Lineage-specific determination of ring neuron circuitry in the central complex of *Drosophila*

**Running title:** *Drosophila ellipsoid body formation*

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**Key words:** *Drosophila*, brain, stem cell lineage, neural circuit, ellipsoid body, central complex.

**Summary statement**

The formation of ring neurons is described from a pair of embryonic stem cells to *engrailed* and *Poxn neuro*-dependent lineage-specific determination of neural circuits in the central complex of *Drosophila*. 
ABSTRACT

The ellipsoid body (EB) of the Drosophila central complex mediates sensorimotor integration and action selection for adaptive behaviours. Insights into its physiological function are steadily accumulating, however the developmental origin and genetic specification have remained largely elusive. Here we identify two stem cells in the embryonic neuroectoderm as precursor cells of neuronal progeny that establish EB circuits in the adult brain. Genetic tracing of embryonic neuroblasts ppd5 and mosaic analysis with a repressible cell marker identified lineage-related progeny as Pox neuro (Poxn)-expressing EB ring neurons, R1-R4. During embryonic brain development, engrailed function is required for the initial formation of Poxn-expressing ppd5-derived progeny. Postembryonic determination of R1-R4 identity depends on lineage-specific Poxn function that separates neuronal subtypes of ppd5-derived progeny into hemi-lineages with projections either terminating in the EB ring neuropil or the superior protocerebrum (SP). Poxn knockdown in ppd5-derived progeny results in identity transformation of engrailed-expressing hemi-lineages from SP to EB-specific circuits. In contrast, lineage-specific knockdown of engrailed leads to reduced numbers of Poxn-expressing ring neurons. These findings establish neuroblasts ppd5-derived ring neurons as lineage-related sister cells that require engrailed and Poxn function for the proper formation of EB circuitry in the adult central complex of Drosophila.
INTRODUCTION

The Drosophila central complex is a composite of midline neuropils that include the protocerebral bridge, the fan-shaped body, the ellipsoid body, the noduli and the lateral accessory lobes (Hanesch et al., 1989). These neuropils are interconnected in a modular way whereby columnar projection neurons leading to and from the central complex connect all its components that are themselves intersected by tangential layers of neural processes, which together form functional modules each representing a segment of sensory space (Strausfeld, 2012). Functional studies have identified specific roles for the central complex in higher motor control, courtship and orientation behaviours, visual memory and place learning, as well as sleep, attention, arousal and decision-making (Strausfeld and Hirth, 2013; Pfeiffer and Homberg, 2014; Turner-Evans and Jayaraman, 2016).

In contrast to expanding insights into the physiological role of the central complex in regulating behaviour, its developmental origin and genetic specification has largely remained elusive. Earlier work described a primordial central complex at late larval/early pupal stages, which becomes fully formed by 48 h after puparium formation (Renn et al., 1999; Young and Armstrong, 2010). Genetic studies have identified several alleles of as yet unidentified genes (Strauss and Heisenberg, 1993), as well as orthodenticle (Hirth et al., 1995), Pax6/eyeless (Callaerts et al., 2001), Pox neuro (Poxn) (Boll and Noll, 2002; Minocha et al., 2017), tay-bridge (Strauss and Heisenberg, 1993; Poeck et al., 2008), roundabout (Nicolas and Preat, 2005), Pdm3 (Chen et al., 2012), and semaphorin (Xie et al., 2017) as genes involved in normal formation of central complex sub-structures (for review see Furukubo-Tokunaga et al., 2012; Strausfeld and Hirth, 2013).

Here we investigate the origin and formation of ellipsoid body (EB) ring neurons R1-R4 in the developing and adult brain of Drosophila. We identify bilateral symmetric neuroblasts ppd5 in the embryonic procephalic neuroectoderm as founder cells of neuronal progeny that constitute R1-R4 subtypes of tangential ring neurons in the adult EB. Mutant analysis and targeted genetic manipulations reveal a lineage-specific requirement of engrailed (en) and Poxn activity that determines the number and identity of ppd5-derived progeny and their EB ring-specific connectivity pattern in the adult central complex of Drosophila.
RESULTS

Ellipsoid body ring neurons are lineage-derived progeny of embryonic neuroblasts ppd5

To gain insights into the origin and formation of the EB, we followed the expression of the Pax2/5/8 homologue Poxn which is expressed in the developing and adult EB as revealed by full enhancer analysis (Boll and Noll, 2002). In the embryonic protocerebrum, Poxn expression can be found at the protocerebral/deutocerebral neuromere boundary, which is also characterised by Engrailed-expressing cells (Hirth et al., 2003). These Engrailed-expressing cells derive from neuroblasts ppd5 and ppd8 (Urbach and Technau, 2004), which are distinguishable by dachshund (Dac) expression that is restricted to ppd8. Ppd5/8 neuroblasts can be visualised with en-Gal4 (Kumar et al., 2009) when combined with UAS-mCD8::GFP expression (Fig. 1) which reveals that neuroblasts ppd5/8 form bilaterally symmetric lineages in the embryonic brain. The resulting neural progeny of ppd5/8 start to express Poxn which can be visualized with en>mCD8::GFP (Fig. 2A-D) but also with Poxn>mCD8::GFP which reveals that Poxn-Gal4+ cells in the embryonic brain are labelled by Engrailed (Fig. 3A, B, arrowheads).

During larval development, Poxn expression is maintained in these lineages as demonstrated by Poxn>mCD8::GFP (Fig. 3C-I) and anti-Poxn immunolabelling (Fig. S1). By larval stage late L2/early L3, Poxn>mCD8::GFP labelled neurons can be identified that send projections towards the midline of the central brain (Fig. 3E, J). In the adult brain, Poxn>mCD8::GFP labels EB ring neurons (Fig. 3 K-M, arrowheads) that no longer express Engrailed (Fig. 3M). Together these data suggest that Poxn-expressing EB ring neurons might be clonally related progeny of en-expressing neuroblasts ppd5.

To test this hypothesis, we used a combination of Gal4/UAS and FLP/FRT cassettes (Roy et al., 2007) allowing the inheritance of a traceable, membrane-tethered marker (mCD8::GFP) which identifies progeny that share a common origin and are therefore clonally related. We first utilised the en-Gal4 driver line with Gal4 expression detectable from early embryogenesis in the procephalic neuroepithelium (Fig. 1) and that remains active throughout development and in the adult (Fig. S2). Analysis of en>mCD8::GFP flies co-labeled with anti-En revealed expression of endogenous Engrailed always within mCD8::GFP-labeled cells, including neuroblasts ppd5 (Fig. 1C-E) and their progeny in the embryonic (Fig. 2C, D), larval (Fig. S2A-E) and adult brain (Fig. 4A-F and S2F-I).
These data establish that *en>*mCD8::GFP recapitulates the spatio-temporal pattern of endogenous *engrailed* expression.

