Shaking hands is a homeodomain transcription factor that controls axon outgrowth of central complex neurons in the insect model Tribolium

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Summary statement: We present the expression of Shaking hands, a novel homeodomain transcription factor, in the Tribolium brain. Shaking hands is required for the formation of the central complex primordium.

Abstract

Gene regulatory mechanisms which specify subtype identity of central complex (CX) neurons are the subject of intense investigation. The CX is a compartment within the brain common to all insect species and functions as a “command center” which directs motor actions. It is made up of several thousand neurons with more than 60 morphologically distinct identities. Accordingly, transcriptional programs must effect the specification of at least as many neuronal subtypes. We demonstrate a role for the transcription factor Shaking hands (Skh) in the specification of embryonic CX neurons in Tribolium. The developmental dynamics of Tc-skh expression are characteristic for terminal selectors of subtype identity. In the embryonic brain, Tc-skh expression is restricted to a subset of neurons, many of which survive to adulthood and contribute to the mature CX. Tc-skh expression is maintained throughout the lifetime in at least some CX neurons. Tc-skh knock-down results in axon outgrowth defects thus preventing the formation of an embryonic CX primordium. The as yet unstudied Drosophila skh shows a similar embryonic expression pattern suggesting that subtype specification of CX neurons may be conserved.
**Introduction**

The insect brain contains a large number of neurons with distinct identities. Cell identity is manifest in specific structural and functional features which together define a neuronal subtype. Subtype specification is already completed in the newborn neuron and during differentiation it effects axon pathfinding thus facilitating the formation of specific neural connections. Neuronal subtypes express distinct sets of differentiation genes which together bring about all the characteristic features of the cell. Transcription factors that regulate the expression of differentiation genes are the endpoint of hierarchical gene regulatory cascades that act earlier during development. (Hobert, 2008; Hobert, 2011; Allan and Thor, 2015; Hobert and Kratsios, 2019). The early regulatory cascades which govern neuronal subtype specification have been intensively investigated in the insect model *Drosophila melanogaster*, reviewed in (Skeath and Thor, 2003; Lin and Lee, 2012; Crews, 2019). All cells of the *Drosophila* brain derive from embryonically born stem cells, called neuroblasts (NBs). Each NB gives rise to a stereotyped and invariant lineage of neurons and glia. Each NB has a unique identity that is manifested in the expression of a unique combination of transcription factors (Urbach and Technau, 2003). NB identity is determined by overlapping spatial information in the procephalic neuroectoderm. Additional neuronal diversity is generated by a temporal cascade: each NB expresses distinct transcription factors in an invariant temporal series. Temporal factors are inherited by the NB progeny and establish neuronal cell fates characteristic for a given temporal window (Kohwi and Doe, 2013; Lin and Lee, 2012; Rossi et al., 2017; Doe, 2017). The expression of temporal transcription factors can be transient, thus making them unlikely regulators of differentiation genes which need to be expressed throughout the life of a neuron. In the *Drosophila* ventral nerve cord (VNC), spatial and temporal factors converge to activate the expression of transcription factors that function as terminal selectors of neuronal subtype identity: these factors regulate the lifelong expression of effector genes that together bring about all features of the differentiated cell type (Allan and Thor, 2015; Hobert and Kratsios, 2019).

The specification of the subtype identity of neurons whose trajectories build up the central complex (CX) is one topic of current interest (Boyan and Reichert, 2011; Sullivan et al., 2019; Hartenstein et al., 2020). The CX is a compartment in the center of the brain common to all insect species. It functions as a multi-modal information processing center which commands locomotor behaviors (Strauss and Heisenberg, 1993; Pfeiffer and Homberg, 2014;
Anatomically, the adult CX is an ensemble of interconnected paired and unpaired neuropils (Hanesch et al., 1989; Strausfeld, 1999). Core components are the protocerebral bridge (PB), the fan-shaped body (FB), the ellipsoid body (EB) and the noduli (NO) (schematic depiction of the CX: Fig. 1E). The PB is located at the dorsoposterior cell body-neuropil interface wedged between the two calyces of the mushroom bodies (MB). The PB consists of 16 glomeruli arranged in the shape of a handlebar. The FB is located anterior-ventrally and forms the largest neuropil of the CX. Within the FB, neuronal trajectories are organized to form substructures of horizontal strata and vertical subdivisions. Just anteriorly to the FB lies the EB, a ring-shaped neuropil that is structured into radial sectors and concentric zones. While the PB, the FB and the EB are midline spanning neuropils, the ventral most module, the NO, are paired. Two further, paired, modules are closely associated with the CX: the bulbs (BU) and the lateral accessory lobes (LAL).

Neurons whose projections make up the neuropils of the CX are classified as either small-field or large-field neurons (Hanesch et al., 1989; Young and Armstrong, 2010; Yu et al., 2013; Wolff et al., 2015). A well-studied group of small-field neurons are the columnar neurons. They form eight sets of isomorphic cells within each brain hemisphere whose somata reside within the pars intercerebralis (PI). The projections of a subgroup of columnar neurons form dendritic tufts giving rise to the 16 glomeruli of the PB. Further anterior-ventrally, columnar neurons project four prominent bilateral pairs of fiber bundles (w, x, y and z tracts). These tracts connect the PB to the FB by inter-hemispheric crossings before they extend further anterior-ventrally to establish the columnar structure of the FB. Large-field neurons provide input from other brain areas into the core of the CX. Some large-field neurons project perpendicular to the columnar neuron tracts and effect the horizontal stratification of the FB. For example, such a projection pattern is characteristic for a subset of neurons of the anterior optic tubercle (AOTU): they project first medially and then ventrally to innervate the uppermost stratum of the FB. Another well-studied group of large-field neurons are the ring neurons whose somata reside ventrolaterally to the CX and whose trajectories innervate the EB.

The architecture of the adult CX and its internal connectivity are well documented in several insect species (Loesel et al., 2002). By contrast, little is known about the regulatory mechanisms which specify subtypes of CX neurons. A few studies have addressed the roles of spatial and temporal factors in the specification of Drosophila CX neurons. For example,
ring neurons arise from within a spot of engrailed expressing procephalic neuroectoderm and loss of Engrailed results in the loss of embryonic ring neurons (Bridi et al., 2019). Recently, the temporal transcription factor Eyeless and its target Twin of Eyeless were shown to specify features of a subset of CX neurons (Sullivan et al., 2019).

While the overall architecture of the CX is well conserved across different insect species, the size and shape of its neuropils vary reflecting evolutionary adaptations (Loesel et al., 2002; Strausfeld, 1999; Immonen et al., 2017). We study the regulatory mechanisms that underlie CX development in the red flour beetle Tribolium castaneum (He et al., 2019; Farnworth et al., 2019). Tribolium is an insect model well suited to the study of gene regulatory pathways: its genome is fully sequenced (Herndon et al., 2020) and Tribolium is amenable to genetic manipulation, including enhancer trapping (Trauner et al., 2009). Additionally, “parental RNA interference” (RNAi) is well established as a means to study gene function (Bucher et al., 2002; Schmitt-Engel et al., 2015). General features of embryonic neurogenesis are remarkably well conserved between Tribolium and Drosophila (Wheeler, 2003; Biffar and Stollewerk, 2014).

