DROSOPHILA EMBRYONIC TYPE II NEUROBLASTS: ORIGIN, TEMPORAL PATTERNING AND CONTRIBUTION TO THE ADULT CENTRAL COMPLEX

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DISSERTATION ABSTRACT

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The large numbers of neurons that compose the adult brain display an immense amount of diversity. Repeated divisions of a relatively small pool of neural stem cells generate this neuronal diversity during development. To increase progress towards medical treatments for neurodegenerative diseases, it is of interest to understand both how neural stem cells generate the assortment of neurons and how these neurons come together to form a functional brain. Brain assembly occurs sequentially across time with early events laying the foundation for later events. Drosophila neural stem cells, neuroblasts (NBs), are an excellent model for investigating how neural diversity is generated and what roles early and late born neurons have in shaping the stereotypical adult brain structure. Generation of neural diversity, begins with specifying the diverse population of stem cells, called spatial patterning, and continues with diversifying neurons made from the diverse stem cells, called temporal patterning. Drosophila NBs exhibit both spatial and temporal patterning. Drosophila NBs have three types of division modes: type 0, type I and type II. Type II NBs expand the number of neurons made with progeny that exhibit a transit-amplifying division pattern, similar to that of mammalian outer subventricular zone (OSVZ) progenitors. Additionally, type II NBs exhibit temporal patterning across both the NB and their progeny to generate a large diversity of

neurons that populate a conserved region of the brain responsible for many sensory and motor functions, called the central complex.

Type II NBs have only been identified and studied during later stages in development, with nothing known about their origin or early divisions. In this dissertation, I describe the early lineages of the type II NBs within the Drosophila embryo. I show that type II NBs and lineages originate early in development, exhibit temporal patterning across both the NB and transit-amplifying progeny, and produce neurons that survive into the adult brain to innervate and potentially serve as a foundation within the adult central complex. Additionally, I explain how live imaging of the developing Drosophila brain can answer questions not easily addressed through other methods.

This dissertation includes unpublished co-authored material.

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

Drosophila Neurogenesis as a Model System: From Embryo to Adult 1

Neural Diversity with Drosophila Neuroblast Lineages 3

Spatial and Temporal Patterning 3

Diversity of Drosophila Neuroblast Division Patterns 5

Drosophila Type II NBs as a Model for Diversity Generation 6

Type II Lineages Role in Neurogenesis 8

II. EMBRYONIC TYPE II NEUROBLASTS: ORIGIN, TEMPORAL PATTERNING, AND CONTRIBUTION TO THE ADULT CENTRAL COMPLEX 12

Introduction 12

Results 15

All type II neuroblasts arise during embryogenesis 15

Embryonic type II neuroblasts generate INPs, GMCs, and neurons during embryogenesis 16

Embryonic type II neuroblasts and INPs undergo quiescence 23

Embryonic type II neuroblasts undergo a late temporal transcription factor cascade 23

Embryonic INPs undergo a truncated temporal transcription factor cascade 26

Embryonic-born INPs contribute to the adult central complex 29

Chapter Page

Discussion 33

Materials and Methods 36

Fly stocks 36

Immunofluorescent staining 36

Clones 37

FLEX-AMP immortalization of embryonic INPs 37

Cell proliferation analysis 38

Imaging 38

Bridge to Chapter III 36

III. LIVE IMAGING OF DROSOPHILA EMBRYOS: NEUROECTODERM AND TYPE II LINEAGES 40

Embryonic Type II formation from the neuroectoderm 40

Embryonic Type II division profile 46

Microscope options for live imaging 48

Technical considerations for live imaging 51

IV. CONCLUSION 55

Specification of type II identity within the embryo 55

Diversity within the type II NB population 56

Differences between embryonic and larval INPs 57

Integration of temporal factors in embryonic NBs and INPs 59

Function of the embryonic-born neurons born within the embryo 59

Integration of temporal factors in embryonic NBs and INPs 59

Chapter Page

APPENDIX: SUPPLEMENTRAL MATERIAL FOR CHAPTER II 63

REFERENCES CITED 64

LIST OF FIGURES

Figure Page

CHAPTER II

1. Eight type II neuroblasts arise during embryogenesis 17

2. Clonal analysis shows that type II neuroblasts make INPs, GMCs and neurons during embryogenesis. 18

3. Embryonic INPs undergo asymmetric cell division 21

4. INPs undergo quiescence across the embryo-larval transition 25

5. Embryonic type II neuroblasts undergo a late temporal transcription factor cascade 26

6. Embryonic INPs express the Dichaete temporal transcription factor 28

7. Embryonic INP progeny contribute to the adult central complex 32

CHAPTER III

1. Embryonic type II neuroblasts arise from PntP1 positive neuroectoderm 42

2. Embryonic PntP1 positive neuroectoderm has placodal morphology 44

LIST OF TABLES

Table Page

CHAPTER II

1. Type II neuroblast clones contain INPs, GMCs, and neurons 20

2. Dichaete is expressed in embryonic INPs 29

CHAPTER III

1. Gal4 lines used to identify embryonic type II neuroblasts 46

2. Markers used for live imaging..... 48

3. Genotypes imaged on live imaging microscopes 51

CHAPTER I

INTRODUCTION

Developmental biology aims to 1) understand how organisms self-assemble using only the instructions within DNA and proteins inherited from the parental egg and sperm and 2) use this knowledge base to contribute to medical advancements in disease treatment. An understanding of how the immense neuronal diversity and intricate circuitry of the brain forms during neurogenesis may lead to stem cell based therapies for neurodegenerative diseases. A large number of neurons with unique identities are required for an organism's survival and success within its environment. Repeated divisions of a relatively small pool of neural stem cells during neurogenesis create these neurons, but much remains unknown about this process. How does the neuronal diversity arise during development? How do the neurons and glia arrange themselves to build a properly functioning brain? Drosophila neural stem cells, neuroblasts (NBs) are an excellent model to ask these developmental questions.

Drosophila Neurogenesis as a Model System: From Embryo to Adult

Drosophila neurogenesis occurs in two distinct regions (the brain and the ventral nerve cord (VNC)), in two distinct phases (embryonic and larval), from repeated divisions of NBs that produce unique and invariant lineages of neurons and glia (Bossing et al., 1996; Schmid et al., 1999). The short embryonic wave of neurogenesis begins with the formation of a cell layer called the neuroectoderm (NE). Every NB responsible for producing the neurons and glia of the brain and ventral nerve cord (VNC) forms from the NE. Following embryogenesis, a five-day larval stage of neurogenesis produces a vast

majority of the adult neurons followed by intense morphogenesis and circuit assembly during the pupal stage (Doe, 2008).

Detailed work in the VNC has revealed the gene regulatory network involving the Notch signaling pathway within proneural clusters of NE cells responsible for bestowing a NB identity onto a stereotyped number of cells delaminating from this NE layer (Cabrera, 1990). Upon delamination from the NE, the NBs divide in a self-renewing fashion to produce neurons and glia until the end of embryogenesis when all but five central brain (CB) NBs enter a period of quiescence lasting until early larval stages (Egger et al., 2008). Entry into quiescence varies among the NB population, with NBs entering at different, yet consistent, times during late embryogenesis (Datta, 1995; Lai and Doe, 2014). After this quiescent period, NB divisions resume for the remainder of larval life until the NBs either differentiate or die during the remodeling that occurs during pupal stages (Sousa-Nunes et al., 2010; Voigt et al., 2002).

As quiescent NBs are mitotically inactive, small in size, and transcriptionally silent for a period of 30 hours, it has been difficult to link the embryonic neuroblasts to their larval counterparts (Datta, 1995). For many larval NB lineages, the identity of the embryonic counterpart and function of embryonic-born neurons remain to be determined. To date, ~30 VNC NB lineages and the four mushroom body NBs in the central brain (CB) are the only larval NBs with identified embryonic equivalents (Kunz et al., 2012; Shepherd et al., 2016). To overcome this, more permanent ways to identify NBs are needed, such as unique molecular signatures, genetic immortalization strategies, dye based labeling, or more detailed understanding of the lineages of individual NBs.