Anatomical and immunohistochemical analysis of *en>*mCD8::GFP brains revealed that GFP-labeled En-expressing cells extend projections during larval development towards the midline of the central brain ([Fig. S2A, B]), which in the adult brain of *en>*mCD8::GFP flies terminate in the superior protocerebrum (SP) ([Fig. S2F]). In addition to cell-specific labelling of *en>*mCD8::GFP projection patterns, cell and lineage-specific labelling using *en*-mediated activation of the constitutively active tubulin driver *tub*-Gal4 (*en>*tub>*mCD8::GFP) ([Fig. S3]), invariably visualised the EB neuropil and EB-specific ring neurons ([Fig. 4G-L and Fig. S4]; n=77 brains). Labelling of *en>*tub>*mCD8::GFP expressing cells in the posterior protocerebrum revealed neuronal projections that terminate in the superior protocerebrum ([Fig. 4H, arrows] as well as in the ellipsoid neuropil ([Fig. 4H, arrowheads]). Labeling *en>*mCD8::GFP brains with anti-Poxn showed hardly any overlap between GFP and Poxn expression ([Fig. 4C-E]), which is detectable immediately adjacent to En expressing cells ([Fig. 4E]). However, *en>*tub>*mCD8::GFP brains immunolabeled with anti-Poxn revealed that protocerebral Poxn-expressing cells were co-labeled with *mCD8::GFP and were located immediately adjacent to cells expressing GFP and En ([Fig. 4I-K, compare to C-E]). These data suggest that Poxn-expressing neurons labelled with *en>*tub>*mCD8::GFP share a common lineage relationship with Engrailed expressing cells.

To corroborate these findings, we carried out mosaic analysis with a repressible cell marker (MARCM) (Lee & Luo, 1999) utilising a tubulin-Gal4 driver. Neuroblast lineage labelling was induced in early L1 and adult brains were screened for GFP expression in both Engrailed-expressing cells projecting to the SP and Poxn-expressing cells projecting to the EB. Following this protocol, we identified Engrailed and Poxn-expressing MARCM labelled cells, both of which initially project together anterior-medially, before Engrailed-expressing cells branch off to the superior protocerebrum and Poxn-expressing cells project to the EB ring neuropil ([Fig. S5]). MARCM thus demonstrates that Engrailed and Poxn-expressing cells in the posterior protocerebrum are clonally related. Together with lineage tracing using *en>*tub>*mCD8::GFP, our findings identify Poxn-expressing EB ring neurons and neighbouring SP-projecting Engrailed-expressing cells as clonally-related progeny that constitute two hemi-lineages derived from Engrailed-expressing neuroblasts ppd5.
ppd5 neuroblast-derived progeny form part of EB R1-R4 ring neuron circuitry

We next wanted to know which ring neuron subtypes these Poxn expressing EB-precursor cells give rise to. Adult EB neurons are classified as large-field ring neurons based on their subtype-specific stereotypical pattern of synapse formation (Hanesch et al., 1989; Renn et al., 1999; Young and Armstrong, 2010). Previous reports identified and visualised R1-R4 neurons using subtype-specific Gal4 driver lines (Renn et al., 1999; Wang et al., 2002; Martin-Peña et al., 2006; Young and Armstrong, 2010; Shaw et al., 2018) which combined with mCD8::GFP reveal that axon terminals of R1-3 neurons enter via the EB canal and synapse outwardly at different positions within the EB ring, whereas R4 projections reach the EB at the distal surface and synapse in the outer ring ([Fig. S6]). We made use of these Gal4 lines to investigate whether Poxn-expressing cells comprise different EB ring neuron subclasses.

In the adult brain, Poxn expression is detectable in GFP-labeled ring neurons ([Fig. S6]) of c105>mCD8::GFP, c819>mCD8::GFP, and c507>mCD8::GFP. We also tested other Gal4 strains, including EB1-Gal4 (Wang et al., 2002), c232-Gal4 and c42-Gal4 (Renn et al., 1999) as well as 796-Gal4 (Martin-Peña et al., 2006) that label ring neuron subtypes partially overlapping with c105, c819 and c507. In all cases examined, we detected anti-Poxn immunoreactivity in nuclei of mCD8::GFP labeled cells ([Fig. S6B-H]), which altogether demonstrates that Poxn expression can be found in ring neuron subtypes R1-4. These data suggest Engrailed-expressing neuroblasts ppd5 give rise to Poxn expressing progeny that comprise ring neuron subtypes R1-R4 of EB-specific circuitry.

Embryonic formation of Poxn expressing lineages requires engrailed function

Our lineage analysis identified Poxn expressing ring neurons as progeny of Engrailed expressing neuroblasts ppd5, suggesting that engrailed might be required for their development and/or specification. To investigate these hypotheses, we first analysed two different alleles affecting engrailed function. en^CX1 affects embryonic patterning but does not completely remove the engrailed orthologues en and invected (inv) (Heemskerk et al., 1991). Df(2R)en^E is a deficiency removing the entire en locus and the majority of the inv locus, resulting in the absence of en and inv gene products, which is therefore considered to be a null allele of engrailed (Tabata et al., 1995).
Analysis of the embryonic brain and ventral nervous system of Df(2R)en\(^E\) homozygous mutants revealed severe patterning defects including absent or fused commissures, fused or broken connectives and a disrupted peripheral nerve pattern. Anti-Poxn immunolabelling of these mutant brains revealed a complete absence of Poxn-labeled neurons in 94.7\% (n = 19) of all cases examined that developed beyond stage 13 (Fig. 2H, I). These data suggest that en\textit{grailed} is required for the formation of Poxn-expressing progeny in the embryonic protocerebrum.

**Determination of ring neuron identity depends on lineage-specific Poxn function**

The extended post-embryonic phase of EB lineage development made it necessary to bypass embryonic lethality associated with recessive lethal mutations, as seen for Df(2R)en\(^E\) homozygous mutants. Moreover, previous studies had shown that Poxn mutants are adult viable but present with an affected EB neuropil (Boll and Noll, 2002; Minocha et al., 2017). We therefore used lineage-specific genetic manipulations to gain insights into the mechanisms of en\textit{grailed} and Poxn mediated EB development. To this end we used UAS-mediated overexpression and RNA interference (RNAi) targeted by en\textit{-Gal4} and co-expressed Dicer-2 (Dcr2) to enhance RNAi efficiency (Dietzl et al., 2007). We first tested whether on its own, en\textit{-Gal4} mediated UAS-Dcr2 expression interfered with lineage formation and EB development. For this we analysed adult brains of en\textit{>mCD8::GFP} controls and en\textit{>mCD8::GFP,Dcr2} co-immunolabeled with anti-Poxn to visualise Poxn-expressing ring neurons, and with anti-En to visualize adjacent hemilineage neurons projecting to the superior protocerebrum. This data revealed indistinguishable patterns of Poxn and Engrailed expression in the brains of both genotypes (Fig. 5A-G, compare to Fig. 4A-E and Fig. S7A-F), suggesting that en\textit{-Gal4} driven ectopic activation of Dcr2 does not affect neuroblast ppd5 lineage formation and EB development.

We next studied whether overexpression of en\textit{grailed} and Poxn might interfere with lineage formation and EB development. Analysis of en\textit{>mCD8::GFP,en} brains revealed projections patterns and anti-Poxn and anti-Engrailed immunolabelling (Fig. 5H-N) indistinguishable from controls (Fig. 4A-E). In contrast, we were not able to analyse adult brains of en\textit{-Gal4} mediated, lineage-specific overexpression of UAS-Poxn due to early developmental lethality of en\textit{>mCD8::GFP,Poxn} flies. We then analysed the brain phenotypes of RNAi-mediated knockdown of en\textit{grailed} and Poxn. Again, we were not
able to analyse adult brains of \textit{en>mCD8::GFP,Dcr2,en-IR} animals due to early developmental lethality.

