and contain more intermediate progenitors, enabling the relative earlier development of the central complex. Finally, we mapped these lineages to the domains of early expressed head pattering genes. Notably, *Tc-otd* is absent from all type-II neuroblasts

and intermediate progenitors, whereas *Tc-six3* marks an anterior subset of the type-II-lineages. The placodal marker *Tc-six4* specifically marks the territory where anterior medial type-II neuroblasts differentiate.

Conclusions: Homologous type-II neuroblasts show a conserved molecular signature between fly and beetle. Enhanced activity of the embryonic beetle neuroblasts-type-II and intermediate progenitors is associated with an earlier central complex development when compared to the fly. Our findings on the differentiation of beetle type-II neuroblasts and on specific marker genes open the possibility to decipher the cellular and molecular mechanisms acting at the stem cell level that contribute to evolutionary divergence in developmental timing and neural morphology.

Introduction

Neural development of insects allows to study molecular and cellular principles in easy to manipulate invertebrate models. The fruit fly *Drosophila melanogaster* (*Drosophila* hereafter) has mostly been used for his purpose, making it one of the best understood models for neurogenesis [1,2]. However, it has remained a puzzle, how the huge ecological diversity of insects and the divergent neural anatomies adapted to different niches [3,4] evolved. Furthermore, *Drosophila* may in many instances not be a good representation of insect development and some processes are derived in the fly lineage [5–7]. For these reasons the beetle *Tribolium castaneum* (*Tribolium* hereafter) has been introduced as an additional model for insect neural development [8–11] and many molecular genetic manipulation and labelling methods have been established [12–16]. Specifically, labelling of neural cell types in *Tribolium* has been facilitated by the advent of CRISPR-Cas9 [12,15,17]. Another factor that makes *Tribolium* an informative model for the understanding of insect brain development is the more conserved development of the head neuroectoderm facilitating the identification of specific neurogenic domains and allowing cross-species comparisons [11,18,19].

The insect central complex is an anterior, midline spanning neuropile and constitutes an important brain centre for the processing of sensory input, coordination of movement and navigation [20,21]. Between the two insect models *Drosophila* and *Tribolium* a temporal shift in the emergence of central complex neuropile was observed with *Tribolium* developing a functional larval central complex during embryogenesis whereas it develops only at the onset of adult life in *Drosophila* [3,10]. It is however not known how these temporal differences are established during development on a cellular and molecular level.

The entire insect central nervous system including the brain is produced by neural stem cells, the neuroblasts (NBs) [22]. While there are evolutionary modifications in the relative position of trunk NBs and their gene expression profiles between fly and beetle, the core determinants specifying their role as neural progenitors are conserved [8]. NBs undergo repeated divisions producing rows of ganglion mother cells (GMCs) which divide one more time to produce neurons and/or glia (see Fig. 1, top panel). This leads to neural cell lineages that include the neuroblast itself and its progeny [23,24]. Several NBs of the anterior-most part of the neuroectoderm contribute to the CX and compared to the ventral ganglia

produced by the trunk segments, it is of distinctively greater complexity [25,26]. Intriguingly, a neuroblast subtype, type-II neuroblasts (type-II NBs), were found to prominently contribute to the formation of the central complex in *Drosophila* and the grasshopper *Schistocerca gregaria* [27,28]. These specific neuroblasts generate more offspring by producing another class of neural precursors, the intermediate progenitors (INPs), which also divide in a stem cell like fashion [29,30] (Fig. 1, lower panel). While type II NBs were found in both grasshopper and flies, only in *Drosophila* they have been characterized molecularly.

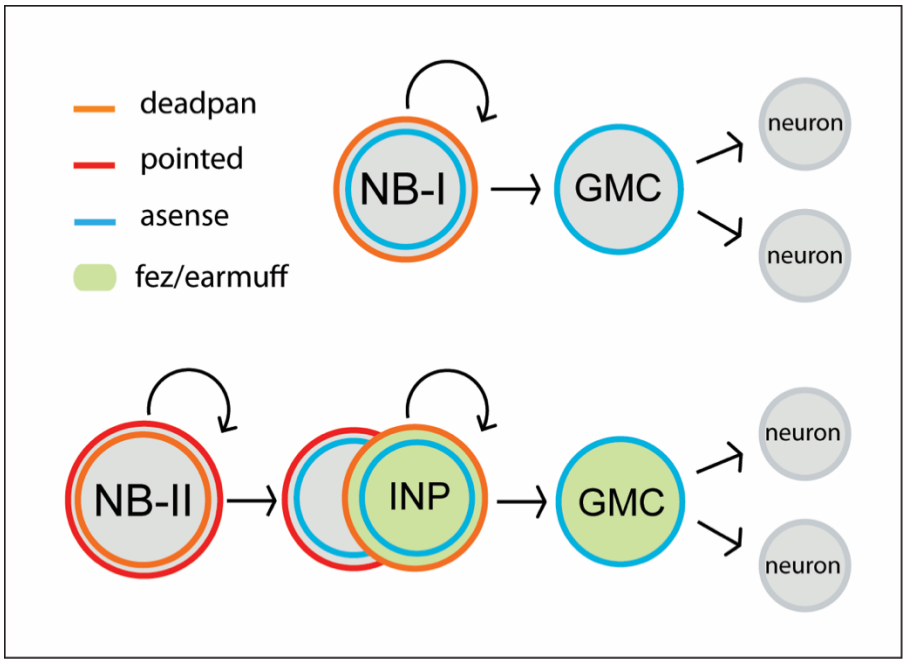

Figure 1) Neuroblasts of the *Drosophila* nervous system. Type-I neuroblasts (type-I NBs) (top panel) and type-II (type-II NBs) (lower panel). Type-I NB undergo stem-cell-like divisions with each round producing a ganglion mother cell (GMC) that divides one more time to produce two neurons or glia cells. In *Drosophila*, all type-I NBs express *dpn* and *ase* whereas GMCs express only *ase*. Type-II NBs express *dpn* and *pnt* but not *ase*. They also undergo repeated divisions, with each round producing an intermediate progenitor cell (INP) which also is a proliferating progenitor and expresses *ase*. INPs undergo a maturation process during which *pnt* is still expressed initially, but not *dpn*. Mature INPs express *dpn*, *ase* and *fez/erm* whereas GMCs express *ase* and *fez/erm*. Type-II NB lineages are only found in the anterior brain, they produce central complex neurons and glia [30,31].

The dramatically increased number of neural cells that are produced by individual type-II lineages, and the fact that one lineage can produce different types of neurons, leads to the generation of increased neural complexity within the anterior insect brain when compared to the ventral nerve cord [27,30]. Type-II NBs have attracted a lot of attention because intermediate cycling progenitors have also been described in vertebrates, the radial glia cells, which also produce a variety of cell types [32,33]. In addition, in *Drosophila* brain tumours have been induced from type-II NBs lineages [34], opening up the possibility of modelling tumorigenesis in an invertebrate brain, thus making these lineages one of the most intriguing stem cell model in invertebrates [35,36].

Based on descriptions of large proliferative lineages in the grasshopper *Schistocerca gregaria,* a hemimetabolous insect distantly related to flies, it is widely believed that type-II NBs and INPs are conserved within insects [25,37]. However, molecular characterisation of such lineages in another insect but the fly and a thorough comparison of type-II NBs lineages and their sub-cell-types between fly and beetle are still lacking.

The characterization of type-II NBs in *Drosophila* has in large parts been based on a specific reporter line in which eGFP expression is driven by an *earmuff (fez/erm)* enhancer element and marks INP-lineages [30,38,39]. *Tribolium earmuff* has been first described as *Tc-fez*, referring to the vertebrate *earmuff* ortholog *fez* [19]. We will use *Tc-fez/erm* in the following. A defined sequence of gene expression has been described in *Drosophila* type-II NBs, INPs and GMCs. The ETS-transcription factor *pointed* (*pnt*) marks type-II NBs [40,41], which do not express the type-I NB marker *asense* (*ase*) but the pro-neural gene deadpan (*dpn*) (Fig. 1). Intermediate progenitors express *fez/erm* and *ase* and at a more mature stage also *dpn*, whereas *prospero* (*pros*) marks mature INPs and GMCs [30,42]. *Six4* is another marker for *Drosophila* type-II NBs and INPs [43].

In the present work we characterize type-II neuroblasts in *Tribolium* with respect to their number, their location and the conservation of the molecular markers known from *Drosophila*. We also wanted to test, in how far the earlier emergence of the central complex in beetles would be reflected by a change of division activity of type-II NBs or intermediate progenitors. To that end, we created a *Tc-fez/erm* enhancer trap line to characterize embryonic *Tc-fez/erm* -expressing cells including INPs and GMCs of type-II NBs lineages and combined this with other labelling methods including multicolour hy*bridisation chain reaction* (HCR) [44,45]. We found that the *Tribolium* embryo produces 9 *Tc-pnt*-expressing

type-II NBs compared to only 8 type-II NB lineages in the *Drosophila* embryo [46]. We show that these lineages produce central complex cells and confirm a largely conserved molecular code that differentiates INPs of distinct maturation stages and GMCs. Intriguingly, we found that lineage sizes in *Tribolium* embryos are considerably larger and include more mature INPs than in the *Drosophila* embryo [46], thus providing the material for the embryonic early central complex formation in *Tribolium*. We also show that the placodal marker gene *Tc-six4* characterizes the embryonic tissue that gives rise to the larger anterior group of type-II NBs, whereas *Tc-six3* is marking only an anterior subset of these lineages. Interestingly there is a part of the central complex precursors expressing neither *Tc-six3* nor *Tc-otd,* which is absent from all type-II NBs lineages.

Results:

A CRISPR-Cas9-NHEJ generated enhancer trap lines marks *Tc-fez/erm*-expressing cells at the embryonic and larval stage.

We created an enhancer trap line driving eGFP that reflects *Tc-fez/erm* gene expression in the embryo. We named the line *fez magic mushrooms* (fez-mm-eGFP) because it marks the mushroom bodies of the larval brain (Fig. 2 A-C). The reporter construct was inserted 160 bp upstream of the *fez/erm*-transcription start site using the *non-homologous end joining* (NHEJ) repair mechanism (see supplementary Fig. S1 for scheme). Analysis of eGFP co-expression with *Tc-fez/erm*-RNA at the embryonic stage revealed that fez-mm-eGFP co-localized with most *Tc-fez/erm*-expressing cells (Fig. 2, panel D). The fez-mm-eGFP line marked several progenitor cell types including cells that we characterize as type-II NB-derived intermediate progenitors (see below). Based on these analyses, we use the fez-mm eGFP expression as a reporter for *Tc-fez/erm* expression.