Embryonic-born neurons constitute only ~10% of the adult neuronal population (Ito and Hotta, 1992; Truman and Bate, 1988). For this reason, it is thought embryonic neurons carry out larval survival tasks such as navigation, prey evasion, or feeding and die before adult stages (Marin et al., 2005; Truman et al., 2004). However, embryonic neurons may serve other developmental roles, such as a pioneer role in guiding larval-born neurons to their correct targets, or a scaffolding role in building adult neuropil structures (Lin et al., 14AD; Raper, J. A., Bastiani, M. J. and Goodman, 1984; Riebli et al., 2013). Alternatively, some embryonic neurons may survive to have an important role in adult brain function (Kunz et al., 2012). As the adult brain requires an astounding diversity of neuronal and glial cells arranged in a precise fashion to properly function, both embryonic and larval NB lineages are excellent models for understanding the generation of neuronal diversity and the sequential stages required to assemble the neurons into a functional adult brain.

Neural Diversity with Drosophila Neuroblast Lineages

Spatial and Temporal Patterning

Generation of neuronal diversity occurs along multiple axes. First, transcription factor expression within the NE specifies unique lineage information within each NB (McDonald and Doe, 1997; McDonald et al., 1998; Weiss et al., 1998). For example, intermediate neuroblasts defective (ind) expression is required to specify the fate of the a column of VNC NBs; misexpression within another column or removal from the VNC results in misspecification of NB identity (Weiss et al., 1998). Furthermore, differences in spatial factor expression between embryonic mushroom NBs is thought to produce the

differences in lineages between the four NBs within the embryo and larva (Kunz et al., 2012). Additionally, single spatial factor changes within the NB can convert NB lineage identity. A single transcription factor, orthodenticle (otd) within a larval NB is essential for production of progeny with correct identity (Sen et al., 2014). However, as there is more diversity of neuronal cell types than NB progenitors, spatial patterning alone does not account for the observed neuronal diversity.

A second axis of patterning within single NB lineages specifies different types of neurons and glia along a temporal axis. Many temporal patterning molecules have been identified in both embryonic and larval NB lineages. In the embryonic VNC, NBs express a sequence of transcription factors Hunchback (Hb) > Krüppel (Kr) > Pdm > Castor (Cas) > Grainy head (Grh) temporal transcription factors (Isshiki et al., 2001). This sequence is expressed in nearly every NB with each NB expressing one factor at a time and producing one progeny in each window. Additionally, NB temporal patterning sequences have been identified within the optic lobe, larval NBs, and others (Li et al., 2013).

Although the factors are common among lineages, the progeny produced in each window differ among lineages. NB7-1 produces a U1 motoneuron during the Hb window, whereas NB 4-2 produces RP2 motoneuron within the same window, suggesting spatial and temporal patterning are both independently required for diversity generation (Isshiki et al., 2001). Neither the spatial factors nor the temporal factors alone are sufficient to produce the observed neuronal diversity. The observed diversity suggests a requirement for integration of the spatial and temporal patterning molecules, which has recently been shown within the optic lobe (Erclik et al., 2017).

Diversity of Drosophila Neuroblast Division Patterns

NB lineages exhibit diversity in the identity of the progeny produced, but also in the cellular division profile of its progeny. Type 0 NBs divide to directly generate a progeny that differentiates into a single neuron or glia (Baumgardt et al., 2014). Type I NBs divide to generate a ganglion mother cell (GMC) intermediate that divides only once to produce two neurons or glia (Doe, 2008). A third type of NB creates more neurons and glia per division than a type 0 or type I. Type II NBs divide to generate four to six neurons or glia per division through transit-amplifying intermediates, termed intermediate neural progenitors (INPs) (Ali Bayraktar et al., 2010; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009). The majority of NBs in the CB and VNC have type I division profile, with rare type 0 divisions having been identified only in the late embryonic VNC from a switch in division pattern from type I lineages (Baumgardt et al., 2014)..

Regulation of how type 0, I or II division profiles originate within NBs is poorly understood, but the transcription factors responsible for type I vs. type II divisions are well studied. All NBs require the transcription factor Deadpan (Dpn) to maintain the ability for self-renewing divisions (Doe, 2008). All type I NBs express the neural precursor gene Asense (Ase), responsible for regulating mitotic activity in NBs and GMC progeny (Brand et al., 1993; Doe, 2008). Type II NBs do not express Ase, but instead express the EGF pathway transcription factor isoform, PointedP1 (PntP1) (Xie et al., 2016; Zhu et al., 2011). PntP1 has been shown to be necessary for generation of INP progeny and repression of Ase in type II NBs (Zhu et al., 2011). Misexpression of PntP1 within larval type I NBs leads to loss of Ase expression and generation of small progeny

with an INP molecular profile. Type I NBs are thus Dpn positive, Ase positive and PntP1 negative, whereas type II NBs are Dpn positive, Ase negative and PntP1 positive. Within type II lineages, the INP progeny resemble an intermediate between type I and type II NBs. Studies within larval type II lineages have reported that INPs have a maturation period of ~six hours before their first division (Homem et al., 2013). Immature INPs have a molecular profile that is PntP1 positive, Ase negative, Dpn negative but with maturation PntP1 expression declines and Dpn expression begins (Zhu et al., 2011).

Drosophila Type II NBs as a model for diversity generation

Type II NBs are an active area of interest for multiple reasons. Only 16 type II NB lineages exist in the larval CB with just one reported within the embryonic brain (Ali Bayraktar et al., 2010; Bello et al., 2006; Boone and Doe, 2008; Bowman et al., 2008; Hwang and Rulifson, 2011). Per lobe, six larval type II NBs reside along the midline (named DM1-6) and two reside more laterally (DL1-2) (Ali Bayraktar et al., 2010; Bayraktar and Doe, 2013). Despite the small number of type II NBs, type II lineages contribute a disproportionate number of neurons and glia to the adult brain. A single type II NB lineage produces ~500 progeny during larval stages (Izergina et al., 2009). The neuronal and glial progeny are not only high in number, but have diverse identities and functions within the adult brain making them an attractive model for diversity generation (Ali Bayraktar et al., 2010; Wang et al., 2014).

Larval type II progeny compose a majority of the neurons innervating the central complex (Ali Bayraktar et al., 2010). The central complex is a set of neuropil regions within the adult Drosophila brain responsible for a diverse set of behaviors including

spatial memory, sleep, mating, flight, locomotion and feeding (Neuser et al., 2008; Strauss, 2002; Young and Armstrong, 2010). Four distinct neuropil regions, evolutionarily conserved among insect species, include: the protocerebral bridge (PB), the ellipsoid body (EB), the fan-shaped body (FB) and the noduli (NO) (Wolff et al., 2015). These regions have been found to contain around 60 different neuronal cell types making type II-derived central complex neurons an excellent model for temporal patterning (Wang et al., 2014; Wolff et al., 2015).

Recent work has revealed larval type II lineages exhibit combinatorial temporal patterning along intersecting NB and INP axes (Bayraktar and Doe, 2013; Syed et al., 2017) . Larval type II NBs express multiple temporal patterning factors in early larva with a Dichaete/Cas expression window at 24 hours after larval hatching to a Seven up positive window by 48 hours (Bayraktar and Doe, 2013). Later in development, larval type II NBs transition into a Chinmo/Imp positive expression window followed by Syncrip/Broad/E93 expression, dividing continuously during each expression window (Syed et al., 2017). In addition to the NB temporal cascade, each larval INP expresses a transcription factors sequence of Dichaete (D) > Grh > Eyeless (Ey) (Bayraktar and Doe, 2013). These domains of intersecting NB and INP temporal factor expression expand the diversity within the prolific type II lineages. For example, progeny from D positive young INPs born from late NBs generate Bsh positive neuronal progeny, in contrast to Ey positive old INPs born from early NBs which make glia (Bayraktar and Doe, 2013). Expanding the methods to identify individual progeny will aid in uncovering further combinations of temporal patterning molecules within the developing brain.