In contrast, RNAi-mediated knockdown of \textit{Poxn (en>mCD8::GFP,Dcr2,Poxn-IR)} revealed a striking EB phenotype (Fig. 5O-U). Although \textit{en-Gal4} normally does not target EB ring neurons in the adult brain (Fig. 4A-E, Fig. 5A-G and Fig. S7A-F), we observed GFP-expressing cells projecting to the EB in \textit{en>mCD8::GFP, Dcr2, Poxn-IR} brains (Fig. 5O,P and Fig. S7G-L). These \textit{en>mCD8::GFP,Dcr2,Poxn-IR}-expressing neurons revealed the typical morphology and projection pattern of tangential ring neurons, in that they send axons via the lateral triangles to terminate in the EB neuropil. Immunohistochemical analysis detected \textit{Poxn} expression clusters, however GFP expression was now also seen in cells immunolabelled with anti-\textit{Poxn} (Fig. 5Q-U) despite the fact that the utilised \textit{UAS-Poxn-IR} led to knockdown of \textit{Poxn} to levels undetectable by immunohistochemistry (Fig. S8). Notably, anti-\textit{En} immunostaining identified GFP-labelled EB ring neurons that express both Engrailed and \textit{Poxn} (Fig. 5U, compare with Fig. 5E-G), which is normally never seen for \textit{en-Gal4} labelled adult neurons typically projecting to the superior protocerebrum (see Fig. 4A-E), nor for \textit{Poxn}-expressing ring neurons that usually do not co-express Engrailed (Fig. 4I-K and Fig. 5E-G). Furthermore, in \textit{en>mCD8::GFP,Dcr2,Poxn-IR} brains we could not detect GFP-labelled cells projecting to the superior protocerebrum (Fig. 5O, P and Fig. S8G-L), that are normally seen in \textit{en>mCD8::GFP} brains (Fig. 4A, B, arrows), in related \textit{en>mCD8::GFP,Dcr2} controls (Fig. 5A, B), and also detectable in genetically traced \textit{en>tub>mCD8::GFP} brains (Fig. 4G, H, arrows). Instead, GFP-labelling of \textit{en>mCD8::GFP,Dcr2,poxn-IR} brains frequently revealed a ventrally open EB ring neuropil (Fig. S7G-L), devoid of the toroidal ring shape that is normally seen in \textit{Poxn::GFP} and genetically traced \textit{en>tub>mCD8::GFP} brains (Fig. 4G, H). These findings suggest that \textit{en-Gal4} mediated knockdown of \textit{Poxn} transforms the identity of \textit{ppd5}-derived hemi-lineages from Engrailed-expressing SP-projecting neurons to ring neurons that send terminal projections to the EB neuropil.

**Specification of ring neuron number requires \textit{Poxn} and \textit{engrailed} function**

The observed ventrally open EB ring phenotype in \textit{en>mCD8::GFP,Dcr2,poxn-IR} brains suggested that \textit{ppd5}-derived progeny devoid of \textit{Poxn} may not adopt a proper ring neuron identity. To test this hypothesis, we carried out experiments utilising a brain-specific \textit{Poxn-Gal4} driver we generated, \textit{Poxn} \cite{757}, that shows activity in only a subset of
Poxn-expressing ring neurons (Fig. 6A), thus allowing for analysis at single cell resolution. To potentiate RNAi-mediated knockdown we again co-expressed Dcr2. Similar to en>mCD8::GFP,Dcr2 brains, analysis of Poxn-Gal4(P757)>mCD8::GFP,Dcr2 brains revealed GFP-labeled EB ring neuron morphology and projections into the ring neuropil (Fig. 6B). We then analysed GFP-labeled ring neurons targeted by RNAi-mediated knockdown of Poxn which identified a ventrally open EB ring neuropil in Poxn(P757)>mCD8::GFP,Dcr2, Poxn-IR brains (Fig. 6C) comparable to en>mCD8::GFP,Dcr2,poxn-IR (Fig. S7G-L). Moreover, the number of GFP-labelled ring neurons (Fig. 6E and Table S1) in Poxn(P757)>mCD8::GFP,Dcr2,Poxn-IR brains was significantly increased (Mean=33, SEM=0.71; n=18) compared to Poxn(P757)>mCD8::GFP,Dcr2 controls (Mean 29, SEM=1.21; n=18). These data suggest a lineage-specific requirement for Poxn to specify the number and identity of EB ring neurons.

The observed phenotypes indicate a rather late function of Poxn in EB ring neuron specification and we wondered whether engrailed might also have a later function in addition to its early requirement for embryonic lineage formation. We therefore carried out Poxn-Gal4(P757) driven RNAi-mediated knockdown of engrailed which identified typical Poxn(P757)>mCD8::GFP labelled ring neurons and their projections into the EB ring neuropil, as shown for Poxn(P757)>mCD8::GFP,Dcr2,en-IR brains (Fig. 6D). However, the Poxn(P757)>mCD8::GFP,Dcr2,en-IR labelled EB ring neuropil was lacking the outer synapse layer typical for R2/4 neurons (Fig. 6D, compare to A and B). Moreover, the number of Poxn(P757)>mCD8::GFP labelled ring neurons (Fig. 6E and Table S1) in Poxn(P757)>mCD8::GFP,Dcr2,en-IR brains was significantly reduced (Mean=17; SEM=1.81; n=10). Together these data demonstrate that engrailed functions in Poxn(P757)-Gal4 targeted cells and is required for the specification of the number and identity of R2/4 neurons to establish the outer layer of the EB ring neuropil.

DISCUSSION

Lineage-specific formation of ellipsoid body ring neuron circuitry

Previous studies suggested the Drosophila EB as part of the central complex develops from precursor cells that differentiate during larval development and during pupal stages generate the EB neuropil (Hanesch et al., 1989; Renn et al., 1999; Ito and Awasaki, 2008; Yu et al., 2009; Bayraktar et al., 2010, Young and Armstrong, 2010; Omoto et al.,...
Our lineage analysis demonstrates that at least part of its origin can be traced back to the embryonic procephalic neuroectoderm. We identified Engrailed-expressing neuroblasts ppd5 as embryonic stem cells that give rise to Poxn expressing progeny which ultimately differentiate into EB ring neurons. Genetic tracing with en-Gal4 identified R1-R4 ring neurons, suggesting that embryonic neuroblasts ppd5 are the major source of Poxn expressing progeny leading to EB ring neurons detected in our study. Based on their position, morphology, gene expression patterns and axonal fasciculation, our findings suggest that ppd5-derived larval lineages (Fig. 3) correspond to previously described larval lineages variously called 'EB-A1/P1' (Ito and Awasaki, 2008; Ito et al., 2013; Yu et al., 2013; Yang et al., 2013), 'DALv2/3' (Spindler and Hartenstein, 2011; Lovick et al., 2013; Omoto et al., 2017), 'MC1' (Kumar et al., 2009), or 'DM' (Bayraktar and Doe, 2013; Yang et al., 2013). We previously demonstrated that these larval lineages express Poxn and give rise to gamma-amino butyric acid (GABA)-ergic ring neurons in the central complex of the adult brain (Shaw et al., 2018). We therefore propose to (re-) name them according to their embryonic origin.