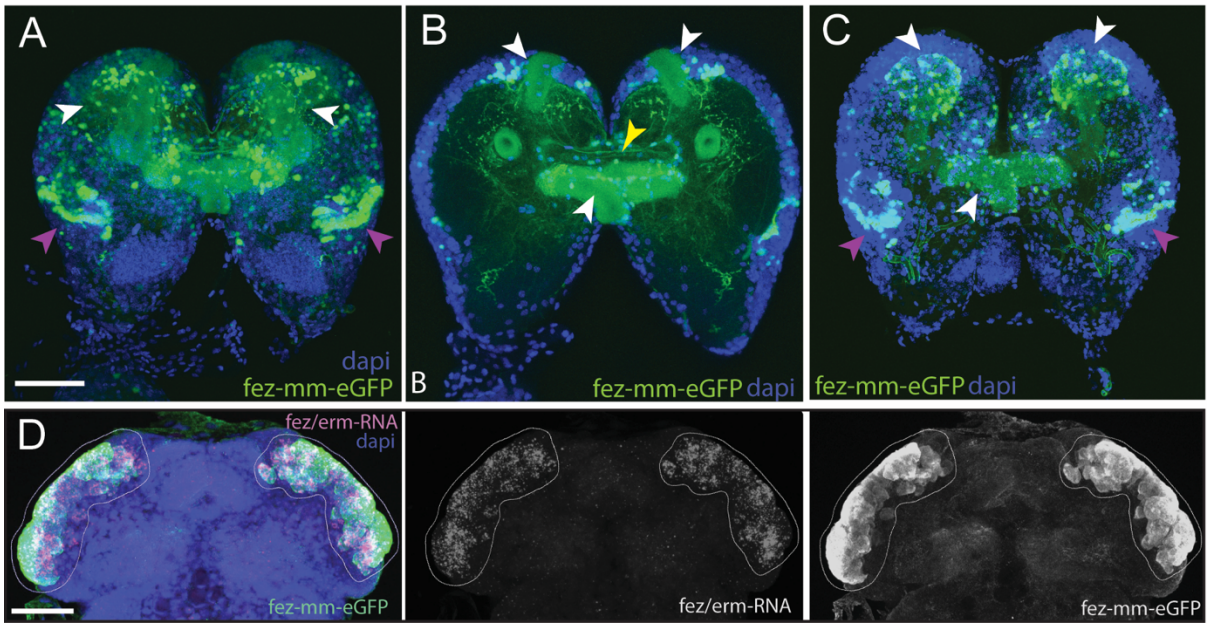

Figure 2) Magic mushrooms (fez-mm-eGFP) reporter line. A-C) eGFP-expression in larval instar brains, D) EGFP antibody labelling in combination with *Tc-fez/erm* RNA *in situ* in the embryonic head, A-D) Dapi labelling of nuclei. Scalebars in A and D are 50 µm. **A)** Full projection of whole brain scan (4th instar larvae): fez-mm-eGPF expression marks many cells of the mushroom bodies (white arrowheads) and of the larval optic lobe (purple arrowheads). Note that we use the terms dorsal and ventral with respect to the neuraxis of the larva. See [10] for orientation. **B)** Central part of the whole brain scan (projection of substack, same brain as in A): mm-eGFP marks the peduncles of the mushroom bodies (white arrowheads) and some central complex ensheathing cells (probably glia cells; yellow arrowhead). **C)** Projection of the dorsal part of same brain as in A shows calyces and peduncles of mushroom bodies (white arrowheads), and optic neuropile (purple arrowheads). **Panel D)** Extensive co-localization of fez-mm-eGFP with *Tc-fez/erm*-RNA in white encircled area. Projection of all planes where expression was detected, embryonic stage NS11.

Tc-pointed marks a population of nine type-II NBs that are associated with fez-mm-eGFP marked INP-lineages.

To identify type II NBs, we searched for cell groups that express the markers known from flies. The transcription factor *pointed* (*pnt*) marks type-II NBs in *Drosophila* and is required for their correct differentiation [40,41]. In both *Tribolium* and *Drosophila*, *pnt* is also expressed in other areas (this work/[47,48]). Therefore, we looked for *Tc-pnt*-expression in

large cells with large nuclei (typical NB morphology) that were closely associated with *Tc-fez/erm*-expressing cells (marking putative INPs). We found a total of 9 such clusters instead of the 8 expected from flies (Fig. 3 A-C). We also found that the large *Tc-pnt* expressing cells as well as fez-mm-eGFP expressing cells are mitotically active (Fig. 3 C-I). Further molecular and cell size analysis corroborated our interpretation that these cells constitute type-II NBs, intermediate progenitors (INPs) at different maturation stages and ganglion mother cells (GMCs) (see details below).

Looking at a series of embryonic stages we determined the emergence and further development of *Tc-pnt* and fez-mm-eGFP expressing lineages as well as their arrangement with respect to the embryonic head and to one another. We first detected conspicuous adjacent expression of *Tc-pnt* and fez-mm-eGFP at stage NS7 (not shown). At stage NS11 seven *Tc-pnt*-clusters are present in a horse-shoe-shaped formation surrounding fez-mm-eGFP positive cells (white arrows in Fig. 3 A, star marks one cluster out of focus). At stage NS13 these groups are found in a position at the medial border of the head lobes and their number has increased to nine. The seven clusters that were already present at stage NS11 are arranged in an anterior group (white arrowheads in Fig. 3B) at stage NS13 while two additional clusters have emerged more posteriorly (yellow arrows in Fig. 3B-I). The clusters are in different depths (reflected by the three optical sections shown in Fig. 3 B-I to B-III, clusters out of focus are visualized by grey circles in Fig. 3 B'I-II, respectively). Fez-mm-eGFP expression not related to type II NB offspring was found in the lateral head lobe from NS13 onwards (blue arrowheads in Fig. 3). At stage NS14 six of the anterior-medial clusters are aligned in one plane along the medial rim of the developing brain (Fig. 3 C-I), whereas the most posterior cluster of the anterior group is located at a separate position and in a deeper plane (Fig. 3C-II). The posterior group is found at about the same focal plane as well (Fig. 3 C-II). The aligned six clusters produce their *Tc-fez/erm*-positive offspring towards laterally whereas the *Tc-fez/erm*-positive offspring of the more posterior cluster is found medially of it (Fig. 3 C-II). The two most posterior clusters produce *Tc-fez/erm*-cells in an anterior-lateral direction (Fig. 3 C-II). The apparent re-arrangement of the *Tc-pnt*-clusters from the anterior rim of the embryonic head to a medial position reflects not active migration but follows the overall tissue movements during head morphogenesis that was previously described in *Tribolium* [19] (Fig. 3 D). Based on their position by the end of embryogenesis (stage NS14) we assign the nine type-II NB clusters into two groups: one anterior median group consisting

of seven type-II NBs and one posterior group consisting of two type-II NBs (see Fig. 3 C and D).

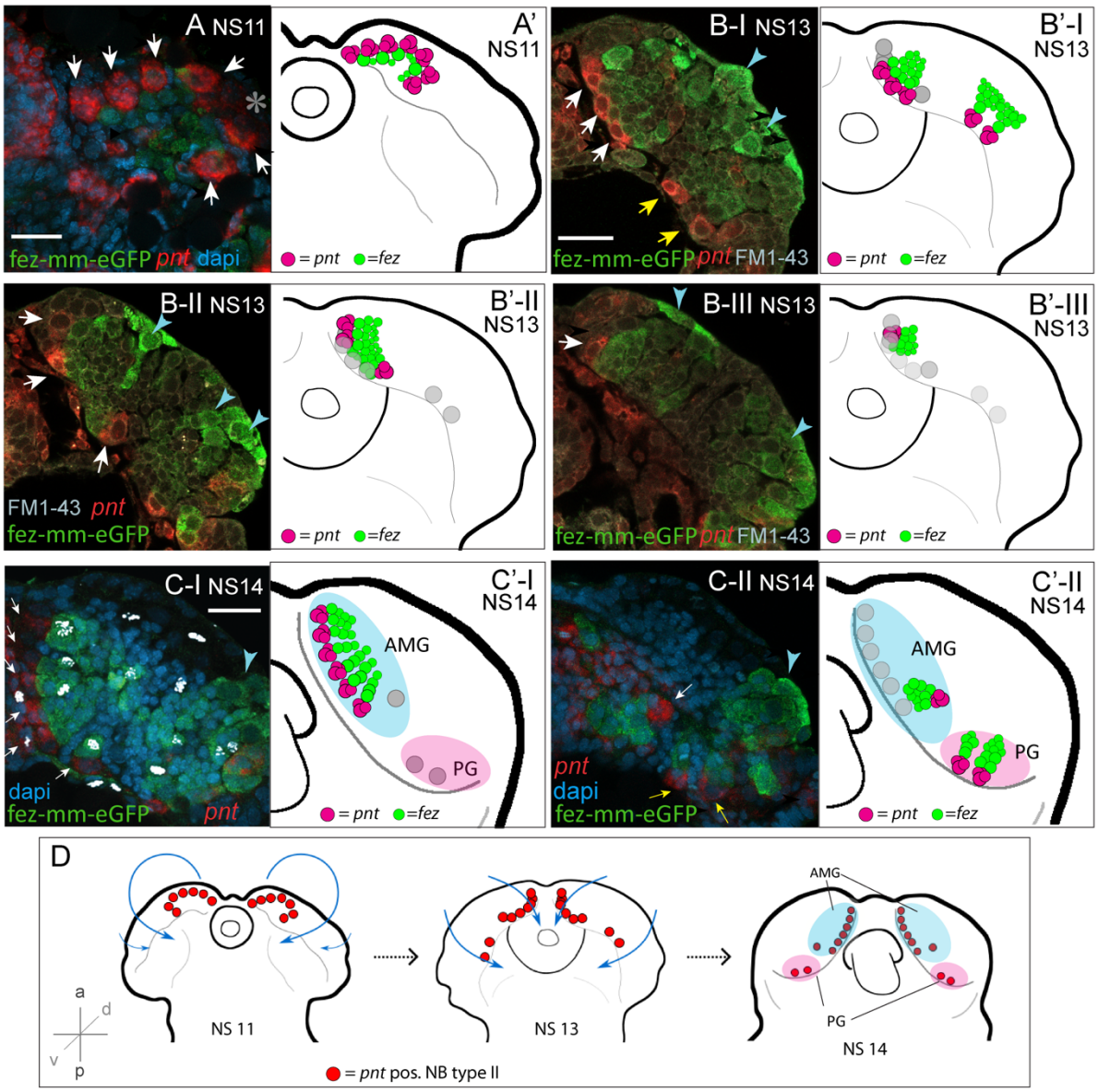

Figure 3) Developmental occurrence and progression of *Tc-pnt*-clusters and associated fez-mm-eGFP cells (type-II NB lineages). A-C) Anti-GFP antibody staining reflecting fez-mm-eGFP expression in combination with *Tc-pnt* RNA *in situ* hybridisation and dapi labelling of nuclei (A, C) or FM1-43 membrane staining (B), single planes from confocal z-stacks of right head lobes. Scalebars are 25 µm. A'-C') Schematic drawing of the head lobe at the respective stages; clusters out of focus depicted by grey circles. D) Schematic depiction of the localization of type-II NBs during *Tribolium* head morphogenesis. **A)** At stage NS11