Here we report a role for the transcription factor Tc-Skh, the Tribolium orthologue of C. elegans Unc-42, in the specification of a subset of CX neurons. The developmental dynamics of Tc-skh expression are characteristic for terminal selectors of neuronal subtype identity. Tc-skh is not expressed in neural progenitors or glia but is expressed in neurons. Many of these neurons survive to adulthood and a subset contributes to the adult CX. Expression of Tc-skh in CX neurons is maintained into adulthood. Notably, Tc-skh is absent from neurons which make up other major compartments of the brain. Tc-skh RNAi results in severe axon outgrowth defects thus preventing the formation of an embryonic CX primordium. In addition, we observe a moderate reduction of Tc-skh expression. The Drosophila orthologue Dm-skh shows a highly similar expression pattern in the embryo suggesting a conserved role in the specification of CX neurons.
Results

The enhancer trap line G10011 labels several neuropils of the *Tribolium* adult CX

To identify genes which play a role in CX development, we screened a collection of enhancer trap lines that express an untagged version of eGFP (Trauner et al., 2009). Analysis of GFP fluorescence in *Tribolium* adult brains led to the identification of the line G10011 in which the CX is heavily labeled (for a 3-D see Fig. S1). G10011 beetles are homozygous viable, fertile and their lifespan is comparable to that of the wild type strain *SB* (www.geku-base.uni-goettingen.de). We did not detect obvious differences in the fluorescence patterns of male and female, young and old adult brains (data not shown). To put G10011-GFP expression into context, we crossed G10011 to Ten-a-Δ-RFP expressing beetles and examined the adult brains of the resulting progeny. Ten-a-Δ-RFP is a derivative of the enhancer trap line Tenascin-a (also called Teneurin-a)-GFP (He et al., 2019). In Ten-a-Δ adult central brains, RFP expression is restricted to the MBs which provide a landmark (Fig. 1A-C). Double-labeled brains show GFP fluorescence in the PB (arrowhead in Fig. 1A), the FB and the EB (Fig. 1B; for a schematic depiction of CX neuropils and coordinates of the axes refer to panels E and G, respectively). G10011-GFP positive somata reside nearly exclusively in the posterior brain. The majority of GFP-positive cell bodies are located in the dorsomedial region where they form several large clusters within the PI and also more ventral areas. Small clusters of large cells are located in the dorsal most region of the PI (Fig. 1A,B). These cells project descending axons which form a chiasma in the dorsal brain and then extend further ventrally to enter the VNC. Based on cell body location and axonal projections, they are likely to be neurosecretory cells. In the dorsolateral brain large clusters of cells reside ventrolaterally to the Kenyon cells (KC) of the MBs (Fig. 1B, arrowhead). In addition, a small number of GFP-positive cells is scattered throughout the lateral regions of the posterior brain. The anterior cortex of the brain contains only a single GFP-positive cluster comprising 6-8 cells which is located ventrolaterally to the EB (arrowhead in Fig. 1C). Finally, a few very large cells within the tritocerebrum project towards the PI (arrow in Fig. 1C). We determined an average number of 320 GFP-positive cells per brain lobe (n=4; Fig. 1F). Notably, G10011-GFP is not expressed in the Kenyon cells and the antennal lobes. We do not know whether GFP expression in the optic lobes is attributable to the G10011 insertion since the transformation marker 3xP3 itself directs GFP expression in the optic lobes (Berghammer
et al., 1999). The VNC shows no GFP-positive somata but contains multiple GFP-positive longitudinal fascicles which originate in the brain (Fig. 1D).

To examine GFP-fluorescence in CX neuropils in more detail, G10011 adult brains were stained with α-Synapsin which facilitates the visualization of brain neuropils (Fig. 2A-D, E-H’). Image analysis at both low (Fig. 2C) and high magnification (Fig. 2G,G’) confirmed that the FB and the EB are strongly labelled by GFP. Within the FB, GFP-fluorescence is observed in all columns and strata. Within the EB, GFP labels all radial segments (Fig. 2G,G’). In addition, all 16 glomeruli of the PB are labelled by GFP (Fig. 2A,B, E,E’). In contrast to the strong GFP-label within the midline-spanning neuropils, GFP-fluorescence within the NO is very weak (Fig. 2F,F’). The CX modules are associated with additional neuropils such as the BU and the LALs. In Tribolium both of these compartments have not been described as yet. We observed a bilaterally symmetric brain area located anterior-ventrally to the FB/EB which we interpret as the LALs. G10011-GFP fluorescence within the putative LALs is weak (Fig. 2H,H’).

Strong GFP-fluorescence in the FB and the EB raises the question as to whether G10011-GFP labels all neuronal projections that make up these neuropils. To address this question, we crossed G10011 beetles to the imaging line 5’rx (retinal homeobox gene) in which the FB and the EB are intensely labelled by RFP (He et al., 2019). Image analysis of the respective progeny revealed that G10011-GFP and 5’rx-RFP fluorescence are largely non-overlapping, indicating G10011-GFP labels only a subset of structures within the FB and the EB (Fig. 2I-J’’).

Anatomical studies in a variety of insects have led to the characterization of small- and large-field neurons whose projections make up the neuropils of the CX (Hanesch et al., 1989; Young and Armstrong, 2010; Yu et al., 2013). Cell body location, morphology and projections of many CX neurons are conserved among different insect species (Pfeiffer and Homberg, 2014). Based on these criteria, we were able to identify one type of small- and two types of large-field neurons that express G10011-GFP. Firstly, sets of neurons whose cell bodies reside within the PI show all properties indicative of columnar neurons (Fig. 2K): their trajectories contribute to the dendritic tufts within the glomeruli of the PB and then extend more ventrally to form four characteristic fiber tracts named z, y, x and w tracts. These tracts connect the PB to the FB by interhemispheric chiasmata before they extend further ventrally to establish the columnar structure of the FB (Fig. 1K). Secondly, in the dorsolateral brain
two large clusters of neurons adjacent to the MB calyces project two major fiber bundles, one of which extends first medially and then ventrally before it enters the uppermost stratum of the FB (Fig. 2L). We interpret these CX neurons as a ventral subset of the AOTU neurons. Thirdly, we identified the ring neurons (R-N) whose somata reside ventrolaterally to the EB and whose projections innervate the EB (Fig. 2M).

**G10011-GFP positive neurons establish the FB primordium**

CX neurons of holometabolus insects are born during embryonic and larval stages while much of the CX connectivity is established in the pupa. We examined the appearance of G10011-GFP labelled cells and the establishment of early CX connectivity at embryonic, larval and pupal stages. Firstly, we addressed G10011-GFP expression during embryogenesis. We observe the embryo staging nomenclature as suggested by Stollewerk which distinguishes 15 stages of neurogenesis: NS1(0%) to NS15 (100% neurogenesis) (Biffar and Stollewerk, 2014) (Fig. S2). The earliest expression of G10011-GFP occurs at NS11 (65% of embryogenesis) in two small clusters of cells in anterior-medial positions of the brain (Fig. 3A). Subsequently, cell numbers within these clusters increase and additional clusters form adjacently in more lateral positions (NS12, Fig. 3B). In addition, clusters of GFP-positive cells appear in posterior-medial regions. Post-NS12, no significant increase of GFP-positive cells in the anterior-medial region takes place. By contrast, in posterior-medial and posterior-lateral regions multiple new GFP-positive cell clusters arise and early-born clusters gain in cell numbers (Fig. 3C–D’, E,F, G,H). The strongest increase in GFP-positive cells is observed during stage NS15. At the end of embryogenesis each brain lobe contains an average number of 362 GFP-positive cells (n=4; Fig. 2J), the vast majority of which reside in the medial area of the dorsoposterior brain. Embryonic expression of G10011 outside of the brain is restricted to the stomodeum and the hindgut and GFP-expressing cell bodies are absent from the VNC (Fig. 3K).