Subclass-specific Gal4 lines together with Poxn expression identifies these lineage-related, ppd5-derived sister cells as R1-R4 ring neurons. Moreover, brain-specific Poxn-Gal4 mediated labeling identifies ring neurons and their axonal projections covering all layers of the EB neuropil, thus suggesting neuroblasts ppd5 give rise to the majority if not all ring neuron subtypes. The ontogenetic relationship between Engrailed-expressing neuroblasts ppd5 and Poxn-expressing EB ring neurons is affirmed by the fact that en-Gal4 and Poxn-Gal4-targeted RNAi-mediated knockdown of Poxn causes similar EB neuropil-specific phenotypes. Together, these data establish that ppd5-derived progeny are clonal units contributing to the ellipsoid body ring neuron circuitry in the central complex in Drosophila.

Lineage-related Poxn and engrailed function specifies EB ring neurons

How are these units specified? In both insects and mammals, the patterning and specification of neural lineages is regulated by genetic programs from neurogenesis to neuronal differentiation (e.g. Skeath and Thor, 2003; Guillemot, 2005; Gao et al., 2013; Allan and Thor, 2015). Our study in Drosophila shows that the development and specification of EB-specific circuit elements is likewise dependent on the lineage-specific activity of developmental regulatory genes. Early formation and maintenance of Poxn
expressing ppd5 lineages requires *engrailed* function as revealed with a deficiency removing both *engrailed* orthologues, *en* and *invected* (Fig. 2H, I). Previous studies showed that, *engrailed*/*invected* are required for the specification of neuroblast identity in the developing nervous system (Bhat and Schedl, 1997), suggesting that *engrailed* is also required for the specification of ppd5. We also found a later, lineage-specific function of *engrailed* in the specification of ring neuron numbers (Fig. 6), which is consistent with its transient expression in Poxn+ lineages in the embryonic brain (Fig. 3A, B) but not at later developmental stages nor in adult ring neurons (Fig. 3M and Fig. 4A-E). *engrailed* codes for a homeodomain transcription factor mediating the activation and suppression of target genes, regulatory interactions that are required for neural lineage formation and specification in the procephalic neuroectoderm (McDonald and Doe, 1997; Gallitano-Mendel and Finkelstein, 1997; Seibert and Urbach, 2010). In contrast, no function for *Poxn* in embryonic brain development has been reported (Awasaki and Kimura, 1997; Awasaki and Kimura, 2001; Boll and Noll, 2002; Minocha et al., 2017), suggesting that Poxn is only during later stages of development required for lineage and/or neuronal specification in the central brain.

Indeed, our experiments identify a postembryonic requirement of *Poxn* in the specification of ppd5-derived progeny. Previous studies showed that zygotic mutations of *Poxn* perturb EB neuropil formation, in that presumptive ring neurons are unable to project their axons across the midline and as a consequence, the EB ring neuropil is not formed (Boll and Noll, 2002; Minocha et al., 2017). In the present study, *en-Gal4* targeted knockdown of *Poxn* reveals Engrailed-expressing cells that project across the midline and form a ring-like neuropil instead of their normal ipsilateral projections to the superior protocerebrum (SP). Significantly, we did not observe any ppd5-derived GFP-labeled cells that project ipsilaterally towards the SP, neurons that are normally detectable with *en-Gal4* targeted GFP expression in the adult brain (Fig. 5B, asterisks). Furthermore, *en>Poxn-IR* targeted, EB neuron-like projections do not form a torroidal ring but are rather characterised by a ventral cleft. These *en>Poxn-IR* cells aberrantly retain Engrailed expression even though their axonal projection and connectivity pattern clearly identifies them as ring neurons that are normally devoid of Engrailed but instead express Poxn (Fig. 4C-E). Together these data suggest that, based on their morphology, Engrailed expression, axogenesis and ring-specific projection patterns,
en>GFP cells normally projecting to the superior protocerebrum have been transformed into EB ring neurons in en>mCD8::GFP,Dcr2,Poxn-IR flies.

The resulting additional ring neurons in en>mCD8::GFP,Dcr2,Poxn-IR flies are accompanied with a ventrally open EB ring neuropil. A comparable phenotype is seen in brains of Poxn(757)>Poxn-IR flies which are characterized by an increased number of Poxn(757)-Gal4 targeted ring neurons, suggesting that increasing numbers of EB ring neurons lead to an arch-like neuropil reminiscent of the arch-like ellipsoid body seen in the majority of arthropods (Strausfeld, 2012). In support of this notion, we previously demonstrated that in vivo amplification of ppd5-derived progenitor cells can lead to fully differentiated supernumerary GABAergic ring neurons that form functional connections often characterised by a ventrally open EB ring neuropil (Shaw et al., 2018). Together, these data identify differential roles of Poxn activity during neuroblast lineage formation, in that Poxn is required for cell identity determination of ppd5-derived progeny, as well as for the specification of cell numbers and terminal neuronal projections of EB ring neurons (Fig. 7).

These Poxn functions in ppd5-derived brain lineages are reminiscent of Poxn activity in the peripheral nervous system (PNS) which mediates the specification of sensory organ precursor (SOP) cell lineages giving rise to external sense organs, the tactile and gustatory bristles, respectively (Ghysen and Dambly-Chaudière, 2000). In these SOP lineages, differential Poxn activity determines progeny fate between chemosensory (gustatory) or mechanosensory (tactile) neuronal identities (Dambly-Chaudière et al., 1992; Awasaki and Kimura, 1997). Furthermore, SOP lineage specific Poxn function specifies the number of these neurons and their connectivity pattern (Nottebohm et al., 1992; Nottebohm et al., 1994; Awasaki and Kimura, 2001). The apparent functional commonalities between Poxn-mediated specification of ppd5 neuroblast derived lineages in the brain and SOP lineages in the PNS, suggest that evolutionarily conserved mechanisms (Alberch, 1991) underlie the development and specification of clonal units as cellular substrates for neural circuit and sensory organ formation.

Clonal units as cellular substrates for neural circuit evolution

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The cytoarchitecture of both the insect and mammalian brain are characterised by neural lineages generated during development by repeated asymmetric divisions of
neural stem and progenitor cells (Shen et al., 1998; Kim and Hirth, 2009; Sousa-Nunes and Hirth, 2016). These ontogenetic clones are thought to constitute building blocks of the insect and mammalian brain (Ito and Awasaki, 2008; Rakic, 2009). In support of this notion, lineage-related progeny constitutes sets of circuit elements of the mushroom bodies (Ito et al., 1997) and antennal lobes in Drosophila (Lai et al., 2008). Clonal relationship also characterizes the lineage-dependent circuit assembly in the mammalian brain, where stem cell-like radial glia give rise to clonally-related neurons that synapse onto each other, as has been shown for cortical columns and GABAergic interneurons in the neocortex (Noctor et al., 2001; Yu et al., 2009; Brown et al., 2011; Xu et al., 2014; Shi et al., 2017) and for striatal compartments of the basal ganglia (Kelly et al., 2018). Our study in Drosophila shows that a pair of bilateral symmetric, engrailed-expressing embryonic stem cells, neuroblasts ppd5, give rise to R1-R4 subtypes of tangential ring neurons that contribute to the layered EB neuropil. Thus, ppd5 neuroblast lineages constitute complete sets of circuit elements intrinsic to the adult central complex in Drosophila (Fig. 7).