seven *Tc-pnt*-clusters (white arrows) are found in an anterior-medial horseshoe-like arrangement with fez-mm-eGFP cells in the centre. Asterisk indicates the position of one *Tc-pnt*-cluster which is outside the focal plane of this image. **B)** At stage NS13 the seven anterior-medial clusters have been moved to the medial margin of the developing brain by morphogenetic movements of the head lobes (white arrows). The clusters are situated in different dorso-ventral levels shown in the different images B-I to B-III. Two additional posterior *Tc-pnt*-clusters have appeared (yellow arrows). Blue arrows point to fez-mm-eGFP positive cells that are not part of the type-II NB lineages. **B-I**: ventral level **B-II** mid-level, **B-III**: dorsal level. **C)** At stage NS14 a group of six clusters are arranged in one plane anterior-medially in the brain lobe (white arrows) (**C-I).** Anti-PH3 labelling marks mitotic cells within the lineages (white signal) and clusters out of the focal plane are indicated by grey circles in C'-I. **C-II** One cluster of the anterior median group (white arrow) is in a deeper plane, as well as the two posterior clusters (yellow arrows). Blue arrowheads point to lateral fez-mm-eGFP expression not associated with type-II NBs. A'-C') AMG =anterior medial group (blue), PG =posterior group (pink). Grey circles in B'-C' indicate position of type-II NBs in different focal planes. **D)** *Tc-pnt*-expressing cells identified as type-II NBs first appear bilaterally in the anterior most part of the embryonic head (stage NS11). During head morphogenesis this anterior tissue folds over such that the type-II NBs end up in a more medial position. Two more type-II NBs emerge posteriorly (stage 13). At the end of embryogenesis (stage NS14) the type-II NBs can be divided in an anterior medial group (AMG (blue); including type-II NBs 1-7, numbering starting from anterior) and a posterior group (PG (pink), including type-II NBs 8-9).

Characterization of cell- and nuclear size of *pnt*-positive type II NBs

We wanted to confirm that each of the *Tc-pnt* and *Tc-fez/erm* expressing lineages contains a neuroblast. Neuroblasts, including type-II NBs, are larger than other cells, have larger nuclei and are mitotically active [37]. Therefore, we determined the cell size of the largest cells of the *Tc-pnt* cluster and compared it to a random sample of cells of the embryonic head. Both samples were taken from stage NS13 and NS14 embryos. We found that the cells that we had assigned as Type-II NBs had a significantly larger diameter (av.: 12.98 µm; n=43) than cells of the reference sample (av.: 6.75 µm; n= 1579) (Fig. 4). We also found that the

diameters of nuclei of type-II NBs were significantly larger (av.: 8.73 µm; n=17) than the ones of a control sample (av.: 5.21 µm; n=1080) (Fig. 4).

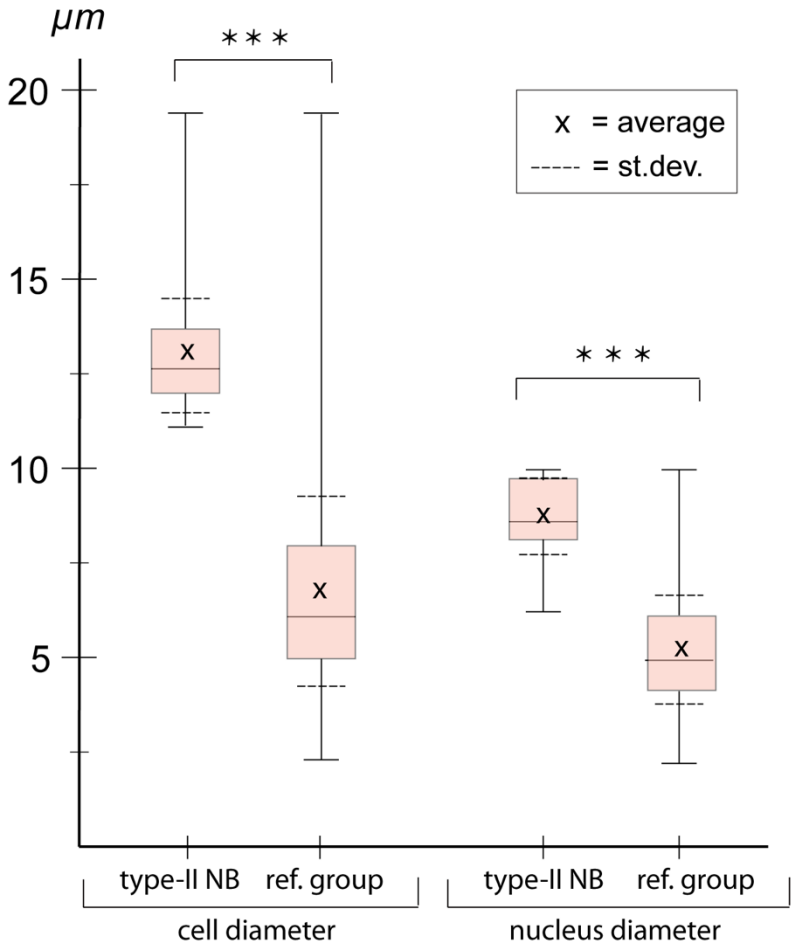

Figure 4) Cell- and nuclear diameter of type-II NBs compared to a control group. Stages NS13-14. Left: diameter of *Tc-pnt*-pos. type-II NBs (average diameter=12.98 µm; n=43) compared to control group (average diameter=6.75, n=1579). Right: average nuclear diameter of type-II NBs (=8,73 µm, n=17) compared to average nuclear diameter of control group (= 5.21, n=1080). Differences between groups are significant (t-test; *** ≙ $P < 0.001$).

Conserved patterns of gene expression mark *Tribolium* type-II NBs, different stages of INPs and GMCs

Drosophila, type-II NBs, INPs and GMCs express a specific sequence of pro-neural genes including *asense* (*ase*), *deadpan* (*dpn*) and *prospero* (*pros*) (Fig. 1) [30]. However, these markers have up to now not been tested for expression in type-II lineages in other organisms. We tested if the respective lineages express these markers in a comparable sequence in the beetle and if they mark distinctive cell types within the lineages (Fig. 5 A-D

and Fig. 6 A, B). We found that the large *Tc- pnt*-positive but fez-mm-eGFP negative cells (i.e. type-II NBs) express the pro-neural gene *Tc-dpn*, in line with expression of this gene in *Drosophila* neural precursors (Fig. 5, panel A). Like *Drosophila* type-II NBs, these cells do not express the type-I NB marker *ase* (yellow arrowhead in Fig. 5, panel D). We further found that, like the type-II NBs itself, the youngest *Tc-pnt*-positive but fez-mm-eGFP-negative INPs neither express *Tc-ase* (Fig. 5D, pink arrowheads). Expression of *Tc-ase* starts in slightly more mature (but still *Tc-pnt*-positive) INPs (Fig. 5 D; blue arrowhead) and is maintained in the *Tc-pnt*-negative INPs that are marked by fez-mm-eGFP (Fig. 5, panel D and E). *Tc-dpn* is absent from the youngest *Tc-pnt*-positive INPs (pink arrowheads) but is expressed in more mature INPs (blue arrowheads in Fig. 5, panel B). Cells that are located at the distal end of the lineages do not express *Tc-dpn* but are positive for fez-mm-eGFP and *Tc-ase* are classified as GMCs (Fig. 5, panels B, C and E). We found the pro-neural gene *Tc-pros* expressed in most fez-mm-eGFP expressing cells of a lineage (in Fig. 6, panel A-B) and infer from the extent of the expression that it is expressed in mature INPs (*Tc-dpn*+) and GMCs (*Tc-dpn*-). Fez-mm-eGFP positive cells at the base of the lineage that do not express *Tc-pros* are young INPs (Fig. 6 A, B; blue arrowheads). In summary, the expression dynamics found in the *Tribolium* type-II NBs lineages are very similar to the one found in *Drosophila.* Therefore these neural markers can be used for a classification of type II NBs (*Tc-pnt*+, *Tc-ase*-), young INPs (*Tc-pnt*+, *Tc-fez/erm*-, *Tc-ase*-), immature INPs (*Tc-pnt*+, *Tc-fez/erm*+, *Tc-ase*+), mature INPs (*Tc-dpn*+, *Tc-ase*+, *Tc-fez/erm*+, *Tc-pros*+), and GMCs (*Tc-ase*+, *Tc-fez/erm*+, *Tc-pros*+, *Tc-dpn*). This classification is summarized in Fig. 7 A-B.