Can temporal patterning within type II NB lineages contribute to understanding mammalian brain development? The large number of progeny produced by type II lineages has attracted attention as a potential model for cortical expansion within the primate brains. Type II NBs share a similar division pattern with mammalian OSVZ neural stem cells, which generate cortical neurons at some points through a transit-amplifying radial glia intermediate (Hansen et al., 2010). Rodents have few OSVZ neural stem cells, making Drosophila a more tractable model to investigate regulation of transit-amplifying progeny. Additionally, both initial specification of NB division pattern and regulation of switching between each pattern (i.e., type 0 > type 1) are still widely understudied. It remains an interesting area of research as some mammalian neural progenitors have been observed to switch between division modes (Hansen et al., 2010; Noctor et al., 2001).

Role of Type II lineages in neurogenesis

Neurogenesis is a step-wise process with each event occurring not in isolation, but as a part of the whole. As current studies on type II NBs focus on larval stages, it is of interest to examine all developmental stages to fully understand how type II lineages contribute to brain development. Work on the development of the central complex neuropils has uncovered a key role for a subset of early born neurons from type II lineages born in forming the central complex (Riebli et al., 2013). By late larval stages, these neurons migrate to the midline where they are proposed to form serve a scaffolding role in the primordium of the future FB neuropil. During pupal stages, the same neurons undergo extensive remodeling and integrate into adult central complex circuitry within

the PB, EB and FB (Riebli et al., 2013). Thus, neurons made early in development may serve important roles in building the foundation for future adult brain structures.

As roles for early born neurons within Drosophila brain emerge, it is important to note that larval stage is only part of neurogenesis. As all NBs form within the embryo, each larval lineage presumably has an embryonic counterpart. Despite only 10% of adult neurons having embryonic origin, even small numbers of neurons can play important roles in shaping the brain. Larval NB lineages form "clonal units" in the brain, with most larval-born neurons remaining undifferentiated and clustered in lineages; whereas most embryonic neurons differentiate quickly extending axons (Dumstrei et al., 2003; Pereanu and Hartenstein, 2006). Embryonic-born neurons serve roles outside establishing proper larval function. The embryonic neurons form primary axon tracts that form the origin of the brain neuropil and can serve as pioneer neurons for guiding the migration of later born neurons (Hidalgo and Brand, 1997; Lin et al., 14AD; Raper and Mason; Taku et al.; Younossi-Hartenstein et al., 2006). As larval type II NBs contribute such a diversity of neurons and glia to the developing brain and central complex, it is essential to understand the entirety of the lineages beginning in the embryo.

Do type II NBs have an embryonic counterpart? This is the central question of this dissertation. Do the 16 type IIs in the larval brain form in the embryo with type II identity? Or, do they arise only in larval stages from a switch from a type I, similar to the type 0> type 1 switch? If they switch, what regulates this switch? Are there more than 16 type II in the embryo? Answering if there are type II NBs present in the embryo is key to understanding their complete lineages, but it remains interesting to speculate if they are the larval counterparts of NB with type I division patterns in the embryo.

If the type II NBs form in the embryo, when and where are they formed? Do the type II NBs also enter quiescence with the type 0 and type I populations? Investigating type II lineages in the embryo would reveal a potential model for how unique division pattern profiles are created within NB populations.

Additionally, with the well-studied NB temporal patterning cascade in the embryonic type I lineages, do the embryonic type II NB lineages express the same Hb > Kr > Pdm > Cas > Grh cascade? Do the type II NBs have the same division pattern such that the INPs are transit-amplifying? It is of interest if they generate transit-amplifying INPs within the embryonic brain. Do the embryonic INPs express the same D > Grh > Ey temporal cascade, or is there another unidentified sequence used to specify embryonic INP progeny? This could provide a model to investigate identified NB temporal patterning molecules in transit-amplifying progeny.

Finally, if there are type II lineage derived neurons or glia within the embryo, what role do they serve in the developing brain? Do they serve a larval function only? Do they serve a pioneering role for the central complex? Do they survive to adulthood?

Here I characterize the embryonic segment of Drosophila type II NB lineages. In Chapter II, I identify the presence of eight type II NBs per brain lobe that form in a stereotyped position along the dorsomedial embryonic brain region that are among the last NBs to form in the CB. I use clonal analysis, gal4 line expression and molecular profiling of asymmetric cell division proteins to establish that embryonic type II NBs indeed generate transit-amplifying INPs similar to larval lineages. Additionally, I show that both embryonic type II NBs and INPs enter quiescence in the late embryo after generating neurons that extend axons during embryogenesis. Furthermore, I show that

embryonic-born type II neurons survive to adulthood to innervate the central complex. This work is submitted to the journal Development and is co-authored with C. Q. Doe. In Chapter III, I address the origin of the embryonic type II NBs from PntP1 positive NE and the attempts to live image both the delamination of type II from the NE layer and the in vivo divisions of embryonic type II NBs and INPs. I then go on to describe the best strategies for live imaging the central brain and neuroectoderm in vivo wholemount Drosophila embryos.

CHAPTER II

EMBRYONIC TYPE II NEUROBLASTS: ORIGIN, TEMPORAL PATTERNING, AND CONTRIBUTION TO THE ADULT CENTRAL COMPLEX

The work in this Chapter was conceived of and written by myself and Chris Q. Doe, while I was in the Doe Laboratory. I performed all of the experiments, analyzed all of the data, and prepared all of the figures.

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Introduction

Drosophila neural progenitors, called neuroblasts, are a model system for investigating stem cell self-renewal versus differentiation (Doe, 2008; Reichert, 2011), as well as how a single progenitor generates different types of neurons and glia over time (Alsio et al., 2013; Kohwi et al., 2013). Drosophila type I neuroblasts have a relatively simple cell lineage: they undergo a series of asymmetric cell divisions to produce a series of smaller ganglion mother cells (GMCs) that typically differentiate into a pair of neurons. There are about 100 type I neuroblasts in each larval brain lobe; they generate progeny during embryogenesis, undergo a period of quiescence, and then resume their lineage in the larva (Truman and Bate, 1988; Datta, 1995; Maurange and Gould, 2005; Sousa-Nunes et al., 2010). Type I neuroblasts have a molecular profile that is Deadpan $(Dpn)^+$, Asense $(Ase)^+$ and Pointed P1 $(PntP1)^-$ (Zhu et al., 2011; Xie et al., 2016). Moreover, many embryonic type I neuroblasts can transition to a simpler "type 0" lineage, in which each neuroblast daughter cell directly differentiates into a neuron (Karcavich and Doe, 2005; Baumgardt et al., 2014; Bertet et al., 2014). In contrast, Drosophila type II neuroblasts

have a more elaborate cell lineage: they divide asymmetrically to bud off smaller intermediate neural progenitors (INPs) that themselves produce a series of 4-6 GMCs that each make a pair of neurons or glia (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009). Type II neuroblasts have a molecular profile that is $Dpn^{+}Ase^{-}$ $PntP1^{+}$ (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009; Zhu et al., 2011). Although there are only eight type II neuroblasts per larval brain lobe, they generate a major portion of the intrinsic neurons of the adult central complex (Bayraktar et al., 2010; Ito et al., 2013; Riebli et al., 2013; Yu et al., 2013), a neuropil devoted to multimodal sensory processing and locomotion (Martin et al., 1999; Renn et al., 1999; Strauss, 2002; Wessnitzer and Webb, 2006; Poeck et al., 2008; Wang et al., 2008; Pan et al., 2009; Bender et al., 2010; Boyan and Reichert, 2011; Ofstad et al., 2011; Seelig and Jayaraman, 2011; Seelig and Jayaraman, 2013; Seelig and Jayaraman, 2015).