In the adult G10011 brain the columnar neurons of the FB are heavily labeled by GFP (Fig. 2K). We asked whether these cells are of embryonic origin and establish the FB primordium (prFB) of the embryonic brain. The prFB is formed by four contra-laterally projecting fiber tracts which emanate from each brain hemisphere and constitute a part of the early commissural system (Farnworth et al., 2020) (schematic illustration in Fig. 3E’’). These fiber tracts are produced by the progeny of four distinct neuronal neuroblasts (DM1-DM4) located
in the posterior-medial brain (Andrade et al., 2019; Farnworth et al., 2020). To visualize the
development of the commissural system, we co-stained G10011 embryos with $\alpha$-acetylated
Tubulin (acTub). At late stage NS11 the first acTub-positive fascicle extends towards the
midline. This fascicle is also labelled by G10011-GFP (Fig. 3A, inset). From NS12 onwards,
GFP-positive fiber tracts make numerous contributions to the commissural system (Fig. 3B-
D'', E-F). At stage NS14 GFP-positive fiber tracts form that are indicative of the prFB: four
contra-laterally projecting fiber tracts enter the commissural system as parallel tracts (Fig.
3E',F). At late stage NS15 these fibers show the characteristic pattern of defasciculation
which initiates the development of the columnar architecture of the FB (Fig. 3G’, schematic
illustration: G’’ and (Farnworth et al., 2020). In Drosophila it has been shown that the contra-
laterally projecting fibers which constitute the prFB pass through a channel formed by glial
membranes (Andrade et al., 2019). We observed a similar arrangement in the Tribolium
embryonic brain (Fig. S3A-A’’). Recently, we have shown that a subset of embryonically-
born columnar neurons express the Retinal homeobox protein (Rx) and establish the prFB
(Farnworth et al., 2020). Double-staining with anti-GFP and anti-Rx revealed a partial
overlap of Rx- and G10011-GFP expressing neurons (Fig. S3B-B’’’). Taken together, we
conclude that G10011-GFP labels embryonically-born columnar neurons which establish the
prFB.

In addition to columnar neurons, we identified subsets of AOTU- and ring neurons in the
G10011-GFP adult brain that contribute to the CX. Studies in Drosophila have shown that
some of these neurons are born in the embryo and persist to adulthood (Lovick et al., 2017;
Bridi et al., 2019). Due to lack of specific markers we are unable to identify these cells in the
Tribolium embryo. However, in the late NS15 brain we observed GFP-positive cells which –
based on morphology and location - we interpret as the same large cells observed in the adult
Tritocerebrum (arrowhead) and the putative neurosecretory cells of the prospective PI
(arrow) (Fig. S3C).

The adult VNC contains GFP-positive fascicles which originate in the brain (Fig. 1D; movie
S1). We observed that some fascicles arise during embryonic stages (Fig. 3 H,H’). For a more
detailed display of the major axon tracts in the late NS15 brain refer to Fig. S4.
G10011-GFP labels immature CX neuropils in the late *Tribolium* larva

During larval development, the brain strongly increases in size and undergoes major morphogenetic movements which together prevent the tracing of individual embryonically-born neurons to late larval stages. The number and distribution of GFP-positive cell bodies in the late larval brain (80-90% larval development) much resembles that of the adult brain (Fig. 4A-L). GFP-positive neurons reside nearly exclusively in the dorsoposterior brain. Most GFP-positive cells are located in the medial brain with exception of two large cell clusters which laterally abut the Kenyon cells (Fig. 4D, D’). As in the adult, G10011-GFP expression is absent from the Kenyon cells. Location, morphology and, in part, axon trajectories allow us to recognize sets of cells which we can also identify in the adult brain: notably, columnar neurons and a subset of AOTU neurons (Fig. 4D-F,D’,E’), putative neurosecretory neurons of the PI (I,J), a subset of ring neurons (J,K) and the large cells of the Tritocerebrum (K,L,L’).

In the late larva, the PB and the FB are clearly labeled by GFP. Fiber tracts emanating from the columnar neurons pass through individual glomeruli of the PB and then extend more ventrally to form tracts with multiple interhemispheric chiasmata before they extend further ventrally to build an immature FB within which a columnar structure is not yet obvious (Fig. 4E’). The overall shape of the FB already resembles that of the adult FB but GFP fluorescence shows no obvious dorsoventral stratification at this stage (Fig. 4G,H,H’). In the late larva, fibers project from the ring neurons to positions just ventral of the FB (arrows in Fig. 4H,H’). However, no structures characteristic of the pupal/adult EB are detectable as yet. In addition, the fibers of the ring neurons do not express Synapsin at this stage (data not shown). In the late larval brain each hemisphere contains on average 384 GFP-positive cells (n=4).

G10011-GFP labels the PB, FB and EB in the late *Tribolium* pupa.

In the late pupal brain, the number and distribution of GFP-positive cell bodies is essentially the same as in the embryonic, larval and the adult brain (Fig. 5A-C). The overall architecture of the pupal CX neuropils closely resembles that of their adult counterparts. The glomeruli of the PB are pronounced but fusion at the midline has not taken place as yet (Fig. 5B). Within the FB, the columnar structure is well established (Fig. 5A,B). In marked contrast to the late larval brain is the appearance of the EB with its radial segmentation (Fig. 5C).
G10011-GFP fluorescence reflects the RNA expression of the transcription factor TC-UNC-42

We mapped the plasmid insertion site of G10011 to the genomic position 6024777 within the first intron of TC008169 (for details see Fig. S5). However, the expression of this gene did not match G10011 GFP fluorescence. Another candidate gene in this region is TC007335 whose putative transcriptional start site is located 18.5kb upstream of the insertion site. To examine whether G10011-GFP reflects the expression of TC007335 in the embryo, we performed fluorescent double-in-situ hybridization with a GFP and a TC007335 RNA probe. The GFP and TC007335 signals colocalize at all embryonic stages, indicating that G10011-GFP faithfully reports TC007335 expression (Fig. 6A-B”, Fig. S5C-C”). Furthermore, TC007335 RNA in situ confirms that expression is restricted to the brain and stomodeum (Fig. S5C). We name TC007335 shaking hands (skh) to highlight the chiasma formed by cells of the PI (Fig. 1B). skh encodes the orthologue of the C.elegans transcription factor UNC-42, a PRD-like homeodomain protein (Baran et al., 1999) (for a phylogenetic tree refer to Fig. S6).

G10011-GFP fluorescence is strong in the adult brain. To investigate whether this reflects GFP perdurance or the continued expression of Tc-skh, we performed skh RNA in situ combined with α-GFP staining in whole-mount adult brains. Most, and possibly all, skh RNA positive cells are also GFP-positive, demonstrating the continued expression of skh transcript (Fig. 6C-C” and Fig. S5 bottom panel, A- E”). However, due to technical limitations, we cannot exclude that a small number of skh RNA positive cells do not express GFP.