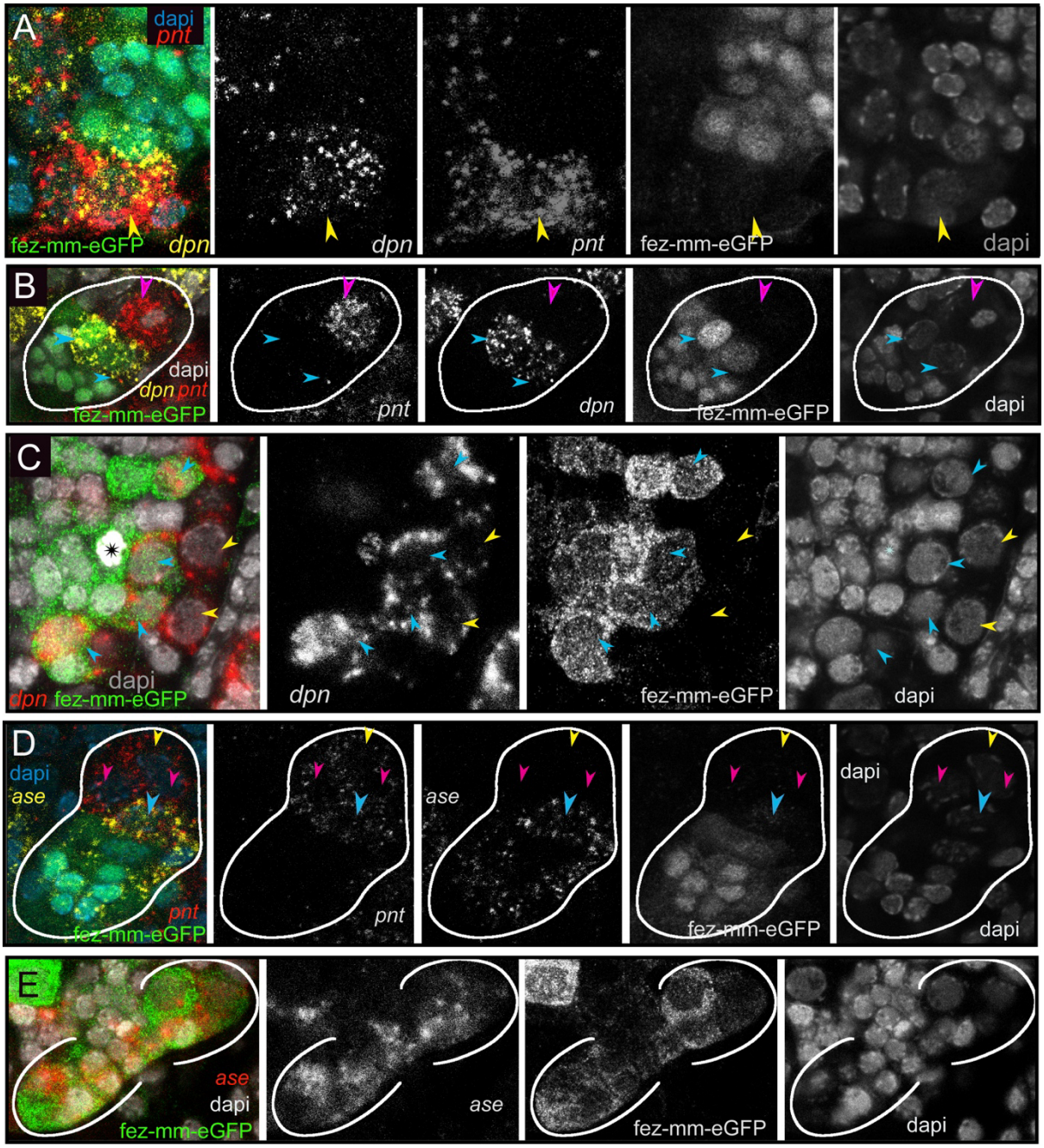

Figure 5: Differential expression of the neural markers *Tc-pnt, Tc-fez/erm, Tc-dpn* and *Tc-ase* in type-II NBs, INPs and GMCs. A, B, D) Anti-GFP staining visualizing fez-mm-eGFP expression in combination with HCR-labelling of further factors. C, E) eGFP antibody staining in combination with RNA *in situ* hybridisation. A-E) Dapi staining of nuclei. All panels are single planes from confocal z-stacks. **Panel A)** Stage NS12, expression of *Tc-pnt* and *Tc-dpn* in type-II NBs (yellow arrowhead) with adjacent fez-mm-eGFP positive INPs. **Panel B)** Stage NS13, white line marks one lineage. Expression of *Tc-dpn* is absent in the youngest INPs *Tc-pnt*+ (pink arrowhead) but present in more mature INPs (blue arrowheads), white line marks one lineage. Type-II NB not in focus of this image. **Panel C)** Stage NS14, expression of *Tc-dpn*

in type-II NBs (yellow arrowheads), and INPs (blue arrowheads). Asterisk marks mitosis in a fez-mm-eGFP cell visible through the condensation of chromatin stained by dapi. **Panel D)** Stage NS13, *Tc-ase*- and *Tc-pnt*-expression in type-II NBs and INPs, white line marks one lineage. Yellow arrowhead: type-II NBs (*Tc-pnt*+), pink arrowheads: immature INP (*Tc-pnt*+), blue arrowhead: young INP (*Tc-fez*+/ *Tc-ase*+/ *Tc-pnt*+). **Panel E)** Stage NS13, expression of *Tc-ase* in fez-mm-eGFP positive cells, location of type-II NBs and young INPs is outside the focus.

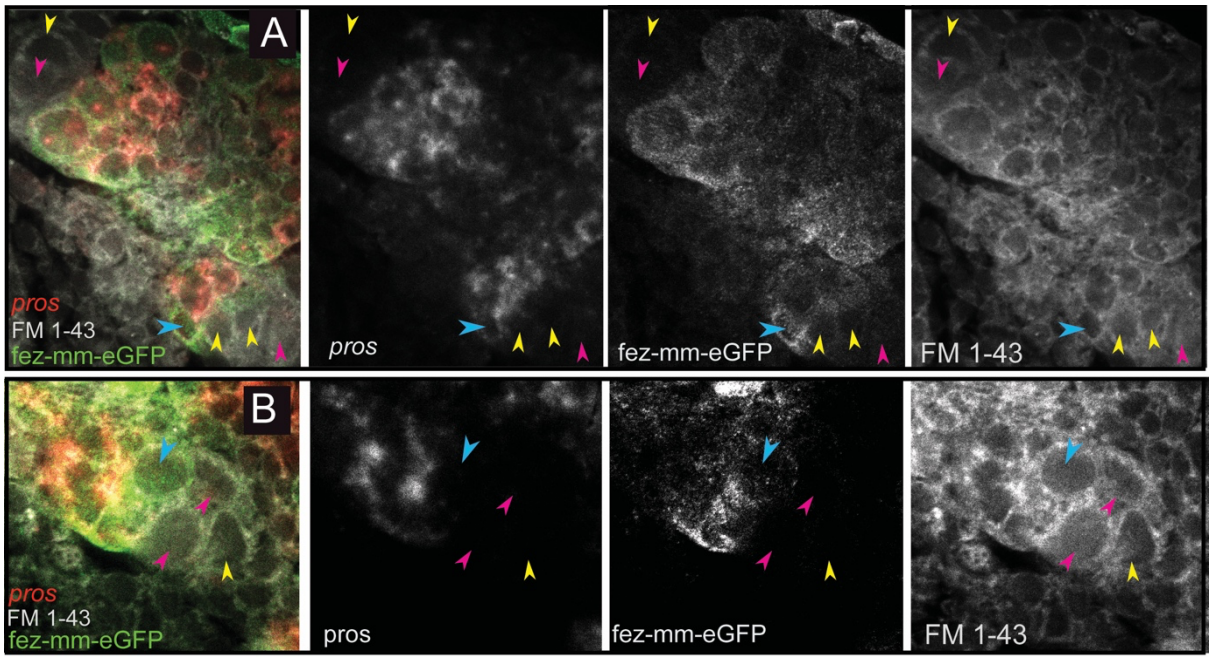

Figure 6) Expression of *Tc-pros* in fez-mm-eGFP cells. A-B) Anti-GFP antibody staining in combination with *Tc-pros* RNA *in situ* hybridization. Membranes stained with FM 1-43. Single planes from confocal z-stacks. **Panel A)** Stage NS13, two type-II NBs lineages producing INPs and GMCs in opposing directions. Expression of *Tc-pros* in mature INPs and GMCs. Cluster of type-II NBs (pink arrowheads) and immature INPs (yellow arrowheads). Young INPs express *Tc-fez/erm* but not *Tc-pros* (blue arrowheads). **Panel B)** Stage NS13, detail of *Tc-pros*-negative type-II NBs (pink arrowhead) and immature INPs (yellow arrowheads) and young INP (blue arrowhead). Note that distinction between type-II NB and immature INPs in **A)** and **B)** is only made based on position.

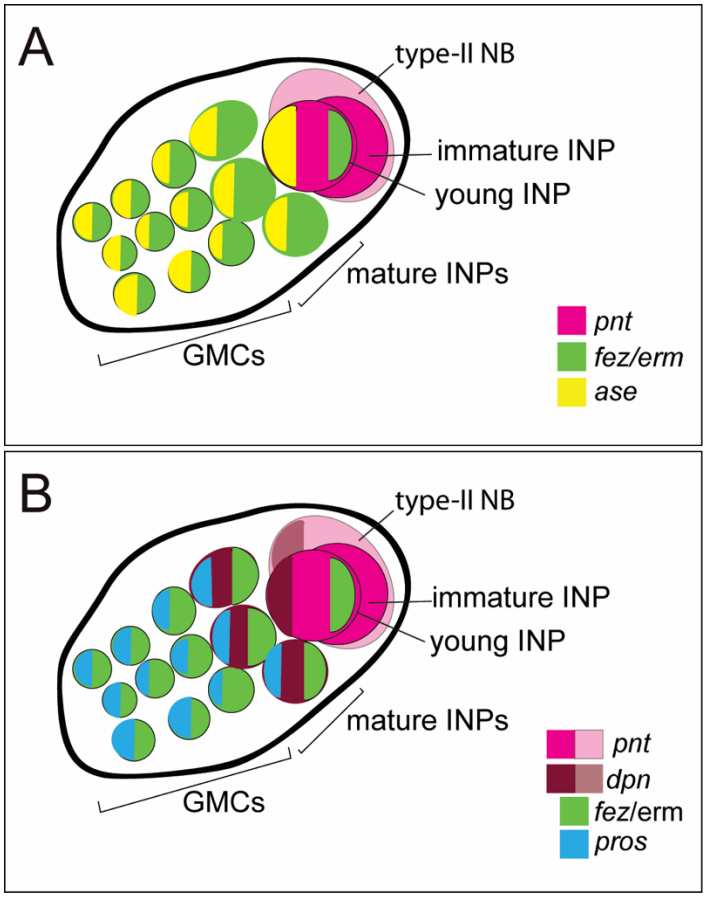

Figure 7) Schematic drawing of expression different markers in type-II NB lineages. A) Subclassification into type-II NBs (*Tc-pnt+*), immature INPs (*Tc-pnt+*), young INPs (*Tc-pnt+, Tc-fez/erm+, Tc-ase+*) and mature INPs and GMCs (*Tc-fez/erm+, Tc-ase+*) (based on staining shown in Fig. 5). **B)** Type-II NB, young and mature INPs express *Tc-dpn*, *Tc-pros* and *Tc-fez* whereas GMCs express only *Tc-fez* and *Tc-pros.* (based on staining shown in Fig. 5 and 6). Note that of the selected markers immature INPs express *Tc-pnt* only.