It has been suggested that clonal expansion of neural lineages contributed to the evolution of complex brains and behaviours (Fish et al., 2008; Enard, 2011; Nielsen, 2015). Key to this hypothetical scenario are ancestral circuit elements in the form of genetically encoded stem cell-derived clonal units, like the ones described in our study here. In such a scenario, lineage-related ancestral circuit elements might have been multiplied and co-opted or diversified during the course of evolution. Multiplication and co-option have been suggested for the evolution of the multiple loop architecture of the basal ganglia that allows processing of cognitive, emotional and motor information (Stephenson-Jones et al., 2011; Enard, 2011). In line with this hypothesis, quantitative control of the transcription factor Prospero is sufficient to cause clonal expansion of ring neuron circuitry in Drosophila (Shaw et al., 2018) that has been implicated in cognitive and motor information processing (e.g. Fiore et al., 2015; Fiore et al., 2017; Kottler et al., 2019) and that resembles extensive correspondences to vertebrate basal ganglia, ranging from comparable developmental genetics to behavioural manifestations and disease-related dysfunctions (Strausfeld and Hirth, 2013).

In contrast to multiplication and co-option, the diversification of stem cell lineages can equally contribute to neural circuit evolution. Our results presented here identify
differential and tightly regulated spatio-temporal functions of *engrailed* and *Poxn* that lead to the differentiation of ppd5 progeny into hemi-lineage specific identities in the adult brain. Loss of *engrailed* affects the formation of precursors cells, whereas its lineage-specific knockdown affects the number of Poxn expressing ring neurons. Correspondingly, en-Gal4 driven lineage-specific knockdown of *Poxn* results in an identity transformation of Engrailed expressing neurons in the adult brain in that they no longer project to the superior protocerebrum, but instead reveal an EB ring neuron identity. This data indicate a binary switch of hemi-lineage identities as the result of a feed-forward mechanism between *engrailed* and *Poxn*. *Engrailed* may activate transcription (directly or indirectly) of *Poxn* which in turn represses *engrailed* to permit differentiation of R1-R4 neurons, thereby regulating the specification of neuronal identities in ppd5 hemi-lineages. This hypothesis is consistent with lineage tracing (Fig. 4) and MARCM experiments (Fig. S5), as well as the transient expression of *engrailed* in embryonic ppd5 lineages but not in adult EB ring neurons. However, further studies are required to elucidate the nature and extend of these putative regulatory interactions between *Engrailed* and *Poxn*.

In summary, our findings presented here establish a causal relationship between a pair of bilateral symmetric embryonic stem cells, *neuroblasts* ppd5 and the lineage-related assembly of their ellipsoid body ring neuron progeny as structural units of the central complex in *Drosophila*. Based on these observations we propose that amplification and diversification of ontogenetic clones together with the repurposed use or exaptation (Gould and Vrba, 1982) of resulting circuitries, is a likely mechanism for the evolution of complex brains and behaviours.

**MATERIALS AND METHODS**

*Drosophila* genetics

All lines were obtained from the Bloomington stock center and raised at 25°C with a 12h/12h light/dark cycle. Embryonic and larval gene expression studies were carried out using *w*^{118}; +; + and *w; en-Gal4, UAS-mCD8::GFP/(CyO); + (en>mCD8::GFP), unless otherwise stated.

To generate *Poxnbrain-Gal4* flies, the *Poxn* brain enhancer (Boll and Noll, 2002) was amplified by PCR from genomic DNA. The PCR product was subcloned into *pPTGal*...
vector using $X_{ba}l$ and $N_{ot}l$ sites, followed by sequencing; the genomic region 2R:11723830 to 11725559 was inserted into $pPT_{Gal}$. Primer sequences are:

forward, 5'-gtcctattaagtaccatgaaa-3';
reverse, 5'-aagcggccgcgttaagtaacgctcggtgg-3'.

Transgenesis was performed by BestGen Inc (CA, USA).

For lineage tracing, the following strains were used: $w^{1118}$ (control), $en\cdot Gal4$ ($en\cdot mCD8::GFP$), $Poxn\cdot Gal4$ or $Dac\cdot Gal4$ were crossed to $UAS\cdot mCD8::GFP$, $tub\cdot FRT\cdot CD2\cdot FRT\cdot Gal4$, $UAS\cdot FLP/CyO\cdot GMR\cdot Dfd\cdot YFP$ (Roy et al., 2007). Offspring were raised at 18°C to suppress leaky or unspecific FLP activity.

For analysis of ring neuron subtypes, the following enhancer trap lines were used: $c42\cdot Gal4$, $c105\cdot Gal4$, $c507\cdot Gal4$ and $c819\cdot Gal4$ (Renn et al., 1999; from S. Goodwin), $EB1\cdot Gal4$ (Wang et al., 2002; from T. Lee), $c232\cdot Gal4$ (Renn et al., 1999; from J.R. Martin), as well as $796\cdot Gal4$ (Martin-Peña et al., 2006; from A. Ferrus) in combination with $yw$; $P[UASmCD8::GFP.L]LL5$; +.

To study specification of ring neuron precursors the engrailed deficiency $f^{36a}_{Df(2R)en}$, $en^E$, $en^E$, $inv^E$/$CyO$; $mwh^1$, $jv^1$, $P[f^{13}]77A/TM2$, was used. RNAi was carried out using $en\cdot mCD8::GFP$ or $Poxn^{(757)}\cdot Gal4$. The lines $UAS\cdot Dcr2$, $UAS\cdot Poxn\cdot RNAi$, and $UAS\cdot en\cdot RNAi$, were obtained from the Vienna Drosophila RNAi Centre (Dietzl et al., 2007) and the Bloomington stock centre; experimental strains carrying $Dcr2$ and each RNAi construct were generated by genetic crosses using the double balancer line $w; If/CyO; MKRS/TM6b,Tb,Hu$. For overexpression of engrailed, we made use of $UAS\cdot en\cdot engrailed$ ($yw\cdot hs.FLP122$; $UAS.en$/ $TM2$; from J. Casal). For overexpression of Poxn, we generated transgenic $UAS\cdot Poxn$ lines using the full-length open reading frame of Poxn ($Poxn$ cDNA clone IP01592, Berkeley Drosophila Genome Project from the Drosophila Genomics Resource Center). cDNA was 6x His tagged at the N-terminus and sub-cloned into $pUAST$. Transgenesis was performed by BestGene Inc (CA, USA).

**Immunohistochemistry and image analysis**

Immunostainings were performed as previously described (Hirth et al., 2003; Diaper et al., 2013; Diaper and Hirth, 2014). Rabbit anti-Poxn antibody was generated using $pUAST\cdot HisPoxn$ derived protein purified by GenScript (New Jersey, USA). Purified Poxn...
protein was injected into rabbits for antibody production by Pab productions (Hebertshausen, Germany).