Tc-skh expression is restricted to neurons

Embryonic and larval brains contain mitotically active and postmitotic cells. Mitotically active cells are NBs and their immediate progeny. To determine whether Tc-skh is expressed in mitotically active cells, we double-stained embryos and larval brains with α-GFP and the mitosis marker α-Phospho-histone-3 (PH-3). Co-localization of α-GFP and α-PH-3 signals were not observed indicating that Tc-skh expression is restricted to postmitotic cells in the analyzed stages (Fig. 7 A-A” and data not shown). This conclusion is supported by the observation that GFP-fluorescence is absent from the superficial, neuroblast, layer of the brain. In addition, we double-stained embryos and adult brains with α-GFP and the glial
marker α-Repo. α-GFP and α-Repo signals did not co-localize (Fig. 7B-C’’). We conclude that Tc-skh expression is restricted to neurons in embryonic and adult brains.

_Tc-skh_ knock-down results in axon outgrowth defects and a reduction of GFP-positive cells.

To explore the effects of reduced Tc-Skh function in the embryo, we performed parental RNAi in G10011 animals using two non-overlapping dsRNA fragments (“frag1” and “frag2”). Knock-down phenotypes were examined by double staining with α-GFP and α-acTubulin. Loss of Tc-Skh has drastic consequences for the outgrowth of all G10011-GFP positive axons: in severely affected embryos, no contra-laterally projecting axons enter the commissural system and hence, no prFB is formed (Fig. 8A-G’’). Axon outgrowth defects occur with high penetrance: RNAi with “frag1” and “frag2” results in severe defects in 71% and 48% of the embryos, respectively. Examination of GFP-fluorescent cells shows that some axon outgrowth still takes place but axons terminate prematurely close to the respective cell bodies (Fig. 8D). Axon outgrowth defects were observed in all GFP-expressing neurons including the progeny of DM1-DM4, which in wild type generate the prFB (Fig. 8G-G’’). Axon extension defects are restricted to GFP-positive trajectories: acTubulin positive but GFP negative axon trajectories form normally (Fig. 8C,D’’,G’’). These results suggest that the requirement of Tc-Skh for axon extension is cell-autonomous.

In addition to axonal defects, we observe a moderate reduction of GFP-positive cells in knock-down embryos (compare Fig. 8A with C; quantification in 8F). Due to the lack of specific markers for G10011-GFP positive cells, we are unable to determine whether loss of GFP is due to apoptosis or the transformation of cell identity. Loss of GFP expression in DM1-DM4 progeny occurs with low penetrance and hence, is unlikely to be the main cause for the loss of GFP-positive commissural fibers which constitute the prCX (of nine embryos with no GFP-positive commissural fibers only three showed loss of one or more GFP-positive DM1-DM4 clusters, Fig. 8G and data not shown).

_Skh_ RNAi animals did not develop to late larval stages thus preventing an analysis of postembryonic CX defects.
The embryonic expression patterns of Tribolium and Drosophila-skh are conserved.

The Drosophila orthologue of Tribolium skh is encoded by CG32532 (see Fig. S6). Its gene product is a homeodomain protein which has remained uncharacterized. We examined the embryonic expression pattern of Dm-skh by RNA in situ hybridization (Fig. 9A-E’). As its Tribolium orthologue, Dm-skh is expressed in the brain but is absent from the VNC and non-neural tissues (Fig. 9A,A’).

To compare the spatial arrangement of Drosophila and Tribolium skh positive cells, we double-labeled Drosophila embryos with skh RNA and α–acTubulin and examined the positions of cell bodies relative to the commissural system. At the end of embryogenesis, the spatial arrangement appears highly similar in both organisms (compare Fig. 9B-B” with Fig. 6B’): small clusters of skh positive cells are located just anteriorly to the commissural system while the vast majority of cells reside posteriorly to the commissural system in the dorsomedial brain. In a few cases we are able to follow the trajectories of skh positive cells and find that some of them enter the commissural system (Fig. 9B-B’’).

Discussion

G10011-GFP is a useful tool for the study of the dynamics of CX development.

Although the anatomy of the adult CX is well described in many insect species, the CX is vastly understudied from a comparative developmental perspective. Beside a large body of work addressing CX development in Drosophila, the CX has been investigated in the desert locust (Heinze and Homberg, 2008; Boyan and Williams, 2011; Boyan et al., 2017) the monarch butterfly (Heinze et al., 2013) and more recently in the dung beetle (Immonen et al., 2017). Developmental studies in non-Drosophila models are hampered by a lack of anatomical information at the single cell level but also by a near complete lack of molecular and genetic tools. We seek to establish Tribolium as an alternative insect model to study CX development (He et al., 2019; Farnworth et al., 2020). G10011-GFP marks the Tribolium CX. As reported before, the Tribolium adult PB is continuous (Dreyer, 2010), a features which is shared with several other insects including the dung beetle (Immonen et al., 2017). Moreover, the subdivision of the PB into eight glomeruli in each brain hemisphere is a shared feature of Tribolium, the locust, the monarch butterfly and the dung beetle but not Drosophila which
has nine paired glomeruli (Heinze and Homberg, 2008; Heinze et al., 2013; Immonen et al., 2017). As described for the dung beetle, the *Tribolium* EB is sausage-shaped and organized in vertical slices (Immonen et al., 2017).

Our results suggest that many GFP-positive CX neurons are born early in development, making G10011-GFP a useful tool for the study of CX formation: G10011-GFP expression confirms and extends the earlier findings that the FB is largely assembled in the larva, while a distinct EB forms later in the pupa (Panov, 1959; Koniszewski et al., 2016; Farnworth et al., 2020). In combination with additional markers, G10011-GFP will be a valuable tool to identify and characterize a subset of CX neurons at the single cell level.

The *Drosophila* orthologue of Tc-skh shows an RNA pattern in the embryo that is highly similar to that of Tc-skh suggesting that early expression is conserved. The generation of a corresponding imaging line should provide a means for a comparative study of *Drosophila* and *Tribolium* CX development at the anatomical level.

**Tc-Skh is a putative terminal selector of neuronal subtype identity.**

Terminal selector expression in neurons is continuous from cell birth to cell death. Therefore, such factors provide excellent markers for subsets of neurons for developmental, molecular and evolutionary studies. With this work, we identify Skh as the first putative terminal selector in neurons that contribute to the CX. Tc-Skh is the orthologue of *C. elegans* Unc-42 whose role in the specification of neuronal subtypes is well described (Wightman *et al.*, 1997). *unc-42* (*uncoordinated -42*) was first discovered by Brenner in his classic screen of mutants which show abnormal locomotion (Brenner, 1974). A later study showed that Unc-42 is required for axon pathfinding in a subset of neurons which facilitate a specific locomotor routine (Baran *et al.*, 1999). Studies of *C. elegans* Unc-42 and other transcription factors have led to the concept of terminal selectors as regulators of neuronal subtype identity (Hobert, 2008). In contrast to developmental genes that are expressed early in the gene regulatory cascade, terminal selectors are the final targets of the cascade. Maintenance of terminal selector expression is accomplished by positive auto-regulatory feed-back loops; accordingly, loss of activity results in the loss of its expression at later stages. The lifelong expression of terminal selectors facilitates the regulation of early aspects of subtype
differentiation, such as axon pathfinding, as well as late aspects like the maintenance of structural and molecular features of the mature neuron.

Our study shows that *Tribolium skh* expression is characteristic for terminal selector genes: We hypothesize that the adult expression of *Tc-skh* reflects the lifelong expression in many embryonically-born neurons; however, due to the lack of genetic tools for permanent cell marking, we can demonstrate this only for embryonically-born columnar neurons and neurons of the PI which can be traced to adulthood. An early aspect of columnar neuron identity is their axonal projection which leads to the establishment of the prFB. Knock-down of Tc-Skh abolishes axon outgrowth indicating that a requirement of Skh for the development of connectivity is conserved between *Tribolium* and *C. elegans*. In late *Tribolium* knock-down embryos we observe a moderate loss of G10011-GFP fluorescence suggesting that maintenance of *Tc-skh* expression by an auto-regulatory feed-back loop may be another conserved feature.