Type-II NBs lineages produce central complex cells that are marked by shaking hands (skh)

To test if the *Tribolium* embryonic type-II NB lineages contribute to the beetle central complex like in the fly, we used the shaking hands- (skh-) reporter line that marks central complex neurons and their postmitotic embryonic precursors [49]. By co-staining for *Tc-pnt* and *Tc-fez/erm* RNA in the background of the skh-line we found that the skh-positive cells are located directly distally of the type-II NBs lineages in a position where the type-II NBs derived neural cells are expected (Fig. 8 panels A-F). Most skh-cells have switched off *fez*-expression, but at the transition between *Tc-fez/erm* and skh cells we found some cells that express both markers (Fig. 8 D, E), supporting the view that many skh-positive central

complex cells stem from the *fez*-expressing GMCs of the type-II NBs lineages (Fig. 8 E). We found skh cells at the end of lineages of the anterior-median group (Fig. 8, panel A) and at the end the two posterior lineages (Fig. 8, B). We therefore conclude that both groups contribute to central complex neuropile (Fig. 8 F). We cannot say whether the lineage of type-II NB seven, which we assigned to the anterior-median group, but which is located with some distance to the other type-II NB of that group (Fig. 2 C, D, grey circle in Fig. 8 F), produces skh+ cells, but we assume that it does.

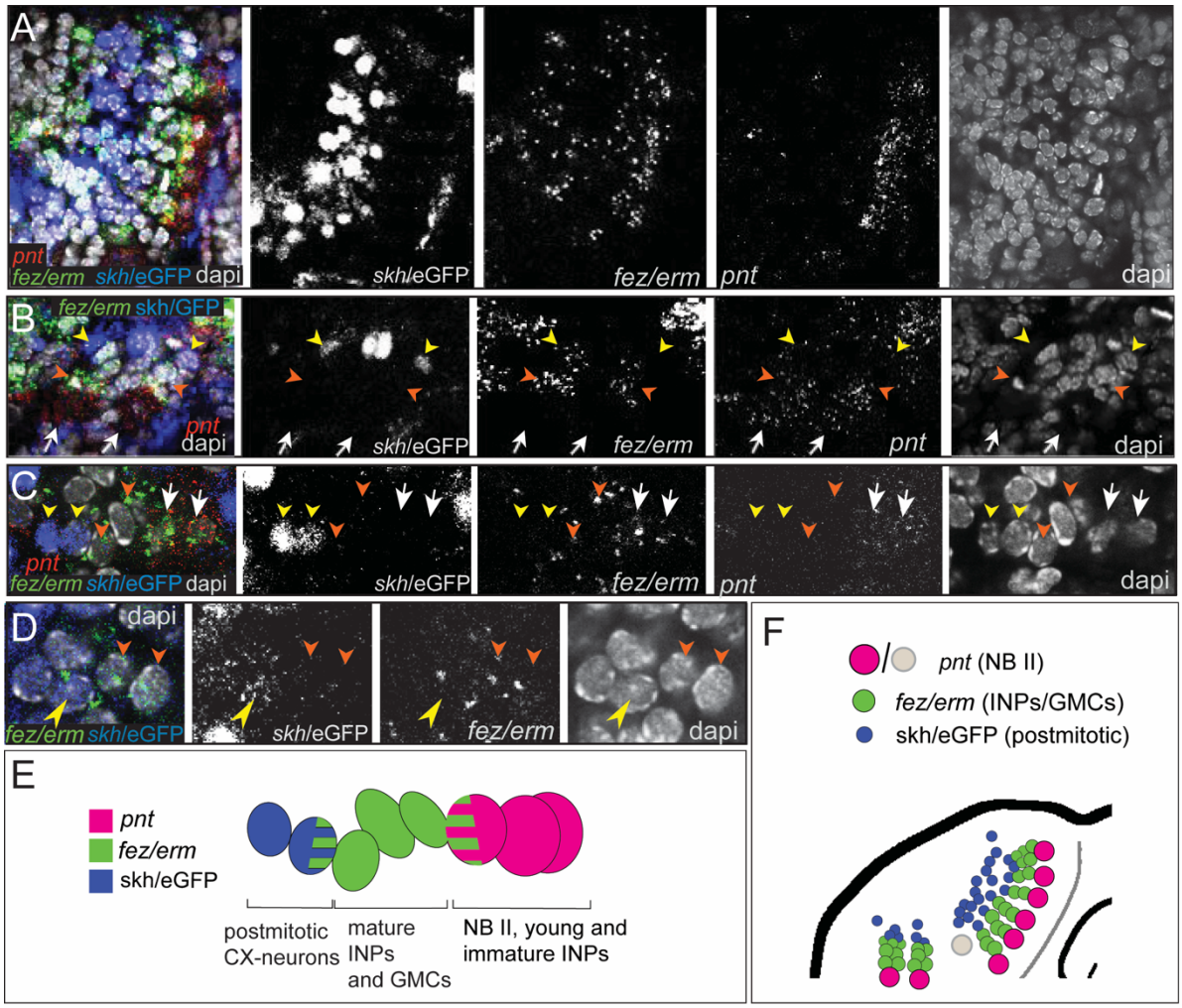

Figure 8) Expression of the transgenic central complex reporter skh/eGFP in relation to type-II NBs lineages. A-D) Antibody labelling of eGFP, which is expressed in the pattern of skh [49] in combination with HCR visualizing *Tc-fez/erm* and *Tc-pnt*, dapi labelling of nuclei, single planes of confocal z-stacks, all stage NS14. **A)** Left head lobe, anterior group of *Tc-pnt*-positive type-II NBs, *Tc-fez/erm*-expressing INPs/GMCs and skh/eGFP positive postmitotic cells. **B)** Left head lobe, posterior group of type-II NBs lineages. *Tc-pnt+* type-II NB or

young/immature INPs (white arrows), *Tc-fez/erm*+ INPs/GMCs (orange arrowheads) and skh/eGFPpostmitotic cells (yellow arrowheads). **C)** Detail of one lineage including type-II NB cluster (*Tc-pnt*+, white arrows), INPs/GMCs (*fez+,* orange arrowheads) and skh/eGFPcells at the end of the lineage (yellow arrowheads). **D)** Detail of transition from *Tc-fez/erm*+ cells (orange arrowheads) to skh/eGFPcells reveals a small area of co-expression of both factors (yellow arrowhead). **E)** Schematic drawing of type-II NBs lineages showing relative positions and markers of type-II NBs, INPs/GMCs and postmitotic central complex forming cells. **F)** Schematic overview of the arrangement of the type-II NBs lineages including skh/eGFP+ central complex forming cells in the head lobe of stage NS 14 (young/immature INPs not shown for simplicity). No data on skh-GFP expression in lineage of type-II NB 7 (marked in grey) is available.

The *Tribolium* embryonic lineages of type-II NBs are larger and contain more mature INPs than those of *Drosophila*

In beetles, a single-unit functional central complex develops during embryogenesis while in flies, the structure is postembryonic [3,21]. As type-II NBs contribute to central complex development, we asked, in how far the embryonic division pattern of these lineages would reflect this heterochronic development. To assess the size of the embryonic type-II NBs lineages in beetles we counted the *Tc- fez/erm* positive (fez-mm-eGFP) cells (INPs and GMCs) associated with a *Tc-pnt*-expressing type-II NBs of the anterior medial group (type-II NBs lineages 1-7). As we were not always able to distinguish between cells belonging to neighbouring INP-lineages we quantified and averaged the cell number of all lineages of the anterior medial group. We also evaluated the proportion of dividing cells in that group based on anti-PH3 staining (table 1).

Table 1: *Tc-fez/erm* positive cell number (NB type II, INPs and GMCs)

Embryo#/stage	*Total number of cells of 7 anterior lineages*	*% dividing cells (PH3 antibody)*	*Cells per lineage*
1/NS 13	146	8.2%	20.9
2/NS 13	111	6.3%	15.9
3/NS 14	139	5.0%	19.9
4/NS 14	96	10.5%	13.7

We found that at stage 13 each lineage consisted on average of 18,4 progenitor cells and at stage 14 of 16.8 progenitors. Between 5-10 % of the cells were marked by the PH3 antibody. As PH3 marks dividing cells for in a specific phase, the portion of dividing cells may be higher. We further quantified the number of young and mature *Tc-dpn*-positive INPs (table 2).

Table 2: Young and mature INPs (*Tc-fez* and *Tc-dpn* positive)

Embryo #/ stage	***Total number of young and mature INPs of 7 anterior lineages***	***% dividing cells (PH3 antibody)***	***Mature INPs per lineage***
1/NS 13	24	n.d.	3,4
2/NS 13	12	n.d.	1,7
3/NS 13	23	n.d.	3,3
4/NS 14	27	3,7%	3,9
5/NS 14	30	13,3%	4,3

We used the numbers from *Tribolium* (table 1, 2) for comparison with type-II NBs lineages from *Drosophila* embryos (data from [29]). We compared our data for the anterior lineages of stages 13 and 14 to the anterior and median cluster in the *Drosophila* stage 15 and 16 embryos. In both species these stages are the two penultimate stages of embryogenesis when all type-II NBs lineages are present. We found that the beetle lineages were significantly larger than the corresponding ones in *Drosophila* (Fig. 9; *Drosophila* data taken from [29]). Due to a lower quality of the image data from this cluster which is in a deeper layer (see Fig. 3) we were not able to quantify cells of the *Tribolium* posterior group. In *Drosophila,* the posterior lineages are larger (av. 10 cells [29]) than the anterior and median ones, but they are still smaller than the anterior lineages of *Tribolium* at the corresponding stages. We also found that the embryonic type-II NBs lineages in *Tribolium* comprised significantly more mature INPs (***Tc-****dpn*+/ ***Tc-****fez*+) than the corresponding lineages in *Drosophila* (Fig. 9). We conclude that the embryonic development of a functional central complex in beetles is associated with an increased activity of type-II NBs and INPs during embryogenesis.