Primary antibodies used were: mouse anti-Dachshund, 1:20 (mAbdac2-3, Developmental Studies Hybridoma Bank, DSHB); mouse anti-Engrailed, 1:2 (4D9, DSHB); rabbit and chicken anti-GFP, 1:500 (Thermo Fisher/Invitrogen – A6455 and Abcam – Ab13970, respectively); goat anti-HRP (Cy3 conjugated- 123-165-021, Cy5 conjugated – 115-175-146), 1:50 (all Jackson ImmunoResearch Labs); rabbit anti-Poxn, 1:200 (Boll and Noll, 2002; from M. Noll); mouse anti-Poxn, 1:100 (Hassanzadeh Gh, et al., 1998; a kind gift from A. Ghysen); rabbit anti-Poxn, 1:400 (generated as described above); mouse anti-Synapsin, 1:50 (3C11, DSHB); mouse anti-Brp, 1:20 (nc82, DSHB); rabbit anti-Miranda, 1:200 (Shaw et al., 2018); mouse anti-Prospero, 1:5 (mAbMR1A, DSHB); rabbit anti-pH3, 1:400 (Sigma-Aldrich, 06-570);. Secondary antibodies were Alexa fluorochromes at 1:150 (Invitrogen). Embryos, larval CNSs and adult brains were mounted in Vectashield with DAPI (H-1200, Vector Laboratories).

Fluorescence samples were scanned and recorded either with a Leica TCS SP5 or A1R Nikon confocal microscopes in sequential scanning mode. Leica TCS SP5 was equipped with Leica Application Suite Advanced Fluorescence (LAS-AF) software, HCX PL APO lambda blue 20.0x 0.70 IMM UV 0.70 numerical aperture (NA) and HCX PL APO CS 40.0x1.25 OIL UV 1.25 NA objectives. A1R Nikon confocal was equipped with Elements Confocal software, Plan Fluor 40x oil DIC H N2 NA 1.3 and Plan Apo VC 20x DIC N2 0.75 NA objectives. Whole mount adult brains were scanned using the same confocal settings for each genotype. Z-projections were created and analysed using FIJI. Neurons expressing UAS-mCD8::GFP were counted using the ImageJ Cell Counter Plugin (http://rsbweb.nih.gov/ij/plugins/cell-counter.html). Images were processed using Adobe Photoshop and figures constructed in Adobe Illustrator.

**Statistics**

Statistical analysis was carried out using GraphPad prism 6. Comparison of means from multiple experimental conditions (>2) with one independent variable was performed using the one-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparisons post-hoc test. The alpha level for all tests was 0.05; for details see Supplementary Table S1.
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Competing interests
The authors declare no competing or financial interests.

Author contributions
Z.N.L. and J.C.B. carried out experiments; Z.N.L., J.C.B. and F.H. analysed the data; F.H. directed the study and prepared the manuscript.

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Supplementary information
Supplementary information includes one table and 8 figures available online at http://

REFERENCES


**FIGURE LEGENDS**

**Fig. 1. Engrailed-expressing embryonic neuroblasts ppd5 and ppd8.** (A) Schematic of stage 11 embryo showing Engrailed (En) expression in the ectoderm (grey areas: hs, head spot; as, antennal stripe; is, intercalary stripe; md, mandibular stripe; mx, maxillary stripe) and (B), in the neuro-ectoderm from which brain neuroblasts delaminate (B, grey dots); these include neuroblasts ppd5 and ppd8 (B, green dots) that derive from the En head spot; lateral views, anterior to the left. (C-H) at stage 11, en>mCD8::GFP (green) visualises expression patterns that mimic endogenous En expression, including the head spot (C, F, dashed areas) as well as neuroblasts ppd8 (D) and ppd5 (E) that both express mCD8::GFP (green) and En (magenta). (F) Dachshund (Dac, magenta) expression in the anterior head ectoderm is also found in the En head spot (F, dashed area) and in neuroblast ppd8 (G) but not in neuroblast ppd5 (H, arrowhead), both of which express en>mCD8::GFP (in H, ppd8 is highlighted with asterisk). D, E, are enlargements of the dashed area in C at different focal planes; G, H, enlargements of the dashed area in F at different focal planes. C, F, projections of confocal sections; D, E, G single sections; H, two confocal sections. n > 20 for each condition; scale bar, 25μm.

**Fig. 2. Neuroblasts ppd5/8 derived neural lineages in the embryonic brain express Poxn and require engrailed function.** (A, C, E, G) Stage 14 embryonic w¹¹¹⁸ control brains labelled with anti-HRP (A, C, E, magenta), dorsal views, anterior up. (B, D, F) en-Gal4 driven UAS-mCD8::GFP expression visualises progeny of neuroblasts ppd5/8 in the posterior protocerebrum (magenta), single confocal sections. (A, G) Poxn expression (green) is detectable in two clusters in the brain (HRP, magenta), in the protocerebrum (asterisks) and deutocerebrum; note that A and G show the same brain. (B) anti-Poxn immunolabelling (green) identifies Poxn expression in en>mCD8::GFP labelled ppd5/8 progeny (white arrows). (C) En expression (green) can be seen in 3 clusters in the embryonic brain; in the anterior protocerebrum, posterior protocerebrum (asterisks) and posterior deutocerebrum. (D) anti-En immunolabelling (green) identifies En expression in en>mCD8::GFP labelled ppd5/8 progeny (white arrows). (E) Dachshund (Dac, green) is expressed in several areas of the embryonic brain including the posterior protocerebrum (asterisks). (F) anti-Dac immunolabelling (green) identifies Dac expression in en>mCD8::GFP labelled ppd5/8 progeny (white arrows). (G) Single-channel image of A showing Poxn-expressing cells, including posterior protocerebral
cluster (arrowheads). (H) Embryonic brain of homozygous deficiency Df(2R)enE labelled with anti-HRP (magenta); arrowheads indicate the position of the posterior protocerebrum, which is devoid of Poxn immunolabelling. (I) Single channel showing absence of Poxn expression in the brain. n > 20 for each condition; scale bars, 50μm.

Fig. 3. Ellipsoid body precursor cell expansion and axogenesis of Poxn::GFP labelled ring neurons. (A-M) Poxn-Gal4>UAS-mCD8::GFP labelled brains visualise GFP-expressing neurons in the posterior protocerebrum of the embryonic brain at stage 14 (A, B), during larval stages L1 (C, C'), L2 (D, D'), L3 (F-J) and in the adult (K-M). (A, B) Poxn>mCD8::GFP expression visualises protocerebral Poxn+ lineages (blue) in the embryonic brain (anti-HRP, magenta); co-immunolabelling with anti-Engrailed (green) reveals that embryonic Poxn-Gal4+ cells co-express Engrailed (arrowheads; A frontal view, B lateral view). (C-E') Immunolabelling with anti-pH3 (magenta) visualises phosphorylated Histone H3 as a marker of mitotic activity. (G-J) Immunolabelling with anti-Miranda (anti-Mira) visualises precursor cells (G, I, asterisks) and reveals that Poxn>mCD8::GFP cells are devoid of Miranda expression. Immunolabelling with anti-Prospero (anti-Pros) labels differentiating neurons (H-J) in the larval brain (Br) and ventral nerve cord (VNC); posterior protocerebral Poxn>GFP cells co-express Prospero and during larval stages L2/L3 send neuronal projections towards the midline (J, arrowheads). Poxn>mCD8::GFP visualises adult EB ring neurons (K-M, arrowheads) that are immunoreactive for anti-Poxn (L) but not for anti-Engrailed which labels cells adjacent to Poxn>mCD8::GFP positive ring neurons (M). n > 20 for each condition; scale bar, 50μm.