The term terminal selector derives from studies in *C. elegans* where some transcription factors directly co-regulate differentiation genes which together bring about all the specific features of a distinct neuronal subtype. The target genes of *Tc-skh* are currently unknown. In early development they are likely to include differentiation genes required for axon pathfinding. The question of whether *Tc-skh* coordinately directs the expression of a battery of effector genes at any developmental stage remains to be investigated.

*Tc-skh* expression is not restricted to one particular neuronal subtype but found in many neurons with different morphological features. Therefore, we expect additional transcription factors to act in parallel to or in combination with Tc-Skh to specify distinct identities.

Comparing the expression patterns and the Skh target genes in *Tribolium* and *Drosophia* CX neurons will contribute to a better understanding of CX formation and may uncover a molecular basis for anatomical differences of the CX in these species.
Material and Methods

Animal husbandry

*Tribolium castaneum* (NCBI:txid7070) beetles were maintained on standard wholemeal wheat flour (type 1050) at 28°C. To obtain embryos, the beetles were transferred to fine wheat flour (type 405) and kept at 32°C. Egg lay was allowed for 24 hrs. Subsequently, the embryos were separated from the beetles, aged for an additional 24-48 hrs at 32°C and then collected for fixation. The San Bernadino strain was used as wild type.

*Drosophila melanogaster Oregon R* flies were maintained at 18°C on standard cornmeal agar supplemented with dry yeast flakes. To obtain embryos, flies were placed in collection cages with apple juice-agar plates smeared with fresh yeast paste and placed at 25°C. Egg lay was allowed for 4 hrs. Then, the apple juice plates were removed from the cages, aged for an additional 16 hrs at 25°C and then collected for fixation.

Fixation

*Tribolium* embryos were collected, fixed and stored as described (Buescher et al., 2020). *Tribolium* larval, pupal and adult brains were dissected in ice-cold 1x phosphate-buffered saline (PBS) for up to 30 min. Then methanol-free formaldehyde was added to a final concentration of 4% (v/v). Fixation was performed for 30 (larval-), 45 (pupal-) or 60 (adult-brains) min on ice. Subsequently, the brains were washed 3x for 20 min each with ice-cold 1x PBST (PBS including 0.1% Triton-X100). In the second wash, DAPI was added to a final concentration of 1 ng/μl. Brains not dedicated to immunohistochemistry were mounted in VectaShield H-100 and imaged immediately. Brains dedicated to immunohistochemistry were placed into blocking solution containing 3% (w/v) bovine serum albumin (BSA) and 0.05% sodium azide. Adult brains dedicated to RNA in situ hybridization were dehydrated by putting them through an ethanol series: 25% ETOH:75% PBS, 50% ETOH:50% PBS, 75% ETOH:25% PBS for 5 min incubation each. Finally, the brains were placed in 100% ETOH and kept at -20°C for several days prior to in situ hybridization.
Immunohistochemistry

*Tribolium* and *Drosophila* embryos: Methanol was discarded from the fixed embryo collections. Subsequently, the embryos were washed 3x with 1x PBST for 20 min each at RT. Embryos were blocked for 1-2 hrs in 3% BSA (w/v) (containing 0.05% sodium azide) at RT. Primary antibodies were added at the indicated concentrations and incubation was performed overnight on a rotating wheel at 4°C. Then the primary antibodies were removed and the embryos were washed 3x with 1x PBST for 30 min each at RT. Secondary antibodies were added at a dilution of 1:1000 and incubation was performed on a rotating wheel for 90 min at RT. Subsequently, the embryos were washed 3x for 20 min each with 1x PBST (PBS containing 0.1% Triton-X100). In the first wash, DAPI was added to a final concentration of 1 ng/µl. Finally as much liquid as possible was removed and VectaShield was added. *Tribolium* germ bands were freed of yolk with the help of a fine brush, mounted with the dorsal side up and imaged. *Drosophila* embryos were pipetted onto microscope slides and imaged as whole-mounts.

Immunohistochemistry with adult brains was performed essentially as with embryos except for the following modifications: the concentration of Triton X-100 was raised to 0.5%, the incubation period with the primary antibody was extended to about 40 hrs and incubation with the secondary antibody was performed overnight at 4°C.

See Table 1 for a list of all primary and secondary antibodies used in this study.

FISH

Single- and double-fluorescent RNA in situ hybridization and RNA in situ hybridization followed by antibody staining of *Tribolium/Drosophila* embryos was performed as described (Buescher et al., 2020). To generate a skh specific RNA in situ probe, a DNA fragment was generated by PCR using wildtype embryonic cDNA (*Tribolium/Drosophila*) as templates (for details see list of reagents). The PCR products were cleaned up by gel-electrophoresis, extracted and used as template for an additional round of amplification using the same gene-specific primer pairs but with the modification of an added T7 RNA transcriptase binding site at the 5’end of the reverse primer. Dioxigenin- or fluorescein- labeled RNA probes were produced using the Roche RNA labelling kit. For RNA in situ hybridizations, the probes were used at a concentration of 4ng/µl (total hybridization volume: 50-100 µl).
RNA in situ hybridization in *Tribolium* adult brains was performed essentially as in embryos, except for the following modifications: the concentration of Triton X-100 was raised to 0.5%, the RNA hybridization period was prolonged to 48 hrs and incubation with the respective antibodies was performed for 48 hrs at 4°C.

**Image acquisition**

Confocal serial scanning images were acquired at 1.5-2 μm intervals using a LSM 510 microscope (Carl Zeiss) using either a 20x 0.5 Plan-Neofluar or a 40x 1.4 Plan-Neofluar objective (Carl Zeiss). Stacks were processed using the Zeiss LSM Browser software and whole or parts of stacks were visualized as maximum intensity projections. Brightness, contrast, size and resolution of the images were processed in Adobe Photoshop CS. The video image of the G10011 adult brain was generated with the ImageJ software (Fig. S1).

**Nomenclature used in anatomical analysis**

For *Tribolium* and *Drosophila* embryos the axes used for anatomical analysis in this study are the body axes (in Fig. 3A and K “b-A” indicates anterior with respect to the body axis). For *Tribolium* postembryonic stages (larva, pupa, adult) the axes used for anatomical studies are neuraxes. According to the neuraxes, the protocerebral bridge and the fan-shaped body are located n-dorsal of the ellipsoid body. For a detailed description of the body- and the neuraxes in *Tribolium* and *Drosophila* refer to (Farnworth *et al.*, 2020).

**Insertion site mapping of the enhancer trap line G10011**

The genomic location of the plasmid insertion was determined by inverse PCR (Thibault *et al.*, 2004). Genomic DNA was extracted from 3 beetles following a standard protocol. The genomic DNA was digested with the restriction enzyme Sau3A, highly diluted and ligated under conditions which facilitate an intramolecular circularization. Subsequently, the ligation products were amplified by PCR using plasmid-specific primers. The PCR product was cleaned up by gel-electrophoresis, extracted and sequenced. Blasting of the sequence against the *Tribolium* genome (genome release 3.0) indicated the insertion of the plasmid on
chromosome 4 at the genomic position 6024777, which is 18.5 kb upstream of the predicted gene *TC007335* (transcription start site 6006266). Using double-fluorescent in situ hybridization, we confirmed that the GFP-expression of G10011 reflects the RNA expression of *TC007335* in the embryo and the adult brain. Sequence analysis of the predicted coding region indicates that *TC007335* encodes a paired-like homeodomain transcription factor and is the orthologue of *C. elegans* unc-42 and *Drosophila* CG32532. We name *TC007335* and CG32532 *Tc*- and *Dm*-shaking hands (*skh*), respectively.