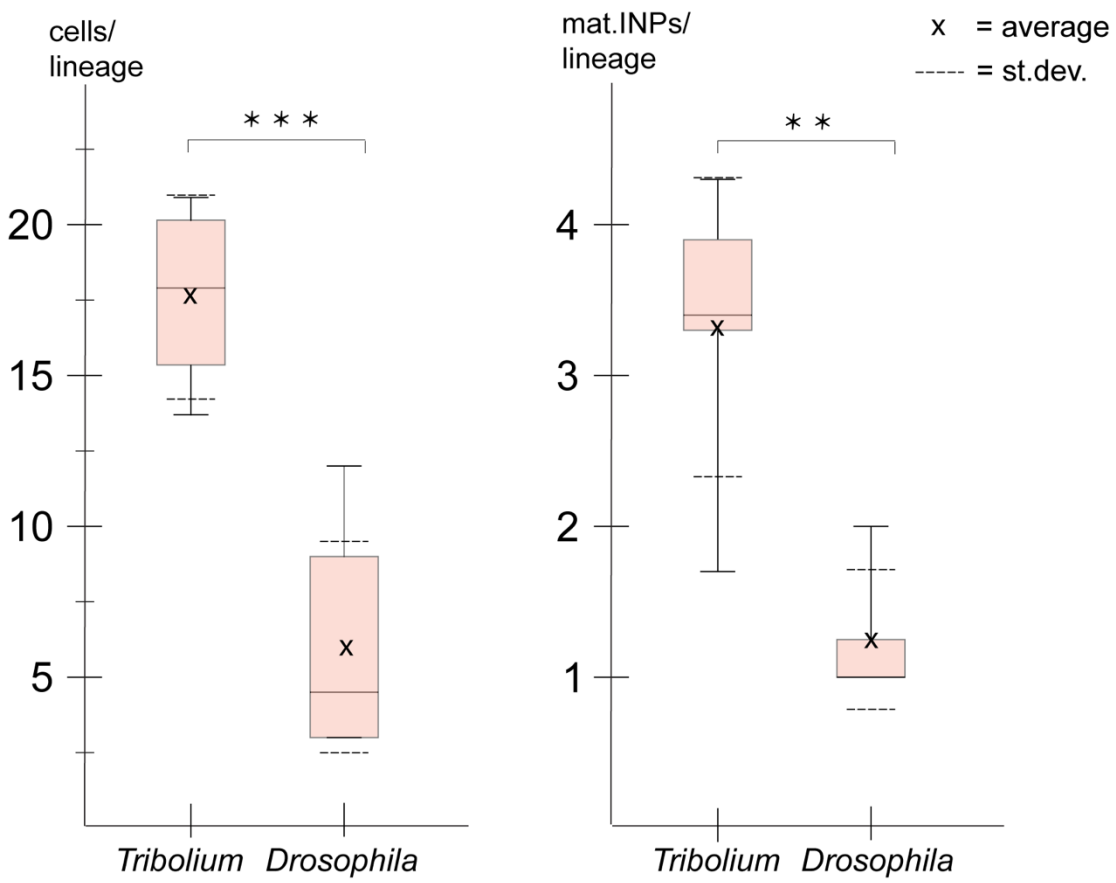

Figure 9) Size comparison of *Tribolium* and *Drosophila* type-II NBs lineages. Anterior and median lineages from *Tribolium* stages 13 and 14 (quantified in this work) compared to *Drosophila* stages 15 and 16 (pooled data from [29]). **A)** Lineage sizes comprising type-II NBs, INPs and GMCs (*Tribolium* n=4; *Drosophila* n=8). **B)** Number of *Tc-dpn*-positive mature INPs (*Tribolium* n=5; *Drosophila* n=8). T-test p-value significance level (t-test; *** ≙ P < 0.001, ** ≙ P < 0.01).

Type-II NBs and their lineages are differentially marked by the head patterning transcription factors *Tc-six4* and *Tc-six3* but do not express *Tc-otd*

All type II lineages show the same developmental trajectory from NB via the INP to the GMC and express the respective marker genes. However, these lineages show at least partially diverging projection patterns [3,17,49]. Hence, we wondered, what genes might give type II NBs different spatial identities. We tested co-expression with previously characterized head patterning transcription factors *Tc-otd*, *Tc-six4*, *Tc-six3*, known to be active in the developing protocerebrum (*Tc-otd*) and in the anterior medial region of the neuroectoderm, where

type-II NB emerge (*Tc-six3*, *Tc-six4*) [11,19,50,51]. We found no co-expression of fez-mm-eGFP marked cells of both the anterior medial and posterior lineages with *Tc-otd*. Rather, *Tc-otd* was expressed in the surrounding embryonic head tissue. *Tc-otd* was also absent from the type-II NBs themselves (Fig. 10 panel A-B). However, *Tc-otd* might be expressed in neural cells derived from these lineages as we can detect expression in the central area of the brain lobes where for instance skh cells are located (Fig. 9 panel A, F; 10 panel A-B). Interestingly, we found that *Tc-six4* is expressed specifically in that area of the head lobe in which the type-II NBs clusters 1-6 of the anterior medial group first differentiate. At stage NS13 *Tc-six4* marks both, type-II NBs and fez-mm-eGFP expressing INPs and some surrounding cells (Fig. 11, panel A). At the following differentiation stage (NS14) *Tc-six4* still marks the type-II NBs clusters of the anterior medial group but is not expressed in the fez-mm-eGFP INPs (Fig. 11 panel B). It is also not expressed in the posterior group of type-II NBs but only marks type-II NBs of the anterior medial group. Finally, we found that *Tc-six3* marks, and is restricted to, the anterior-most lineages 1-4 including both the type-II NBs and the fez-mm-eGFP marked INPs/GMCs (Fig. 11 panel C). These results reveal candidates for distinguishing type-II identity from type-I NBs in the brain. Only the latter is possibly marked by *Tc-otd* as *Drosophila otd* is expressed in type-I brain neuroblasts [52]. By contrast, *Tc-six4* only marks type-II NB and appears to be an early determinant of the anterior medial group. *Tc-six4* is also a candidate for distinguishing the anterior medial from the posterior cluster. The results also reveal a subdivision of the anterior cluster by *Tc-six3*, which marks the four most anterior NB-type II.

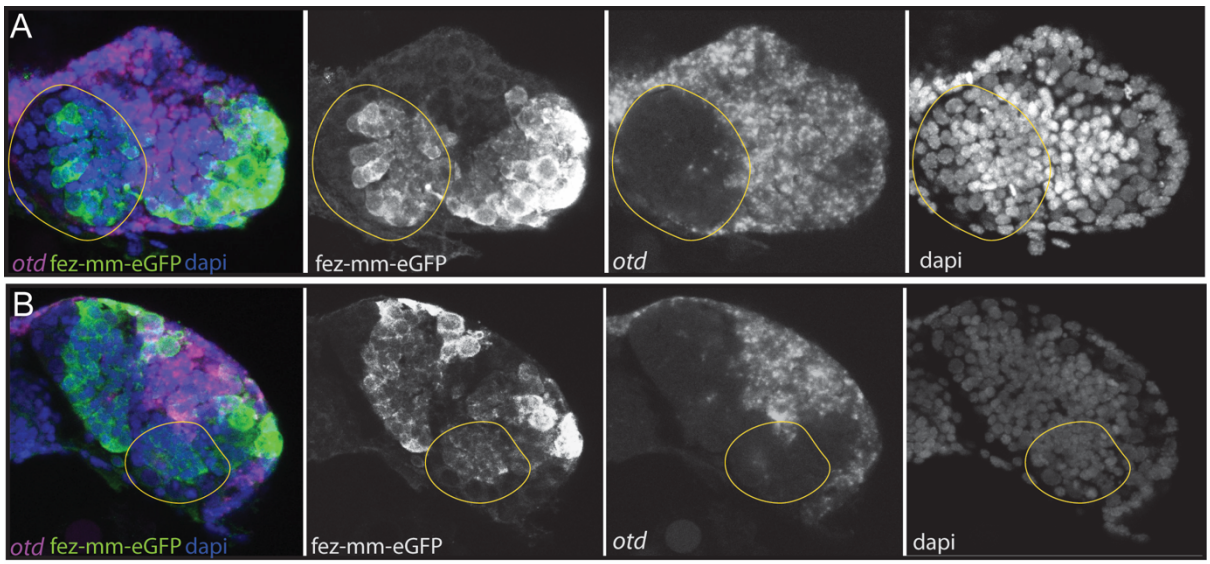

Figure 10) Expression of *Tc-otd* in relation to the fez-mm-eGFP lineages. A/B) RNA *in situ* hybridisation for *Tc-otd* in combination with eGFP antibody staining and dapi labelling of nuclei, A and B show projections of different levels of NS14 embryos, right head lobe. **A)** *Tc-otd* is neither expressed in type-II NBs nor the in the fez-mm-eGFP positive INPs of the anterior medial group (both positioned within encircled area) but it is expressed in the directly surrounding tissue. **B)** Likewise, *Tc-otd* is not expressed in type-II NBs or fez-mm-eGFP cells of the posterior group (encircled area).