A large amount of work over the past two decades has illuminated the general principles for how type I neuroblasts generate neuronal diversity. First, dorso-ventral, anterior-posterior, and Hox spatial patterning cues generate unique neuroblast identities (Chu-LaGraff and Doe, 1993; Prokop and Technau, 1994; Skeath et al., 1995; McDonald et al., 1998; Weiss et al., 1998; Skeath and Thor, 2003; Marin et al., 2012; Estacio-Gomez and Diaz-Benjumea, 2014; Moris-Sanz et al., 2015). Second, the temporal transcription factors Hunchback (Hb), Krüppel (Kr), Nubbin/Pdm2 (Pdm), Castor (Cas) and Grainy head (Grh) specify unique GMC identities within each neuroblast lineage (Brody and Odenwald, 2000; Berger et al., 2001; Isshiki et al., 2001; Novotny et al., 2002; Cenci and Gould, 2005; Kanai et al., 2005; Grosskortenhaus et al., 2006; Mettler et

al., 2006; Urban and Mettler, 2006; Maurange et al., 2008; Tran and Doe, 2008; Tsuji et al., 2008; Ulvklo et al., 2012; Herrero et al., 2014; Moris-Sanz et al., 2014). In contrast, much less is known about type II neuroblasts. Only one of the eight type II neuroblasts has been identified in the embryo (Hwang and Rulifson, 2011); the origin of the other type II neuroblasts has not been reported in existing embryonic brain neuroblast maps (Urbach and Technau, 2003). It remains unknown whether type II neuroblasts arise de novo from the neuroectoderm similar to type I neuroblasts, or whether they arise from a type I > type II transition similar to the type I > type 0 neuroblast transitions (Baumgardt et al., 2014; Bertet et al., 2014). If type II neuroblasts form during embryogenesis, it is unknown whether they utilize the same Hb > Kr > Pdm > Cas > Grh temporal transcription factor cascade to generate neuronal diversity, or whether they make embryonic born INPs that sequentially express Dichaete (D) > Grh > Eyeless similar to larval INPs (Bayraktar and Doe, 2013). Furthermore, if type II neuroblast lineages are initiated in the embryo, it would be interesting to know if their INPs undergo quiescence, similar to type I and II neuroblasts; if so they would be the only cell type beyond neuroblasts known to enter quiescence at the embryo/larval transition. Perhaps most importantly, identifying embryonic type II neuroblasts is essential for subsequent characterization of their early-born progeny, which are likely to generate pioneer neurons crucially important for establishing larval or adult brain architecture.

Here we address all of these open questions. We show that all eight type II neuroblasts form during embryogenesis. We use molecular markers and clonal data to show that embryonic type II neuroblasts give rise to INPs that produce multiple GMCs and neurons during embryogenesis, and that INPs undergo quiescence during the

embryo-larval transition. We find that embryonic type II neuroblasts sequentially express a subset of neuroblast temporal transcription factors (Pdm > Cas > Grh), and embryonic INPs express a subset of the known larval INP temporal transcription factors (Dichaete). Finally, we show that embryonic INPs give rise to neurons that survive to populate the adult central complex.

Results

All type II neuroblasts arise during embryogenesis

Larval type II neuroblasts are PntP1^{+} Dpn^{+} Ase^{-} and here we used these markers to determine if type II neuroblasts exist in the embryo. We found that type II neuroblasts formed internal to the dorsal cephalic neuroectoderm beginning at late stage 11. At this stage, there is one PntP1^{+} Dpn^{+} Ase^{-} type II neuroblast in a stereotyped dorsal posteromedial location; this is always the first type II neuroblast to appear (Fig. 1). By stage 12, the number of type II neuroblasts along the dorso-medial region of the brain increased from four (8h) to six (9.5h), and from stage 15 (12h) to the end of embryogenesis there were reliably eight type II neuroblasts per lobe (Fig. 1), the same number previously observed at all stages of larval development (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009). We reliably found three clusters of type II neuroblasts: an anteromedial group of three neuroblasts, a medial group of three neuroblasts, and a posterior ventrolateral group of two neuroblasts (Fig. 1A; summarized in Fig. 1B). Due to the dynamic morphogenetic movements of head involution, and the close positioning of the type II neuroblasts, we could not reliably identify individual neuroblasts within each cluster.

We tried to link the embryonic type II neuroblasts to the map of embryonic brain neuroblasts (Urbach and Technau, 2003), but were unsuccessful, probably because most type II neuroblasts arise later than the stages described in that study. Based on molecular marker analysis, we conclude that all eight known type II neuroblasts form during embryogenesis and they are among the last neuroblasts to form during embryogenesis.

Embryonic type II neuroblasts generate INPs, GMCs, and neurons during embryogenesis

Here we use molecular markers and clonal analysis to determine whether embryonic type II lineages produce INPs, GMCs, and neurons. We used a *Pnt-gal4* line to make clones; to validate the type II lineage-specific expression of this line, we stained for Pnt-gal4 and type II neuroblast and INP markers (Fig. 2A). We found that Pnt-gal4 is expressed in the parental type II neuroblast, the maturing INPs, and their GMC progeny (Fig. 2B). We did not detect any type I neuroblasts expressing this marker. Next, we generated "flip-out" clones using the heat shock-inducible multicolor flip out method (Nern et al., 2015) crossed to the *Pnt-gal4* line. When we assayed clones relatively early in embryogenesis (stage 13) we detected small clones containing a single type II neuroblast and one or more INPs (Fig. 2C; Table 1). Allowing the embryos to develop further resulted in larger clones that additionally contained GMCs and neurons (Fig. 2D). We found clones containing one type II neuroblast with up to five INPs at the latest stages of embryogenesis (Table 1). Taken together, these data show that embryonic type II neuroblasts