**skh knock-down**

Parental RNAi in *Tribolium*: 300-400 female G10011 pupae (at 70-80% pupal development) were injected with dsRNA (2 μg/μl) or injection buffer only (control) using a FemtoJet Express (Eppendorf). Injected pupae were placed on fine wheat flour (type 405) for 24 hrs at 28°C. Eclosed beetles were added to approx. 200 male G10011 beetles and maintained for another 24 hrs at 28°C. Then all beetles were collected, placed on fresh fine wheat flour and shifted to 32°C. Eggs were collected for 24 hrs, aged for an additional 48 hrs and then fixated for immunostaining. Eggs were collected for 8 consecutive days. Pupal injections were performed twice with fragment 1 and once with fragment 2.

**Generation of gene-specific dsRNA fragments**

Embryonic cDNA (0-72 hrs), prepared from the *San Bernadino* wildtype strain, was used as template for the generation of gene-specific fragments within the predicted *TC007335* transcribed region. Two primer pairs were used to generate two non-overlapping fragments (fragment 1: 287 bp, fragment 2: 261 bp) by PCR (for details: see list of reagents). The products were cleaned up by gel-electrophoresis, extracted and used as templates for an additional round of amplification using the same gene-specific primer pairs but with the modification of added T7 RNA transcriptase binding sites at both 5' ends. The PCR products were used as templates for large scale RNA synthesis using the MEGAscript T7 Transcription Kit (Invitrogen). The dsRNA was precipitated with LiCl, washed with 70% ethanol, dried and dissolved in injection buffer (1.4 mM NaCl, 0.07 mM Na₂HPO₄ 0.03 mM KH₂PO₄, 4 mM KCl, pH 6.8) to a concentration of 2 μg/μl.
Acknowledgements
We thank Elke Kuester for excellent technical assistance, Dr. Gerd Vorbrueggen for his continued interest in the project and critical reading of the manuscript and Dr. Benjamin Altenhein for providing the α-Repo antibody. The monoclonal antibody α-SYNORF1 was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. We acknowledge support by the Open Access Publication Funds of the University of Goettingen.

Footnotes

Author Contributions: Concept: MB. Investigation: MB, NGP. Methodology: MB. Formal analysis: MB, NGP. Validation: MB, NGP. Resources: MB, GB. Writing, original draft: MB. Writing, review and editing: MB, NGP, GB. Supervision: MB, GB. Funding: GB.

Competing interests: The authors declare that no competing interests exist.

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Data availability: Original image stacks can be viewed at https://figshare.com/projects/Additional_data_for_Garcia-Perez_et_al_Tribolium_shaking_hands_is_a_putative_terminal_selector_and_controls_axon_outgrowth_of_central_complex_neurons/93149
References