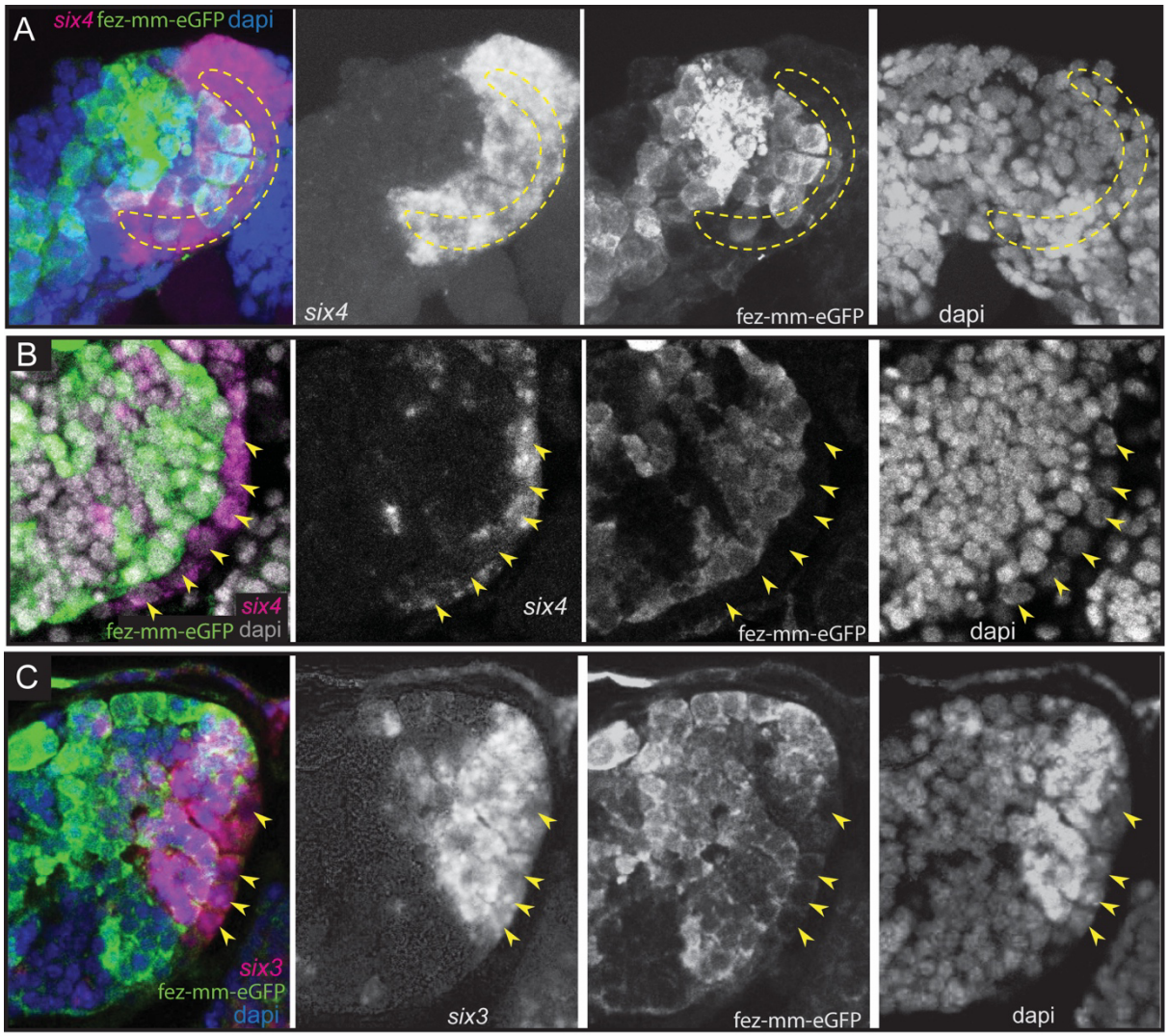

Figure 11) Expression of *Tc-six4* and *Tc-six3* in type-II NBs and INPs. A-C) RNA *in situ* hybridisations of *Tc-six4* and *Tc-six3* in combination with GFP antibody staining reflecting fez-mm-eGFP expression, dapi labelling of nuclei, left head lobes shown. **Panel A)** Stage NS13; ***Tc-six4*** is expressed in an area encompassing type-II NBs (positioned in encircled area) and mm-eGFP expressing INPs. **Panel B)** Stage NS14; *Tc-six4* is now restricted to type-II NBs

and their youngest progeny that do not express fez-mm-eGFP (yellow arrowheads). **Panel C)** Stage NS14; *Tc-six3* specifically marks the four anterior-most lineages. It is expressed in the type-II NBs at the base of the lineages (yellow arrowheads) and in the fez-mm-eGFP positive INPs. It is absent from the remaining lineages of the anterior medial group and is also not seen in the posterior group (not shown).

Discussion

A beetle enhancer trap lines reflects *Tc-fez/earmuff* expression

The discovery of the CRISPR-Cas9 system has brought the possibility of performing targeted genome editing to create mutants and knock-in lines to a wide range of less traditional model organisms [53]. The possibility of creating imaging lines that mark the expression of specific subsets of neural cells in the beetle greatly enhances its suitability as a model for developmental neurogenetics [11,15,17]. We used n*on-homologous end joining* (NHEJ) to insert a reporter construct upstream of the Tc-*fez/erm*-gene and by these means created an enhancer trap [12,15]. Enhancer traps sometimes only reflect parts of a gene expression pattern, which can also make them more cell type specific. However, our line shows extensive co-expression with *Tc-fez/erm* RNA at the embryonic stage, suggesting that it reflects the entire expression of *Tc-fez/erm* at least at this stage.

Evolutionary divergence of number and grouping of embryonic type-II NBs lineages between beetle, fly and grasshopper

We have identified a total of 9 type-II NBs lineages on each side of the *Tribolium* embryonic head. This is surprising as in both, the hemimetabolan grasshopper *Schistocerca gregaria* and the fruit fly *Drosophila melanogaster* only 8 embryonic type-II NBs were identified [29,54]. Our finding of an additional type-II NB lineage at the embryonic stage represents a major evolutionary divergence between these insects. Further research is required to determine if this difference is associated with morphological differences of the brain. Although in the frame of this work we did not perform lineage tracing we can speculate about which NB might be lacking in fly embryos based on our data. In the hemimetabolan *Schistocerca* there is no obvious clustering of type-II NBs and they are all arranged in one row at the medial rim of the head lobes. In *Tribolium* we have observed an arrangement of the type-II NBs into two groups per side, one large anterior medial group containing seven

type-II NBs clusters and one posterior group of two type-II NBs. The anterior six of the anterior medial group are arranged in one row like in the grasshopper. In *Drosophila* there are three described groups referred to as the anterior, the middle and the posterior cluster [29]. The posterior cluster consists of two type-II NBs and therefore most likely corresponds to the posterior two type-II NBs in *Tribolium*. The *Drosophila* middle and anterior cluster are most likely equivalent to the anterior medial group of *Tribolium.* However, the type-II NB 7, which is we assigned to the anterior medial group but which is a bit separated and produces offspring into the opposite direction (see Fig. 2 C-II, white arrow) might be the one that does not have a homologue in the fly embryo The identification of more specific spatial markers for type-II NBs (in addition to *Tc-six3* and *Tc-six4*) or lineage tracing tools are required to identify the neuroblast, which is not present in fly embryos. Subsequently, it would be especially interesting to see what the role of the additional type-II NBs in the beetle is and which neuropile it contributes to.

Summing up, there is a tendency towards grouping of type-II NBs into subsets in the holometabolan models that was not observed in the only hemimetabolan studied so far. A ninth type-II NBs was only found in the *Tribolium* embryo represents a striking developmentally divergent feature, but its role and the homologisation of individual type-II NBs between the different insects requires further studies.

Gene expression identifies homologous cell types and suggests conservation of gene function between fly and beetle type-II NB lineages

The genes defining the different stages of differentiation in type-II NB lineages have been identified and intensively studied in *Drosophila,* but it had remained unclear, in how far the respective patterns and mechanisms were conserved. Our results show a large degree of conservation of expression and probably also function – at least in holometabola. We identified type-II NBs by their larger than average size [37] and the expression of the signature marker *Tc-pnt* adjacent to a group of *Tc-fez/erm* expressing cells (INPs and GMCs). In *Drosophila* both these factors have key functions in defining the developmental potential of type-II NBs and INPs: Pnt suppresses Ase in type-II NBs and promotes the formation of INPs [41]. Loss of *pnt* expression leads to a de-differentiation of INPs as *fez/erm* is no longer repressed in young INPs [40]. Fez/Erm controls proliferation of INPs by activating Pros and prevents dedifferentiation of INPs into type-II NBs [39]. We show that also in *Tribolium* INPs

undergo a maturation process, during which expression of *Tc-pnt* stalls and expression of *Tc-fez/erm* is switched on. Despite both factors characterising different stages of INP maturation they are not completely mutual exclusive and we observed a small window of overlap in young INPs, highly suggestive of a conserved role of Pnt in the activation of *fez/erm* [40] (Fig. 12). *Drosophila dpn* is a neural marker expressed in type-II NBs and again in more mature INPs, leaving a gap of expression in immature INPs. *Drosophila ase* is repressed in type-II NBs but expressed together with *fez/erm* in INPs and GMCs where its expression overlaps with *pros* [55]. We found identical dynamics of gene expression in *Tribolium* (see Fig. 12) suggesting conservation of gene function within the lineages and a conserved process of INP maturation.

In conclusion, expression of the genes examined in this study in type-II NBs and their lineages is highly conserved in fly and beetle, which is implicit of conserved gene function and a conserved process of central complex formation.

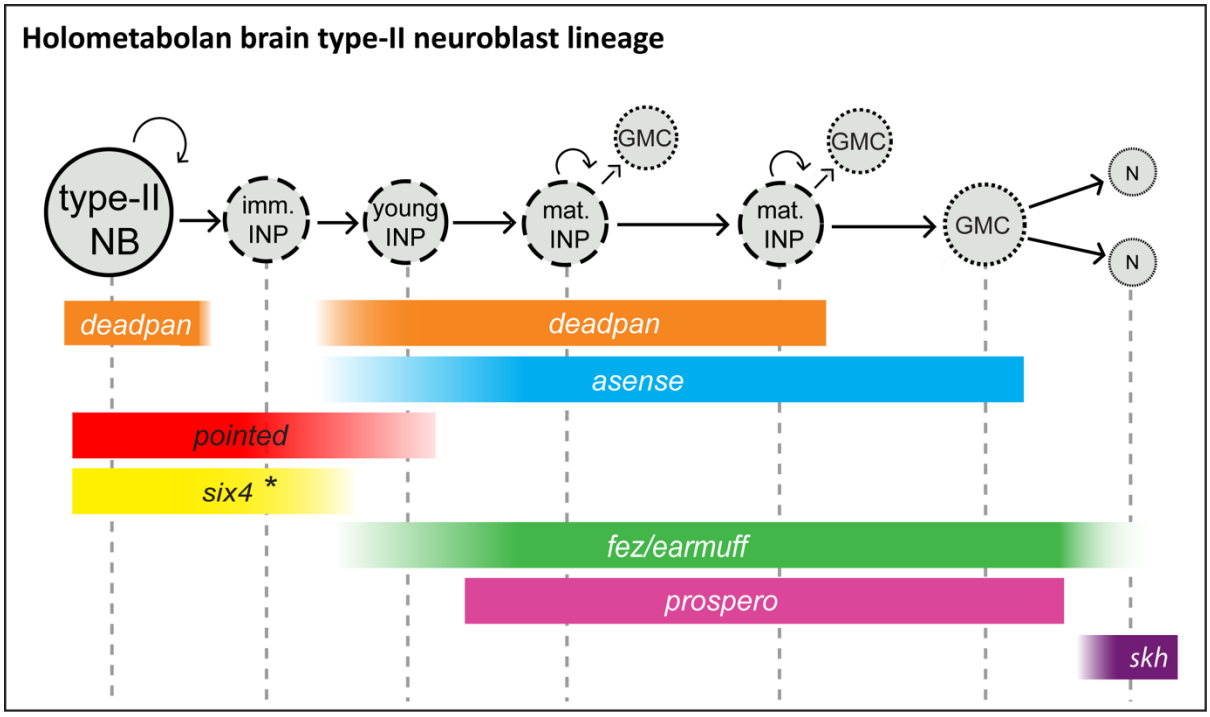

Figure 12) Conserved aspects of gene expression in type-II NB lineages of the holometabolan models *Drosophila* and *Tribolium*. Overview on the different cell types within one lineage (type-II NBs, INPs, GMCs and CX neurons), their mitotic activity, and summary of the expression of key factors in the respective cell types. INPs are divided into immature, young, and mature INPs with each subtype having a unique expression profile.