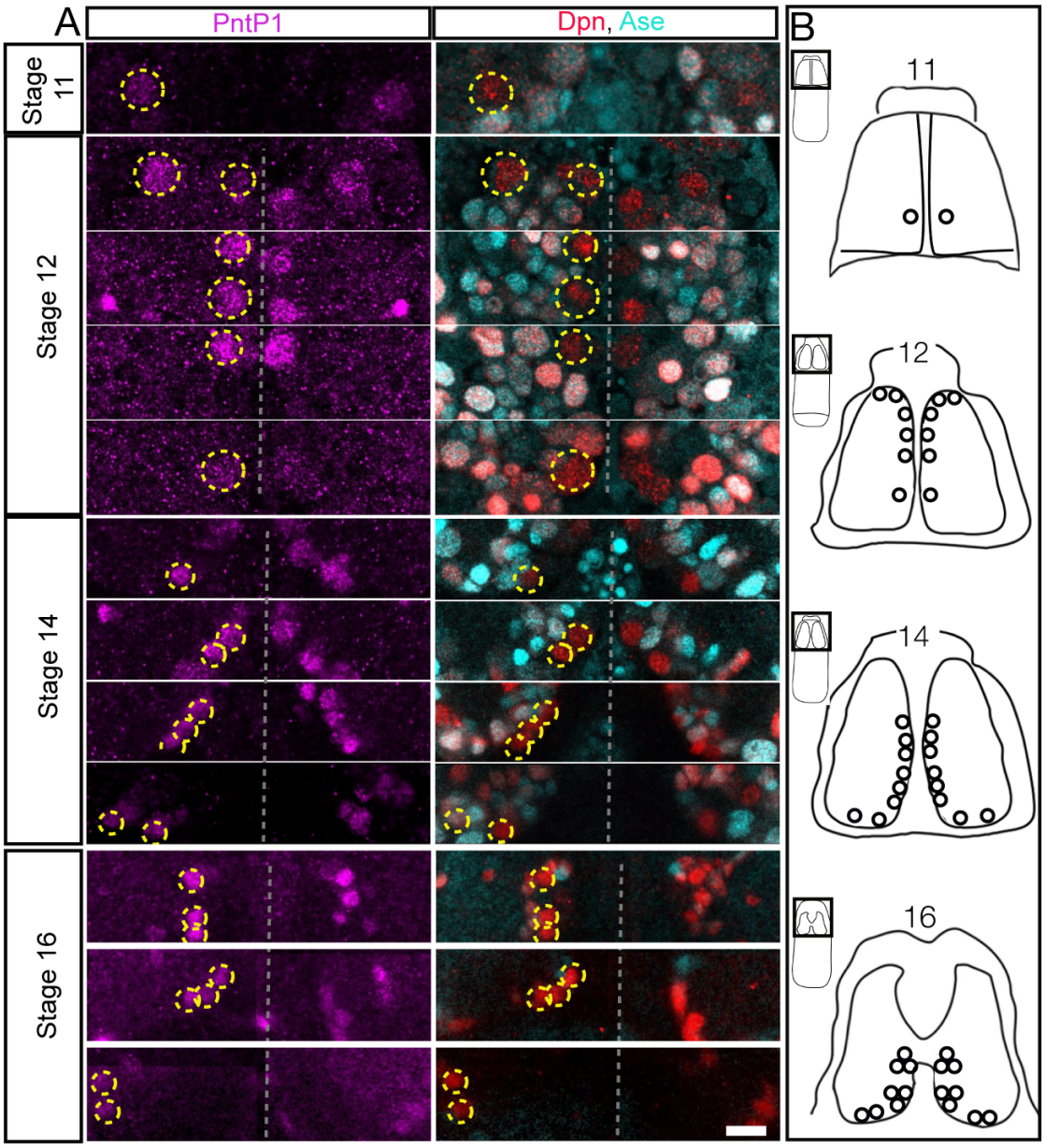

Figure 1. Eight type II neuroblasts arise during embryogenesis.
(A) Embryonic type II neuroblasts (yellow circles on left brain lobe; unlabeled on right brain lobe) are PntP1+ (magenta) Dpn+ (red) Ase- (cyan)., Each stage shows multiple focal planes from anterior to posterior (top to bottom in the figure) to clearly visualize each type II neuroblast, except for stage 11 where there is a single type II neuroblast.
(B) Summary of type II neuroblast formation; due to rapid morphogenetic movements it is not possible to identify individual type II neuroblasts from stage to stage, but beginning at stage 14 it is possible to recognize three clusters of neuroblasts. All panels are dorsal views with the dorsal midline in the center of the panel, anterior up. Scale bar = 10 μm

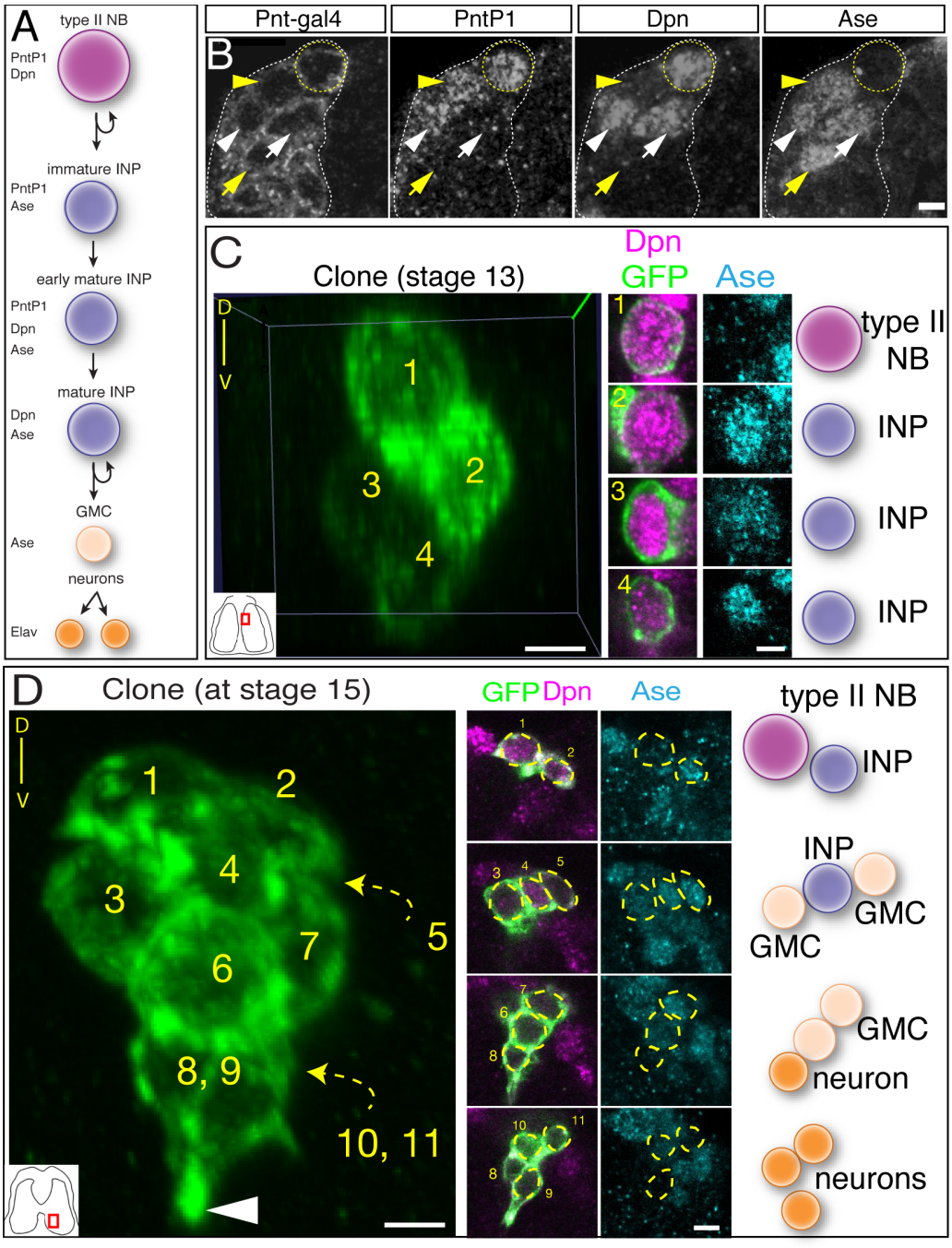

Figure 2, Walsh and Doe

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Figure 2. Clonal analysis shows that type II neuroblasts make INPs, GMCs and neurons during embryogenesis.

(**A**) Molecular markers used to identify cell types within type II lineages, neuroblast (NB).