Fig. 4. Genetic tracing of engrailed-expressing ppd5 lineages reveals Poxn-expressing ellipsoid body ring neurons in the adult brain. (A) en>mCD8::GFP expression in the adult brain (dashed area enlarged in B). (B) en>mCD8::GFP expressing cells (arrowheads) in the posterior protocerebrum; they project towards and arborise the superior protocerebrum (arrows) dorsal to the ellipsoid body neuropil (outlined as dashed circle). (C-E) en>mCD8::GFP-labeled cells located in the posterior protocerebrum express Engrailed (C) but not Poxn (D), showing no overlap of anti-En and anti-Poxn (E). (F) Schematic of en>mCD8::GFP targeted cells and their projections in the adult brain: En-expressing cells (blue) reside adjacent to Poxn-expressing cells (red) and send axons (grey) to the superior protocerebrum. (G) Genetic tracing of en>tub>mCD8::GFP in the...
adult brain (dashed area enlarged in H) visualises cells and axons similar to, but stronger than *en>mCD8::GFP* (compare to A) as well as ring neurons and the EB neuropil. (H) *en>tub>mCD8::GFP* expressing cells (arrowheads) in the posterior protocerebrum project towards and arborise into the superior protocerebrum (arrows) but also into the ellipsoid neuropil (dashed circle). (I-K) *en>tub>mCD8::GFP* expressing cells located in the posterior protocerebrum express En (I) and Poxn (J). Note that anti-En does not overlap with anti-Poxn immunoreactivity (K). (L) Schematic of *en>tub>mCD8::GFP* targeted cells and their projections in the adult brain; En-expressing cells (blue) reside adjacent to Poxn-expressing cells (red) and send projections (grey) to the superior protocerebrum (SP); Poxn-expressing neurons (red) send projection (grey) along the lateral triangle (LTR) into the EB ring neuropil (EB). *n > 20* for each condition; scale bars, 50μm in G & H; 10μm in K.

**Fig. 5. Lineage-specific genetic manipulation of engrailed and Poxn.** Confocal images of whole-mount adult brains expressing *mCD8::GFP* and immunolabelled either with anti-Syntaxin/3C11 (A, H, O, magenta), or with anti-Engrailed (C, F, G, J, M, N, Q, T, U, magenta) and anti-Poxn (E, F, G, L, M, N, S, T, U, cyan); dorsal is up. (A) In *en>mCD8::GFP,Dcr2* the ellipsoid body (EB) neuropil is not visualised (arrow); (B) GFP expressing cells (arrowheads) send their projections ipsilaterally (asterisks) to the superior protocerebrum (SP), dorsal to EB neuropil (arrow). (C) *en>mCD8::GFP* cells express engrailed and (D) GFP, but (E) not Poxn, which together are (F) expressed in close vicinity but (G) non-overlapping (arrowheads). (H) In *en>mCD8::GFP,en*, UAS-mediated overexpression of *engrailed* reveals (I) GFP expressing cells (arrowheads) sending projections ipsilaterally (asterisks) to the SP (arrow indicates position of EB neuropil). (J) *en>mCD8::GFP,en* cells express engrailed and (K) GFP, but (L) not Poxn, which together are (M) expressed in close vicinity but (N) non-overlapping (arrowheads); compare to corresponding control in Fig. 4A-E. (O) In *en>mCD8::GFP,Dcr2, Poxn-IR*, RNAi-mediated knockdown of Poxn in ppd5/8 lineages reveals GFP-positive EB neuropil (arrow); (P) GFP expressing cells (arrowheads) send their projections contralaterally (asterisks) into the EB neuropil (arrow). (Q) *en>mCD8::GFP,Dcr2, Poxn-IR* cells express engrailed and (R) GFP, some of which (S) also express Poxn, which together (T) are co-expressed in (U) some GFP-expressing ring neurons (arrowheads). *n > 10* for each condition; scale bars, 50μm in A; 10μm in G, N, U.
Fig. 6. Specification of ellipsoid body ring neuron requires *Poxn* and * engrailed*. Confocal images of whole-mount adult *Poxn(757)>mCD8::GFP* brains; dorsal is up. (A) *Poxn(757)>mCD8::GFP* visualises selection of ellipsoid body (EB) ring neurons (arrows) and their projections terminating in R1-R4 layers of the EB ring neuropil (arrowhead). (B) A comparable GFP expression pattern is seen in *Poxn(757)>mCD8::GFP, Dcr2* brains. (C) In *Poxn(757)>mCD8::GFP, Dcr2, Poxn-IR* brains, RNAi-mediated knockdown of *Poxn* reveals more GFP-positive EB ring neurons (arrows) and a ventrally open EB neuropil (arrowhead). (D) In *Poxn(757)>mCD8::GFP, Dcr2, en-IR* brains, RNAi-mediated knockdown of *engrailed* reveals less GFP-positive EB ring neurons (arrows) and a diminished outer layer of the EB ring neuropil (arrowhead). (E) Quantification of *Poxn(757)>mCD8::GFP* targeted EB ring neurons reveals increased cell numbers in *Poxn(757)>mCD8::GFP, Dcr2, Poxn-IR* brains (n=18, mean=33, SEM=0.71, p=0.0186) compared to *Poxn(757)>mCD8::GFP, Dcr2* controls (n=18; mean=29, SEM=1.21), but significantly less cells in *Poxn(757)>mCD8::GFP, Dcr2, en-IR* (n=10; mean=17, SEM=1.81, p<0.0001). P-values were determined with Bonferroni's multiple comparisons test, see Table S1 for details. Scale bar, 10μm.

Fig. 7. *Poxn*-expressing ellipsoid body ring neurons R1-R4 descend from *engrailed*-expressing neuroblasts ppd5. During embryogenesis, *engrailed* expressing neuroblasts ppd5 and ppd8 (large blue circles) derive from the procephalic neuroepithelium; they can be distinguished by Dachshund expression (Dac+) restricted to ppd8. At stage 11, ppd5/8 have produced a small number of Engrailed-expressing progeny (small blue circles). At stage 14, two classes of ppd5/8-derived neuron are visible: En+/Poxn- (small blue circles) and En-/Poxn+ (small red circles). At this stage, cells are already sending axons towards the interhemispheric commissure. The lineages continue to expand during larval and pupal development and acquire their adult morphology during metamorphosis. Genetic tracing and mosaic analysis with a repressible cell marker identify En+/Poxn- (small blue circles) and En-/Poxn+ (small red circles) as hemi-lineages derived from bilateral symmetric neuroblasts ppd5. *Poxn* neuro expression identifies R1-R4 ring neurons of the adult ellipsoid body.
SUPPLEMENTARY INFORMATION

Lineage-specific determination of ring neuron circuitry in the central complex of Drosophila

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Table S1. Quantification of phenotypes and statistical tests used.