Figure 1. The enhancer trap line G10011-GFP labels CX neuropils in the adult

*Tribolium brain.* Brain of an animal with the genotype G10011-GFP;Ten-aΔ-RFP (GFP auto-fluorescence green, and RFP auto-fluorescence magenta). Note that in the central brain Ten-aΔ-RFP expression is restricted to the mushroom bodies (MB; magenta). Serial confocal
sections were combined and visualized as maximum intensity projections to display distinct anatomical features. Scan direction is from the n-dorsal (n-D) to the n-ventral (n-V) surface of the brain (coordinates of the neuraxes are shown in (G)). Depth along the Z-axis is given in µm. (A) GFP-positive cell bodies in the posterior brain. GFP-expression is absent from the Kenyon cells (KC) of the MB. Arrowhead indicates the protocerebral bridge (PB) which is only partially visible (for the PB, refer to Figure 2 A). Arrows indicate descending axon projections which extend longitudinal connectives into the ventral nerve cord (VNC). (B) The fan-shaped body (FB) and the ellipsoid body (EB) are heavily labelled by GFP. Clusters of laterally located cells send their axon trajectories toward the upper layer of the FB body (arrowhead; compare with Figure 2 L). Small clusters of large cells are located in the pars intercerebralis (PI). Based on location and axon projections, they may be neurosecretory cells. Note their descending axon tracts towards the VNC (arrows). GFP-fluorescence is also seen in the optic lobes (OL). (C) The arrowhead marks a single cell cluster of 4-6 GFP-positive cells in the anterior brain. Individual large cells within the Tritocerebrum (TC) project anteriorly towards the PI (arrow). (PC: Protocerebrum, DC: Deutocerebrum) (D) First thoracic segment (T1) of the VNC: multiple axon projections which originate in the brain form longitudinal connectives in the VNC (arrowheads mark the limits of the first thoracic segment T1). Note the absence of GFP-positive somata in the VNC. White line marks the ventral midline. (E) Schematic illustration of CX small-field and large-field neurons. Two types of small-field neurons, pb-fb-eb (green) and pb-fb (blue) are shown. Two types of large-field neurons, a ring neuron (red) and an AOTU neuron (purple) are shown. (F) The average number of G10011-GFP-positive cells is 320 per brain lobe (n=4). (G) Coordinates according to the neuraxes.
**Figure 2. GFP-labeled CX neuropils in the adult G10011 brain.** Serial confocal sections were combined and visualized as maximum intensity projections to display individual anatomical features. (A-H') Adult G10011 brains (green, GFP auto-fluorescence) stained with α-Synapsin antibody (magenta). (A-D) Whole brain imaged at low magnification. Scan
direction is from the n-dorsal to the n-ventral surface of the brain. Depth along the Z-axis is
given in µm. (A-B) GFP-label within the PB. Arrow in (B) indicates the most lateral
glomerulus of the PB. (C) GFP-label within the FB and the EB. (D) GFP-label of a subset of
ring neurons (arrowhead). (E-H’) Close-ups of CX neuropils. (E) PB, (F) NO, (G) FB and
EB, (H) We interpret compartments which are located postero-lateral to the EB as the lateral-
accessory-lobes (LALs). (E’-H’) GFP auto-fluorescence only. Note that the NO and the
putative LALs are only weakly labelled by GFP. (I-J’’) CX of an animal with the genotype
G10011-GFP;5’rx-RFP. (I-I’’ and J-J’’’) depict two different planes of the CX along the D/V
axis. (I’, J’) GFP auto-fluorescence only. (I’’, J’’) RFP only. Note that there is little overlap
of GFP- and RFP-fluorescence. Arrowheads indicate the NO. (K-M) Identified sets of
neurons with projections into the FB and/or the EB. (K) Small-field, columnar neurons
arborize within the PB, form the characteristic z, y, x and w fascicles, decussate in the upper
part of the FB and establish the columnar organization of the FB. Blue stain is DAPI. (L-M)
Adult brain of an animal with the genotype G10011-GFP;Ten-aΔ-RFP. (L) AOTU large-
field neurons and their projections into the FB (arrowheads). (M) Large-field ring neurons
(R-N) projection into the EB body (arrowhead).
Figure 3. Embryonic expression of G10011 and the formation of the embryonic commissural system. (A-H') Developmental series of G10011-GFP brains from stage NS11 (~65% embryogenesis) up to stage NS15 (100% embryogenesis) (for staging see Figure S2). Coordinates are given according to the body axes (b-A arrow in A indicates "anterior up").
Double staining with $\alpha$-GFP (green) and $\alpha$-acetylated Tubulin (magenta) antibodies. (A-C) depict single confocal planes; anterior is up with respect to the body axis (arrow “b-A”). (A, inset) The first continuous commissural fascicle which links both hemispheres of the protocerebrum is established at late NS11. This primary commissural fascicle is labelled by $\alpha$-GFP. (C) Note that multiple GFP-positive cell clusters project their axons from posterior dorsomedial regions towards the primary brain commissure (arrowhead). (D-D’) Serial confocal sections were combined and visualized as maximum intensity projections to depict either superficial (D) or deep lying (D’) regions of a late NS13 brain. (D’) Multiple GFP-positive cell clusters project axons from posterior dorsomedial and dorsolateral regions towards the primary brain commissure (arrows). GFP also labels the stomodeum (asterisk). (D’’) Close-up of (D’) Multiple commissural fascicles have formed; only a subset is GFP-positive (eAC: embryonic anterior commissure). (E-H’) Serial confocal sections were combined and visualized as maximum intensity projections to depict superficial (E-E’) or deep (F-F’) lying regions of a stage NS14 brain. Note that nearly all GFP-positive cell bodies are located in the dorsoposterior brain. (E) White lines indicate four clusters of cells. We interpret these cells as the progeny of DM1-DM4 which differentiate into columnar neurons. (E’) The four clusters of neurons produce four parallel running GFP-positive fascicles which enter the commissural fiber system (white lines). We interpret these fibers as the precursors of the w, x, y, z tracts and hence as the prFB. (E’’) Schematic illustration: the trajectories of 4 cell clusters generate the prFB; dashed line indicates the ventral midline. (F, F’) GFP-positive input into the primary commissure stems largely from cells located in posterior dorsomedial and dorsolateral regions of the brain (arrowhead). (G,G’) Superficial and (H,H’) deep lying regions of the NS15 brain. (G) Note the beginning defasciculation of GFP-positive commissural fiber tracts (white lines). (G’’) Schematic representation of the beginning defasciculation. (H,H’) Multiple GFP-positive fibers exit the brain and project
towards the VNC (arrowheads). (I) Schematic representation of morphogenetic head movements during embryogenesis. (J) The average number of G10011-GFP-positive cells in late stage NS15 brain lobes is 362 (n=4). (K) Dorsal view of a whole-mount NS14 animal. Note that embryonic G10011-GFP expression is restricted to the brain, the stomodeum (asterisk) and the hindgut. Blue stain is DAPI. “b-A” arrow: anterior is left.
Figure 4. GFP expression in the late G10011 larva. (A-L) GFP auto-fluorescence (green) and DAPI (blue) staining. (D’, E’, H’ and L’) GFP only. Serial confocal sections were combined and visualized as maximum intensity projections to display individual anatomical features. Scan direction is from the n-dorsal (A) towards the n-ventral (L) surface of the brain. Depth along the Z-axis is given in μm. (A-C) GFP-positive cell bodies in the posterior brain. Note that the Kenyon cells (KC) do not express GFP. GFP fluorescence within the optic lobes (OL) may reflect the expression of the transfection marker 3xP3-GFP. (D,D’)
Multiple axon tracts originating in the n-antero-medial and –lateral protocerebrum descend towards the VNC (arrows). Arrowheads indicate the dorsal and ventral clusters of AOTU neurons. (E, E’) A subset of columnar neurons (arrows) with their arborizations within the PB and their characteristic z, y, and x axon tracts (the w tract is not in focus). (F-H, H’) The FB is strongly labelled by GFP (arrowheads). Note that distinct elements of the EB are not yet present. (I) Ascending axon tracts originating from cells of the Tritocerebrum (arrowhead) project towards the PI. (J-K) GFP-positive ring neurons (arrows). (L,L’) GFP-expressing cells form multiple dendritic arborizations which enwrap distinct parts of the MBs (arrowheads).
Figure 5. GFP-expression in the late (90% development) G10011 pupal brain. (A-C) GFP autofluorescence (in A combined with DAPI staining, blue). (A) Confocal stack is visualized as maximum intensity projection. The columnar organization of the FB is well established. Note the ring neurons (R-N) and their projection towards the EB (arrowhead). (B) Columnar neurons, their arborizations within the glomeruli of the PB and their axon trajectories z, y, x (the w tract is not in focus). The PB is not yet fused at the midline. (C) The EB is well developed in the late pupa.
Figure 6. G10011-GFP reflects the RNA expression of Tc-shaking hands (s\(h\)) (TC007335). Double fluorescent in situ with a GFP (magenta) and a s\(h\) (green) RNA probe in a stage NS14 embryo. (A-A’) Dorsal view of a whole-mount embryo. Note that the expression of GFP and s\(h\) are restricted to the brain and stomodeum (asterisk). (B-B’’) GFP and s\(h\) RNA expression co-localize in the embryonic brain. White lines indicate the midline. (C-C’’) s\(h\) RNA in situ (magenta) combined with α-GFP antibody staining (green) in an adult G10011 brain. Serial confocal sections were combined and visualized as maximum intensity projections. Note the co-localization of s\(h\) RNA and GFP protein. For additional images see Figure S5.
Figure 7. G10011-GFP expression is restricted to non-dividing, non-glial cells.

Embryonic G10011 brains were stained with α-GFP (green) and α-PH3 (A-A’’) or α-Repo (B-C’’) (both magenta). Serial confocal sections were combined and visualized as maximum intensity projections. (A-A’’) NS13 and (B-C’’) NS14. Note that there is no overlap of GFP- and PH3- or Repo- expressing cells. White lines indicate the midline.
Figure 8. parental RNAi of skh leads to severe axon outgrowth defects in the embryonic brain. G10011-GFP stage NS14 brains double-stained with α-GFP (green) and α-acTubulin (magenta); dorsal views. (A-B'') Control brain (progeny of buffer-injected pupae). (A) Whole brain at low magnification; GFP-positive axon tracts join the commissural system...
linking both hemispheres of the brain (arrow). The arrowhead indicates GFP-positive cell clusters in the posterior brain. (B-B’’) Close-ups; GFP-positive axons project towards the midline (arrows). GFP-positive input into the primary commissure stems largely from cells located in posterior dorsomedial and dorsolateral regions of the brain (arrow). (C-D’’) skh RNAi brain. (C) Whole brain at low magnification; GFP-positive axons fail to join the commissural system. Arrowhead indicates the loss of GFP-positive cell clusters. (D-D’’)