(B) Embryonic type II neuroblasts generate embryonic-born INPs and GMCs. Dorso-medial view of a type II neuroblast cluster in a stage 16 embryo. Type II neuroblast (Pnt-gal4^{+} PntP1^{+} Dpn^{+} and Ase^{-}; yellow circle); immature INP (Pnt-gal4^{+} PntP1^{+} Dpn^{-} and Ase^{+}; yellow arrowhead); mature INP (Pnt-gal4^{+} PntP1^{+} Dpn^{+} and Ase^{+}; white arrowhead); mature INP that has lost PntP1 expression (Pnt-gal4^{+} PntP1^{-} Dpn^{+} and Ase^{+};

white arrow); and GMC (Pnt-gal4^{+} PntP1^{-} Dpn^{-} and Ase^{+}; yellow arrow). Scale bar, 5 μm.
(C) Single neuroblast clone assayed at stage 13; location shown in inset, lower left. Four cell clone containing a type II neuroblast and three INPs. Orientation is dorsal up, with the neuroblast closest to the dorsal surface of the brain.
(**D**) Single neuroblast clone assayed at stage 15; location shown in inset, lower left. Eleven cell clone containing a type II neuroblast, two INP, four GMCs, and four neurons. Orientation is dorsal up, showing that the neurons are sending projections ventrally (arrowhead). Scale bar for (C) and (D) = 10 μm for clone projection, 5 μm for insets

Cluster	**Stage**	**Type II NB** *Dpn+ Ase-*	**INP** Dpn+Ase+	**GMC** *Dpn- Ase+*	**Neuron** *Dpn- Ase-*	**Total Cells**
Anterior	15	1	2	0	0	3
Anterior	15	1	2	0	0	3
Anterior	16	1	1	3	0	5
Anterior	16	1	1	2	5	9
Anterior	16	1	1	1	9	12
Anterior	16	1	1	2	5	9
Middle	15	1	1	1	0	3
Middle	15	1	1	2	0	4
Posterior	16	1	2	1	7	11
Posterior	14	1	6	3	2	12
Posterior	15	1	5	3	3	12
Posterior	15	1	4	1	7	13
Posterior	15	1	2	4	2	9
Posterior	15	1	1	1	0	3

Table 1. Type II neuroblast clones contain INPs, GMCs, and neurons.
Each row represents a single clone that is clearly spatially separate from other clones in the embryonic brain. Stage, time of clone analysis. Markers, molecular marker profile of each cell in the clone.

generate multiple INPs which themselves produce GMCs and neurons prior to larval hatching. A defining feature of type II neuroblasts is their ability to make INPs which undergo a molecularly asymmetric cell division to self-renew and generate a GMC (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008; Izergina et al., 2009). Here we determine if embryonic INPs undergo asymmetric cell division. To identify INPs and

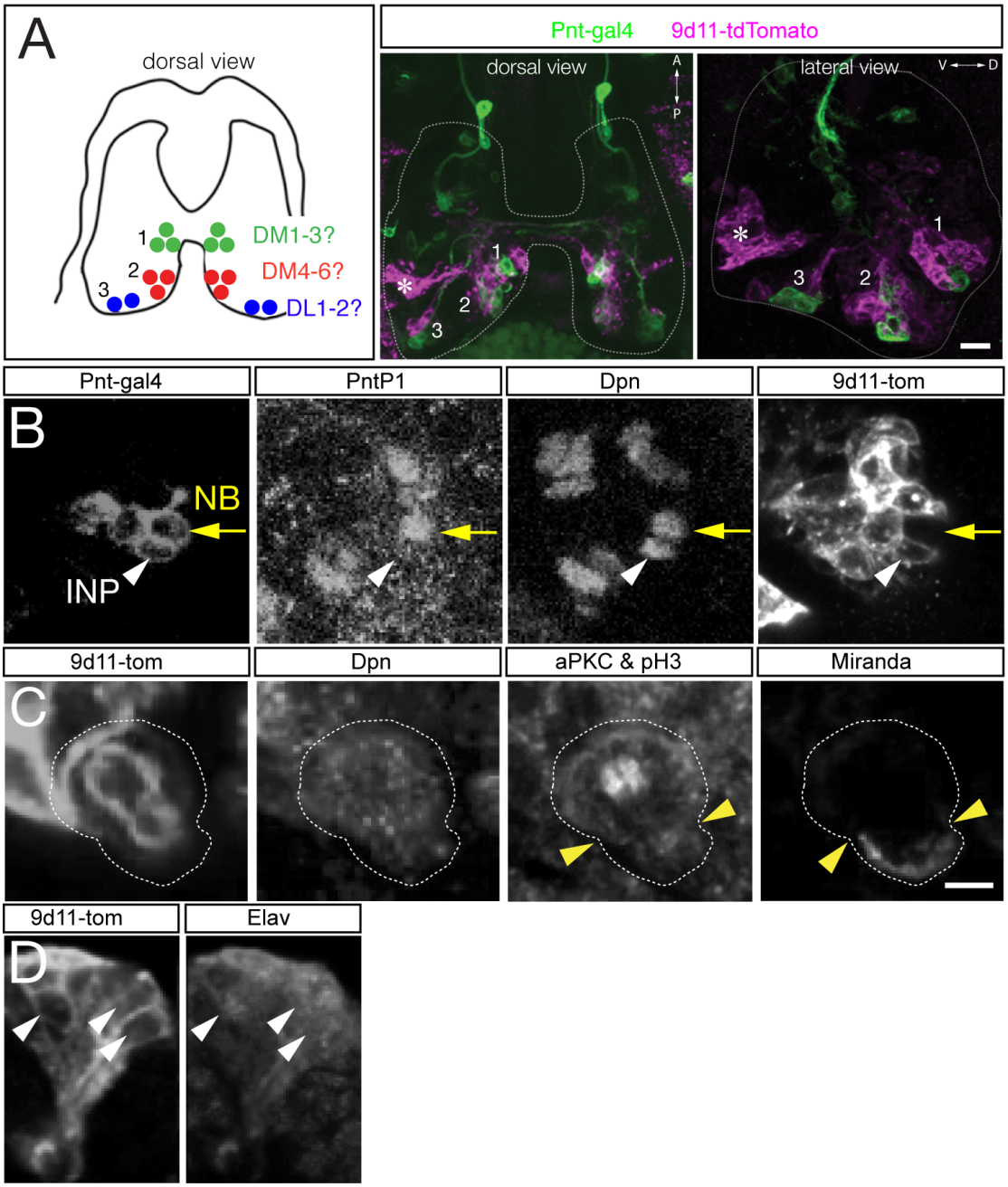

Figure 3, Walsh & Doe

Figure 3. (previous page) Embryonic INPs undergo asymmetric cell division
(A,B) *R9D11-tdTomato* (*9D11-tom*) labels embryonic INPs and their progeny, but not type II neuroblasts. (A) Left: summary of type II neuroblast positions (dorsal view). Center and left panels: dorsal or lateral view of the three type II neuroblast clusters labeled with *Pnt-gal4* (green; type II neuroblasts and progeny) and *9D11-tom* (magenta; INPs and progeny). Note there is *9D11-tom* expression at a deep ventral location that is not near any type II lineage (asterisk). Scale bar, 15 μm. (B) Type II neuroblast (*Pnt-gal4*$^+$ PntP1$^+$ Dpn$^+$ *9D11-tom*$^+$ (yellow arrow); INP (*Pnt-gal4*$^+$ PntP1$^-$ Dpn$^+$ *9D11-tom*$^+$ (white arrowhead) at stage 16. Scale bar, 10 μm.
(**C**) Embryonic INPs undergo asymmetric cell division. INPs were identified as *9D11-tom*$^+$ Dpn$^+$ and positioned within the middle cluster of neuroblasts in the dorsal posterior medial brain lobe. aPKC and pH3 are co-stained: aPKC is localized to the larger apical cell cortex (white cortex above arrowheads; future INP daughter cell) while pH3 decorates the mitotic chromosomes in the middle of the INP. Miranda is localized to the smaller basal cell cortex (cyan cortex below arrowheads; future GMC daughter cell). Scale bar, 5 μm.
(**D**) Embryonic INPs generate embryonic-born neurons. Lateral view of a *9D11-tom*$^+$ cluster in a stage 16 embryo. The post-mitotic neuronal marker Elav is detected in a subset of the *9D11-tom*$^+$ cluster (white arrowheads), and axon projections can be observed (bottom left). Scale bar, 5 μm.