Note that each lineage can have several mature INPs. We strongly assume that glia are also produced from these lineages in *Tribolium*, as they are in *Drosophila* [30]. * In the *Tribolium* embryo *Tc-six4* is only expressed in the anterior medial lineages, and its expression is overlapping with *Tc-fez/erm* at an earlier stage (NS12) whereas it is restricted to type-II NBs and immature INPs, mutually exclusive with *Tc-fez/erm*, at a later embryonic stage (NS14). *Drosophila six4* was found in all 8 larval lineages [43]. The scheme is based on this work and [30,39,40,43,49]. NB= neuroblast; imm. INP = immature intermediate progenitor; mat. INP= mature intermediate progenitor; CX=central complex; N=neuron; Skh=shaking hands (enhancer trap) [49].

Divergent timing of type-II NB activity and heterochronic development of the central complex

Previous work described a heterochronic shift in central complex formation between fly and beetle. In *Tribolium* parts of the central complex develop during embryogenesis. It becomes functional at the onset of the first larval stage, and is presumably required for the more complex movements of the beetle larvae using legs [3]. In both *Drosophila* and *Tribolium* the anterior groups of type-II NBs (anterior medial group of *Tribolium* and anterior and middle cluster of *Drosophila*) are fully present in the last 3rd of embryogenesis, with the beginning of germ band retraction [8,29,56]. However, in the *Drosophila* embryo only a very limited number of INPs and GMCs are produced before they enter a resting stage prior to hatching [29]. By contrast, we show that *Tribolium* type-II NB lineages are much larger compared to *Drosopohila* [29]. They also include more mature, *Tc-dpn* expressing, cycling INPs which have the capability of driving the increase in lineage size. This increased activity very likely contributes to the embryonic development of a functional central body and protocerebral bridge [3]. The upstream factors responsible for the different timing of NB activity and the early formation of a functional central complex neuropile in the beetle remain to be identified.

A number of features remain to be studied in beetle type II lineages. We do not have any information whether *Tribolium* type-II NBs, or *Tribolium* neuroblasts in general, also enter a stage of quiescence at the end of embryogenesis as they do in *Drosophila*. After preformation in the embryo *Drosophila* type-II NBs have their main period of activity in the 3rd larva and the lineages are much larger and contain more INPs than the *Tribolium*

embryonic lineages [30]. It is another open question if *Tribolium* type-II NB lineages are active during larval development and how that relates to the described steps of central complex development. Adult like x, y, and w tracts as well as protocerebral bridge are present at the end of the embryonic stage but a bipartite central body containing both the ellipsoid and the fan shaped body is only differentiated in the pupa [3]. Therefore, it would be interesting to see if type-II NBs lineages are present and active in the late larval brain of *Tribolium*.

The placodal marker *Tc-six4* may be responsible for the differentiation and the spatial identity of the anterior medial group of type-II NBs

The different type-II NB lineages contribute to different parts of the brain. For instance, only the anterior four type-II NB lineages form the w, x, y, and z tracts of the central complex in flies and grasshoppers. Hence, there must be signals that make them different despite the conserved and well-studied sequence of neural gene expression and functional interactions in *Drosophila* (Fig. 12) [30,39,40,57]. In the ventral nerve cord, the different identities of the NBs are specified by the combination of spatial patterning genes expressed in the neuroectoderm at the time of delamination [24]. However, little is known about these signals for type II NBs. In *Tribolium* the domains of head patterning genes in the brain neuroectoderm are well characterized and some highly conserved territories like the anterior-medial *six3*-positive domain were described and suggested to give respective NBs different spatial identities [11,19,50]. The *Tc-six4* expression domain is also largely coincident with an embryonic structure termed insect head placode, a neurogenic, invaginating tissue [51,58] potentially homologous to the vertebrate adenohypophyseal placode marked by *six4* [11,51]. A placodal origin of *Drosophila* type-II NB has been shown for one type-II NB, and it is assumed that the other type-II NB also stem from placodal tissue [29,46,59].

Tc-six4 is also a very interesting factor with regards to the specification of type-II NBs because it is expressed only in the anterior group of type-II NBs. Hence, it could contribute to distinguishing their developmental fate from the posterior group. This is however different in the *Drosophila* larva. Here Six4 is expressed in all eight lineages where it prevents the formation of supernumerary type-II NBs and a premature differentiation of INPs [43]. In the *Tribolium* embryo Six4 may have a similar rate limiting role within the

anterior group type-II NBs. However, its early expression in a small part of the neuroectoderm [51] and the delamination of the anterior-medial type-II NBs group within this domain also hints at an instructive role of *Tc-six4* in the formation of the anterior median group.

Tc-six3 marks a subset of type-II NBs whereas _Tc-otd_ is absent from all lineages

Importantly, we found that in *Tribolium* late embryogenesis, *Tc-six3* is expressed specifically within the lineages of type-II NBs 1-4 of the anterior medial group, but not in the other linages. In grasshopper and fly these anterior 4 lineages (DM1-4) give rise to the z, y, x and w tracts [25,54] and contribute crucially to the development of protocerebral bridge and central body. These tracts form a major midline crossing neuropile of the central body which serves as a scaffold in the development of this structure [3,25]. We conclude that the evolutionary ancient *six3* territory gives rise to the neuropile of the z, y, x and w tracts. Indeed, the central body is also missing in weak *Tribolium Tc-six3*-RNAi phenotypes, suggesting that *Tribolium six3* is required for the formation of this structure [19]. Given that central complex neuropile is marked by additional factors such as *Tc*-foxQ2 (*Drosophila* fd102c [60]) and *Tc-rx*- [3,17], it would be interesting to see the relationship of additional spatial patterning genes with regards to the type-II NB lineages in future studies.

The gene *otd* is a marker of the posterior protocerebrum and *six3* and *otd* are believed to subdivide the embryonic anterior brain into two major domains [50]. This subdivision is also part of the recently revisited concept of an ancestral division of the insect protocerebrum into archicerebrum (*six3*-positive) and prosocerebrum (*otd*-positive) [11]. As discussed above, *Tc-six3* is expressed in the anteriormost four type-II NBs lineages, but unexpectedly, we found that *Tc-otd* is specifically absent from all type-II NBs lineages, suggesting that its inhibition is required for the development of all these lineages. Interestingly, the lineages of type-II NBs 5 and 6 (and presumably of type-II NB 7) of the anterior cluster and the posterior type-II NBs lineages do neither express *Tc-otd* nor *Tc-six3* showing that there are protocerebral structures expressing none of these conserved markers in development.

In summary, our findings are just the beginning of the quest for the genes required for the identity specification of type-II NB lineages. The genes known to be involved in head patterning are an excellent starting point for this purpose [11,18].

Methods

Creation of transgenic reporter line

Using CRISPR-Cas9 we have inserted a transgene into the *Tc-fez/erm* locus that includes *egfp* behind the *Tribolium* basal heat shock promoter (bhsp68), which is not active by itself but can be activated by nearby enhancer elements [61,62]. The transgene also contained the coding sequence of Cre-recombinase (not used in this work) transcribed from the same promoter, and the eye pigmentation gene *Tc-vermilion* behind the eye-specific 3xP3 enhancer element [63] (see Fig. S1 A for map of the transgene). On the repair plasmid the transgene was flanked by CRISPR target sequences derived from *Dm-yellow* and *Dm-ebony*, which were used for *in vivo* linearisation of the transgene.

Using CRISPR Optimal Target Finder [64] we designed three guide RNAs (gRNAs) targeting the genomic region 2.5 kb upstream of the *Tc-fez/erm* transcription start site (see table S1). We produced three plasmids where single gRNAs were transcribed from the *Tribolium* pU6b RNA promoter, as described in [12]. We co-injected the three gRNAs plasmids at 125µg/µl each, gRNAs targeting the *Dm-yellow* and *Dm-ebony* sequence at 125µg/µl each, Cas9 helper plasmid (see [12]) at 500 µg/µl and the repair plasmid at 500 µg/µl. Embryos of the white eyed *Tribolium vermilion-white* (*vw*) strain (G0) were injected, raised and then bred to *vw*-beetles in individual crosses. The offspring of these crosses (F1) were screened for black eyed heterozygous carriers of the transgene. These were again outcrossed to *vw*-beetles and the heterozygous offspring (F2) were further analysed genetically (see table S2) and with respect to fluorescent signal. Siblings of the F2 generation were then crossed to one another to generate the homozygous line (*fez*-mm-eGFP). Details of the insertion can be found in Fig. S1 A.

We also used the shaking hands (skh) enhancer trap line (G10011-GFP) which marks central complex cells [49].

Larval brain dissection, fixation, and staining

Larval brains of the newly generated *Tc-fez/erm* enhancer trap line *fez*-mm-eGFP were dissected and stained as described in [16]. Brains were mounted in *Vectashield* for microscopic inspection. See table S4 for a list of antibodies and staining reagent used.

Embryonic RNA *in situ* hybridisation, hybridisation chain reaction and antibody staining

Gene identifiers and primer sequences that were used for amplification can be found in table S3. The fragments were inserted into a pJet1.2 cloning vector. Standard RNA *in situ* probes were synthesised using the 5X Megascript T7 kit (Ambion) according to the manufacturers protocol. Embryo fixation and single colour RNA *in situ* chain reaction in combination with antibody staining to eGFP or phospo-histone H3 staining to mark mitoses was conducted as described in [65]. Embryos were also stained for DNA or cell membranes. A full list of antibodies and staining reagents can be found in table S4.

Probes for multicolour *hybridisation chain reaction (HCR)* binding *Tc-fez/erm*, *Tc-dpn*, *Tc-ase* and *Tc-pnt* were produced by Molecular Instruments (see table S5). Labelling reactions were performed as described in [66]. Antibody staining to eGFP was performed following the completion of the HCR staining (see [65]). All embryos were mounted in *Vectashield* for microscopic inspection (see table S4).

Image acquisition and analysis

Multichannel image stacks were recorded using a Zeiss LSM 980 confocal laser scanning microscope. Image stacks consisted of 100-300 slices, depending on the specimen. The resolution ranged from 1024 x 1024 to 2048 x 2048 pixels. Plane thickness was optimized according to width of the pinhole and ranged from 0.1 – 1 µm. We used FIJI [67] for 3 dimensional inspection, to export individual planes or to generate maximum intensity projections, as well as for quantification of cell size, cell number and number of mitoses. Statistics on the numerical data were performed using Microsoft Excel 2016. Cropping and adjustment of brightness and contrast was done with GIMP (version 2.10.32) or Adobe Photoshop CS5.

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