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Bonferroni’s multiple comparisons

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**Fig. S1: Engrailed and Poxn expression in the larval central nervous system.**
Confocal images of whole-mount larval CNS' immunolabelled with anti-Poxn and anti-Engrailed; anterior is up. (A) Late L1 larval CNS, 44-48 hrs after egg laying (AEL). Poxn expression can be detected in two clusters of the anterior brain in close vicinity to En expressing cells, including the posterior protocerebrum (dashed squared area, enlarged bottom left). (B) Late L2 larval CNS, 68-72 hrs AEL. Poxn expression is still detectable in the posterior protocerebrum in close vicinity to En expressing cells (dashed squared area, enlarged bottom left). (C) The same applies to late L3 larval CNS, 90-96 hrs AEL. Poxn expression is still detectable in close vicinity to En expressing cells in the posterior protocerebrum (dashed squared area, enlarged bottom left). n > 20 for each condition; scale bars, 50μm.
**Fig. S2.** *en-Gal4, UAS-mCD8::GFP* recapitulates endogenous Engrailed expression in L3 and adult brains. (A) late 3rd instar larval CNS expressing *en>mCD8::GFP* and co-labelled with DAPI to show the outline of the CNS. (B) enlargement of dashed area shown in A. Posterior protocerebral cluster of *en>mCD8::GFP*-expressing cells (dashed box) are also positive for anti-En. (C-E) enlargements of dashed area shown in B. There is complete colocalisation between *en>mCD8::GFP* and anti-En. (F) adult *en>mCD8::GFP* brain labelled with anti-En showing the adult *en>mCD8::GFP* projection pattern. (G-I) enlargements of dashed area shown in F. There is complete colocalisation between *en>mCD8::GFP* and anti-En. Abbrev.: SP, superior protocerebrum; AL, antennal lobes; TC, tritocerebrum. $n > 20$ for each condition; scale bars, 100 µm.
Fig. S3. Posterior protocerebral *engrailed* expressing cells visualised with *en>mCD8::GFP* and *en>tub>mCD8::GFP*. Confocal images of late 3rd instar larval CNS, anterior is up. (A) *en>mCD8::GFP* and DAPI labelling reveals *engrailed* expressing lineages in the larval CNS (same specimen as in Fig. S2A; dashed area illustrates enlarged area in B, but B shows different specimen). (B) *en>mCD8::GFP*-labelled cells in the posterior protocerebrum (dashed box) project anteriorly and towards the midline. (C-E) *en>mCD8::GFP*-labelled cells (C) in the posterior protocerebrum do not express Poxn (D, E). (F) Schematic of cell and lineage labelling using *en>tub>mCD8::GFP*. *en-Gal4* activates expression of *UAS-FLP* which mediates mitotic recombination at FRT sites. This leads to excision of CD2 ‘stop’ element, allowing the *tub* enhancer to activate Gal4, which in turn maintains GFP expression in this cell and all its progeny. (F) Genetic tracing with *en>tub>mCD8::GFP* reveals more GFP-expressing cells than *en>mCD8::GFP* in the posterior protocerebrum (dashed box); neurons project anteriorly and towards the midline. (G-J) *en>tub>mCD8::GFP* labelling reveals GFP-expressing cells (G) that are immunolabelled with anti-Poxn (H-J). *n > 20* for each condition; scale bar, 100μm.
Fig. S4. Genetic tracing with en>tub>mCD8::GFP invariably labels the ellipsoid body in the adult brain. Confocal images of whole-mount adult brains; dorsal is up. (A-I) Genetic tracing with en>tub>mCD8::GFP consistently reveals GFP-expressing ellipsoid body (EB) ring neuron cell bodies (arrowheads) and their projections to the EB ring neuropil (asterisks). n = 70; scale bar, 100μm.
Fig. S5. Lineage analysis of Engrailed and Poxn-expressing neurons in the posterior protocerebrum. Mosaic analysis with a repressible cell marker (MARCM). (A) section of adult central brain showing a posterior protocerebral MARCM clone. The clone includes cells that express Engrailed (anti-en; solid arrowheads, see also B) and project to the superior protocerebrum (solid arrows); and cells expressing Poxn (antiPoxn; open arrowheads, see also C) that send projection to the ellipsoid body (open arrows) that terminate in the ring neuropil (asterisk). (B) anti-engrailed labels GFP-positive cells (arrowhead) of the MARCM clone. (C) anti-Poxn labels GFP-positive cells (arrowhead) of the MARCM clone. (D) Same section as B and C with co-immunolabelling of anti-Poxn adn anti-en showing that Poxn and Engrailed expressing cell clusters are adjacent to one another. Note that the presence of both Poxn and Engrailed expressing cell types in a single MARCM clone demonstrates that their ontogenetic relationship as sister cells. $n > 20$; scale bar, 50$\mu$m.
Fig. S6. Ellipsoid body ring neurons R1-R4 express Poxn. (A) Schematic of R1-R4 subtypes of ellipsoid body ring neurons and their layer-specific projections into the ring neuropil; LTR, lateral triangle. (B) c105>mCD8::GFP visualises R1 neurons. (C) c42>mCD8::GFP labels R2 & R4m neurons. (D) c507>mCD8::GFP visualises R3 and R4d neurons. (E) c819>mCD8::GFP visualises R2 & R4m neurons. (F) 796>mCD8::GFP visualises R3 & R4d neurons. (G) EB1>mCD8::GFP visualises R2 & R4m neurons. (H) c232>mCD8::GFP visualises R3 & R4d neurons. Anti-Poxn immunolabelling (magenta) reveals Poxn expression in all subtypes of R1-R4 ellipsoid body ring neurons. n > 10 for each condition.
**Fig. S7. en-Gal4 UAS-RNAi mediated knockdown of Poxn.** Confocal images of whole-mount adult brains expressing mCD8::GFP and immunolabelled with anti-Syntaxin/3C11 (magenta); dorsal is up. (A-F) In en>mCD8::GFP, Dcr2 brain, the ellipsoid body (EB) neuropil is not visualised (arrow); GFP expressing cells (arrowheads) send their projections ipsilaterally (asterisks) into the superior protocerebrum, dorsal to the EB ring neuropil (position indicated with arrow). (G-L) In en>mCD8::GFP, Dcr2, Poxn-IR, RNAi-mediated knockdown of Poxn in ppd5 lineages reveals GFP expressing cells (arrowheads) that send their projections contralaterally (asterisks) into the EB ring neuropil (arrow) which appears ventrally open to varying degrees. n > 10 for each condition; scale bars, 50μm.
Fig. S8. RNAi-mediated knockdown of Poxn removes Poxn immunoreactivity.

Confocal images of whole-mount adult brains immunolabelled with anti-Poxn (magenta); dorsal is up. (A) In EB1>mCD8::GFP, Dcr2 brains, GFP-labelled ellipsoid body (EB) ring neurons send projections into the EB ring neuropil. (B) EB1>mCD8::GFP, Dcr2, cells express GFP and (C) are immunoreactive for anti-Poxn, revealing (D) that all GFP-positive cells are also labelled with anti-Poxn (arrowheads). (E) In EB1>mCD8::GFP, Dcr2, Poxn-IR brains, RNAi-mediated knockdown of Poxn reveals GFP-labelled cells in the posterior protocerebrum (arrowheads) sending projections to the EB (arrow). (F) EB1>mCD8::GFP, Dcr2, Poxn-IR, cells express GFP in close vicinity to (G) anti-Poxn immunoreactive cells; however (H) the majority of GFP expressing are devoid of anti-Poxn immunoreactivity (arrowheads). n > 10 for each condition; scale bars, 50μm in A, E; 10μm in D, H.