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their progeny, we used the INP marker *R9D11-tdTomato* (henceforth *9D11-tom*) (Bayraktar and Doe, 2013), and confirmed that it is expressed in embryonic INPs (Figs 3A,B). We also detected a deep ventral cluster of unrelated cells that expressed *9D11-tom* but not Dpn, but these can be excluded from analysis due to their distinct position (Fig. 3A, asterisk). Using this marker, we found that *9D11-tom*$^+$ Dpn$^+$ embryonic INPs undergo asymmetric cell division: they partition aPKC and Miranda to opposite cortical domains (Fig. 3C). To confirm that these GMCs generate post-mitotic neurons during embryogenesis, we stained for the neuronal marker Elav, and found that *9D11-tom* clusters contained Elav$^+$ neurons (Fig. 3D). Additionally, axon fascicles from single type II neuroblast lineage clones were visible during embryogenesis (data not shown), confirming the production of embryonic-born

neurons from type II lineages. We conclude that embryonic type II neuroblasts generate asymmetrically dividing INPs that produce GMCs and neurons during embryogenesis.

Embryonic type II neuroblasts and INPs undergo quiescence

Type I central brain and thoracic neuroblast have been shown to undergo quiescence at the embryo-larval transition (Truman and Bate, 1988). Type II neuroblasts also undergo quiescence, because only the four mushroom body neuroblasts and a single lateral neuroblast maintain proliferation during the embryo-larval transition (Egger et al., 2008). In contrast, nothing is known about whether INPs undergo quiescence. To address this question, we counted the total number of INPs over time, as well as the number of mitotic INPs. We identified INPs as *9D11-tom*$^{+}$ Dpn^{+} and mitotic INPs by immunoreactivity for phospho-histone H3 (pH3). We quantified INPs in each cluster independently as well as all INPs in each brain lobe (Fig. 4A). We observed a fairly constant number of INPs in each cluster from embryonic stage 14 to stage 17 (Fig. 4B), yet the number of proliferating INPs declined significantly over time, reaching zero by stage 17 (Fig. 4C). We conclude that the INPs enter quiescence by embryonic stage 17.

If INPs enter quiescence in the late embryo, we should be able to detect them in the newly hatched larvae, prior to production of larval born INPs made from type II neuroblasts that have re-entered the cell cycle. We assayed 0-4h newly-hatched larvae for Dpn and *9D11-tom* to mark the small quiescent INPs (Fig. 4D). We observed an average of 10 ± 2 *9D11-tom*$^{+}$ Dpn^{+} cells in each brain lobe, and none of these INPs were mitotic (n=11; Fig. 4D). We conclude that INPs undergo quiescence in the late embryo and can persist into the larvae. The fate of these quiescent INPs – whether they resume proliferation, differentiate, or die – remains to be determined.

Embryonic type II neuroblasts undergo a late temporal transcription factor cascade

Embryonic type I neuroblasts undergo a well-characterized temporal transcription factor cascade that generates GMC diversity and ultimately neuronal diversity. Most type I neuroblasts sequentially express Hunchback > Krüppel > Pdm > Cas > Grh (Kohwi and Doe, 2013), although late-forming neuroblasts can skip some of the early factors:

neuroblast 3-3 begins the series with Krüppel (Tsuji et al., 2008) and NB6-1 begins the series with Cas (Cui and Doe, 1992). Due to the fact that type II neuroblasts are among the latest to form, it raises the possibility that they do not express any known temporal transcription factors.

We stained embryos for type II neuroblast markers (Dpn^+ Ase^-) and individual temporal identity transcription factors. We did not observe the first two temporal transcription factors, Hunchback or Krüppel, in any type II neuroblasts at any stage of development (data not shown). We next focused on the first type II neuroblast to form, which can be uniquely identified at late stage 11 (see Fig. 1). This early-forming neuroblast showed the temporal cascade of Pdm > Pdm/Cas > Cas > Cas/Grh > Grh (Fig. 5). All later-forming type II neuroblasts exhibited a more truncated temporal cascade of Cas > Cas/Grh > Grh (Fig. 5). We conclude that embryonic type II neuroblasts undergo a late temporal transcription factor cascade.

Figure 4. INPs undergo quiescence across the embryo-larval transition. (next page)
(**A**) Schematic outlining the three pools of type II neuroblast INP progeny assayed in graphs to the right (red box).
(**B**) Total number of INPs per pool at the indicated stages; INPs identified as 9D11-tom^+ Dpn^+ cells. (**C**) Number of phospho-histoneH3 (pH3)-positive mitotic INPs per pool at the indicated stages; INPs are identified as *9D11-tom*$^+$ Dpn^+ cells. Each circle represents the number of INPs in the cluster of neuroblasts shown in A; black bar represents the average, shown with SEM.
(**D**) Quiescent INPs are present in the newly hatched larva. INPs marked with *9D11-gal4 UAS-tdTomato* (green); brain neuroblasts and INPs marked with Dpn (magenta). Anterior up, dorsal midline, dashed. Scale bar = 15 μm.