Close-ups; (D’) GFP-positive axons stall while most acTubulin-positive axons are unaffected (D’’). White lines indicate the midline. (E,F) Quantification of skh RNAi phenotypes. (E) Commissural defects were scored at stages NS14 and NS15. Buffer-injected control (co) n=80 (2 biological replicates) 3% defects, dsRNA frag1 n=85 (2 biological replicates) 71% defects, dsRNA frag2 n=35, 48% defects. (F) Loss of GFP-positive cells in skh RNAi embryos were scored at NS15. Buffer-injected control (co): 362 GFP-positive cells (n=4), dsRNA frag1: 308 GFP-positive cells (n=4). (G-G’’) Loss of GFP-positive commissural fascicles is largely due to fascicle stalling. Superficial layer of a G10011-GFP brain at stage NS14. Note the absence of GFP-positive fibers within the commissure (arrowhead) despite the presence of cell clusters representing the progeny of the neuroblasts DM1-DM4. Insets: deep layer of the same brain: the fascicle emanating from the DM4-derived cell cluster stalls (arrow). Original stacks can be viewed at https://figshare.com/projects/Additional_data_for_Garc_a_P_rez_et_al_Tribolium_shaking_hands_is_a_putative_terminal_selector_and_controls_axon_outgrowth_of_central_complex_neurons/93149
Figure 9. Embryonic expression of Dm skh. (A,A’) skh RNA in situ (red) and DAPI staining (blue). (A,A’) stage 16 whole-mount embryo; anterior is up. (A) Dorsal view; (A’) Ventral view. Note that Dm skh RNA is restricted to the brain. Red fluorescence in the trachea is an in situ artefact (arrow). (B) Spatial organization of skh RNA expressing cells and major axon tracts; skh RNA in situ (magenta) combined with α-acetylated Tubulin (green) staining. (B’) skh RNA only. Compare with Figure 6B: embryonic skh-expressing cells are similarly distributed in Tribolium and Drosophila. (B’’) α-acetylated Tubulin only. White lines indicate the dorsal midline in all panels except (A’) where it marks the ventral midline.
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Genetic reagents used in each experiment

**Tribolium castaneum genotypes**

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**Drosophila melanogaster line**

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**Sequence-based reagents**

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Fig. S1. Embryonic development of *Tribolium*; dorsal view of whole-mount embryos. Neuroblasts (NBs) are visualized by *asense* RNA in situ. *Tribolium* embryogenesis is subdivided into 15 stages from NS1 (0% embryogenesis) to NS15 (100% embryogenesis). NB formation in the brain begins at stage NS4. Subsequently, the number of NBs increases steadily until stage NS13/NS14. The precise number of brain NBs that are generated during embryogenesis is not known but is estimated to be around 100 per lobe. G100111-GFP-positive cells are first observed at stage NS11; that is at approx. 60% of embryonic development (stage 11 is marked by an asterisk).
Fig. S2. Embryonic G10011GFP-positive neurons establish the FB primordium (prFB). (A-A'') Double-immuno-staining with α-GFP (green) and α-Repo antibody (magenta). Cell membranes of midline-associated glia (arrowheads in A'') form a channel through which the DM1-DM4 progeny project their trajectories which constitute the prFB (arrows in A and A';
white line in A marks the midline). (B-D) Double-immuno-staining with α-GFP (green) and α-Rx antibody (magenta). (B) The prFB (arrow) is established by the progeny of four neuroblasts (in Drosophila called DM1-DM4) (white lines) (Andrade et al., 2019). (B’-B’’) Many prFB neurons have been shown to express Rx protein (Farnworth et al.; 2020). A subset of Rx-positive neurons co-expresses G10011-GFP. Arrow in B’ indicates the trajectories of the four neuroblast progeny. (C) We interpret a group of posterior lateral located cells as AOTU neurons (arrow). (D) We interpret a cluster of very large G10011-GFP-positive cells near the midline (white line) as neurosecretory cells of the prospective PI (arrow). A small cluster of prominent cells is labelled with an arrowhead. In Drosophila neurons in this position and with similar morphology and axon trajectories express the neuropeptide HUGIN.
**Fig. S3.** G10011-GFP positive fascicles contribute to several major axon tracts in the embryonic brain. (A-F) G10011 brain at late stage NS15 stained with α-GFP (green) and α-acetylated Tubulin (magenta). Serial confocal sections were combined and visualized as maximum intensity projections to display individual anatomical features. (A’-F’) acetylated Tubulin only. (A’’-F’’) GFP only. (B’-D’’) GFP-positive axon trajectories make multiple contributions to the commissural system (arrows). (E’’’,F’’’) GFP-positive fibres contribute to longitudinal axon tracts which extend towards the VNC (arrowheads).
**Fig. S4. Identification of the G10011 associated gene. Top panel:** (A) Genomic localization of the G10011 plasmid insertion site. The plasmid insertion was mapped to the position 602477 on the fourth chromosome (genome release 3.0). The insertion site is located in the first intron of the predicted gene TC008169 (magenta arrowhead). We found no
experimental evidence for the expression of the first TC008169 exon suggesting that the
plasmid insertion site is inter- rather than intragenic. TC008169 encodes an EF1 hand protein.
(B) To determine whether the expression of G10011-GFP reflects the expression of
TC008169, we performed RNA in situ with a TC008169 probe (magenta) combined with a α-
GFP staining (green). (B”) TC008169 expression is pan-neural in the embryonic brain while
G10011-GFP is expressed only in a subset of cells (B”). We conclude that G10011-GFP is
unlikely to reflect the expression of TC008169. The plasmid insertion site is located 18.5 kb
upstream of the predicted gene TC007335 (transcription start site 6006266, red arrow in (A).
**Mid panel:** (C-C”) The TC007335 RNA in situ signal and the G10011-GFP RNA in situ
signal co-localize in the embryonic brain at the developmental stages NS12 (C’) and NS13
(C’, C”; two different focal planes) indicating that G10011-GFP is a faithful reporter of
TC007335 RNA expression (for additional evidence refer to Figure 6). The asterisk in (C)
marks the stomodeum. Note that the expression of TC007335 RNA in the stomodeum is very
low as compared to the expression of GFP-RNA. **Bottom panel:** (A-A’’) The TC007335
RNA in situ signal and the G10011-GFP protein signal largely (and possibly) wholly co-
localize in the adult brain. (A) asterix: putative neurosecretory cells of the PI; arrowhead:
columnar neurons; arrow: putative AOTU neurons. (A’) skh-RNA. (A’’) G10011-GFP
protein. (A’’) DAPI staining indicates all nuclei. (B-B”) putative neurosecretory cells of the
PI. (B’) skh-RNA. (B”) G10011-GFP protein. (C-C”) Columnar neurons. (C’) skh-RNA.
(C”) G10011-GFP protein. (D-D”) putative AOTU neurons. (D’) skh-RNA. (D’’) G10011-
GFP protein. (E- E”) neurons of the tritocerebrum. (E’) skh-RNA. (E’’) G10011-GFP
protein. Not that skh-RNA and GFP protein signals colocalize in all panels (B- E”).
Fig. S5. Phylogenetic tree. (A) Phylogenetic tree reveals *D. melanogaster* CG32532, *C. elegans* unc42 and *M. musculus* Prop1 as single orthologs of TC007335. The TC007335 protein sequence was used to search the NCBI Ref-Seq databases for these species for the most similar proteins using blastp. Alignment was done using the Muscle algorithm as implemented in MEGA 6 (Tamura et al., 2013). The alignment was trimmed to remove all sequences with unclear alignment or gaps. We used Maximum Likelihood, UPGMA and neighbor joining algorithms as implemented in MEGA 6 with bootstrapping based on 500 replications to construct the phylogenetic tree. With all algorithms, the same orthology group is found (shown is the maximum likelihood tree with bootstrap values based on 500 replicates). (B) Protein sequence comparison of Tc-Skh and Dm-Skh. Note that Tc-Skh (229aa) is considerably smaller than the largest predicted Dm-Skh isoform (RB-D) (691aa).
**Movie 1.** G10011-GFP adult brain