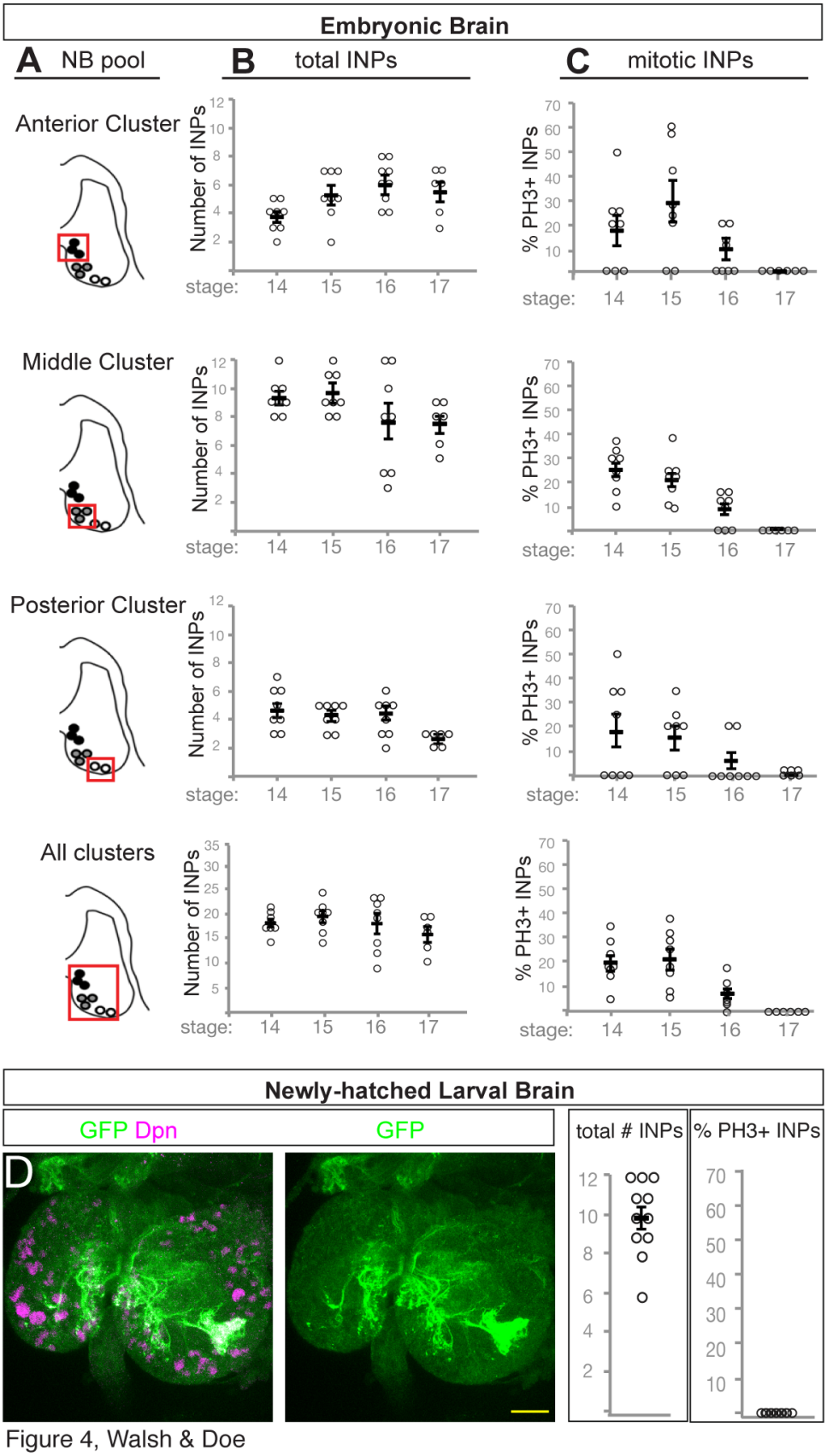


Figure 4, Walsh & Doe

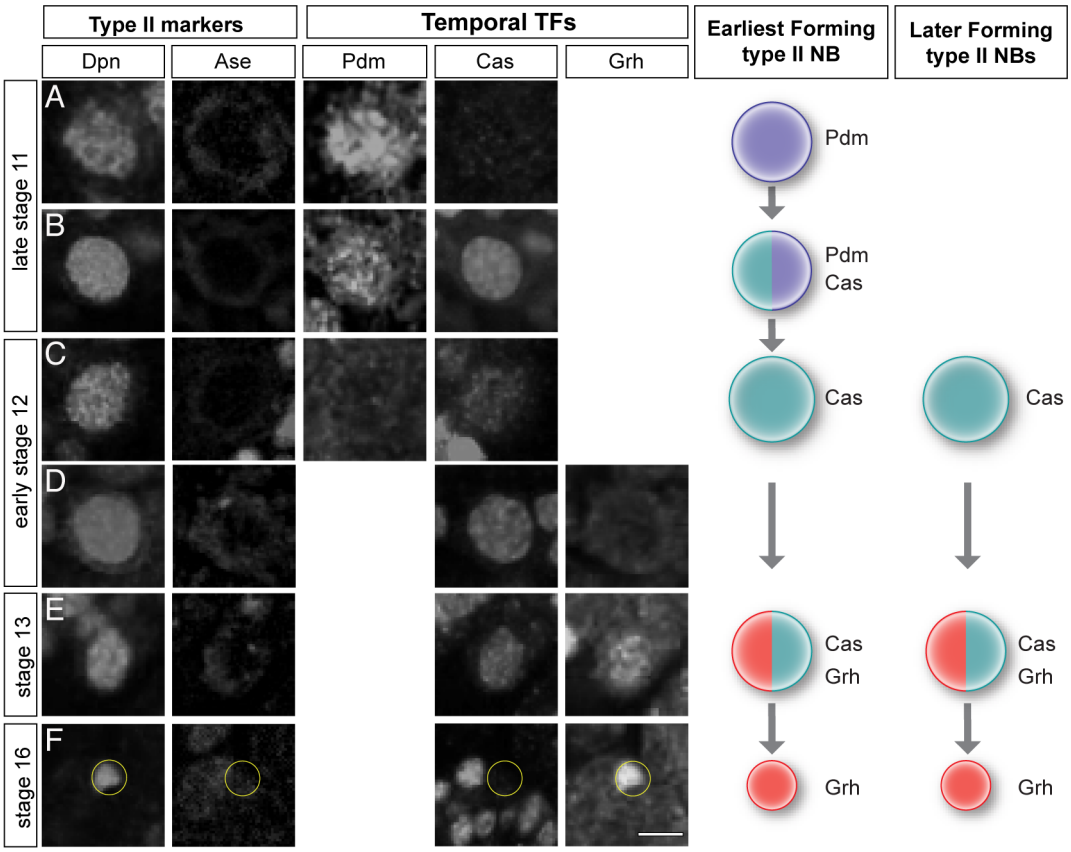


Figure 5, Walsh &Doe

Figure 5. Embryonic type II neuroblasts express late temporal transcription factors.

(A-F) Temporal transcription factor expression in the earliest type II neuroblast to form (posterior-most, see Fig. 1). Type II neuroblasts identified as Dpn^{+} Ase^{-} (left columns); temporal transcription factor expression reveals sequential expression of Pdm^{+} > Pdm^{+} Cas^{+} > Cas^{+} > Cas^{+} Grh^{+} > Grh^{+}. Summarized at left; later-forming type II neuroblasts start the cascade with Cas. Scale bar = 10 μm.

Embryonic INPs undergo a truncated temporal transcription factor cascade.

Larval INPs undergo a temporal transcription factor cascade of Dichaete-Grh-Eyeless over their ~12 hour lifespan (Bayraktar and Doe, 2013). We wondered if the shorter timeframe of embryogenesis may result in shorter temporal transcription factor expression windows, a truncated temporal cascade, or perhaps a lack of all temporal transcription factor expression.

To identify embryonic INPs expressing known INP temporal transcription factors, we generated FLP-out clones using a heat shock FLP in mid-embryogenesis (4h-9h) and assayed brains containing a single type II neuroblast clone. We stained embryos for the clone marker, Dpn, and Ase to identify the neuroblast (Dpn^+ Ase^-) and INPs (Dpn^+ Ase^+), and one of the larval INP temporal transcription factors (Dichaete, Grh or Eyeless). We found that the early temporal factor Dichaete was detected in all INPs within the anterior and middle clusters (n=15 clones, anterior; n=12 clones, middle) (Fig. 6A,B; quantified in Table 2), but the posterior cluster contained no $Dichaete^+$ INPs at any stage (n=9 clones) (Fig. 6C; quantified in Table 2). The middle temporal factor, Grh, was only detected in a single INP next to Grh^+ neuroblasts, but not next to Grh^- neuroblasts, suggesting that it is transiently inherited from the parental neuroblast, as is also observed in larval INP lineages (Bayraktar and Doe, 2013); we never detected Grh in INPs distant from the neuroblasts, as would be expected for a middle temporal transcription factor (data not shown). The late temporal factor Eyeless was never detected in INPs during embryogenesis (data not shown). We conclude that embryonic INPs undergo a temporal cascade that is truncated during the Dichaete window by entry into quiescence (Fig. 6E). It would be interesting to determine whether embryonic-born INPs express the later temporal factors Grh and Eyeless in the larvae, if they re-enter the cell cycle.

Figure 6 (next page). Embryonic INPs express the Dichaete temporal transcription factor.

(A) Anterior cluster clone containing Dichaete $(D)^+$ INPs. Four cell FLP-out clone at stage 16 (left) stained for the clone marker (GFP, green), Dpn (magenta), Ase (cyan) and D (white). The clone contains a type II neuroblast (1), a D^+ INP (2) and two GMCs, one D^+ and one D^- (3,4)

(B) Anterior cluster clone containing D+ INPs. Four cell FLP-out clone at stage 16 stained the same as in (A) containing a type II neuroblast (1), one D^+ INP (4), and two D^- GMCs (2,3).

(C) Posterior cluster clone lacking D+ INPs. Nine cell FLP-out clone at stage 16 (left) stained the same as in (A) containing a type II neuroblast (1), four D^- INPs (2,5-7) and four D^- neurons (3,4,8,9). Scale bar 7 μm in clonal projections, 5 μm in insets.

(D) Model for INP temporal factor expression; top, embryonic INPs from anterior and middle clusters; bottom, larval INP temporal factor expression (Bayraktar and Doe, 2013).

(E) Cell type key for panels above.

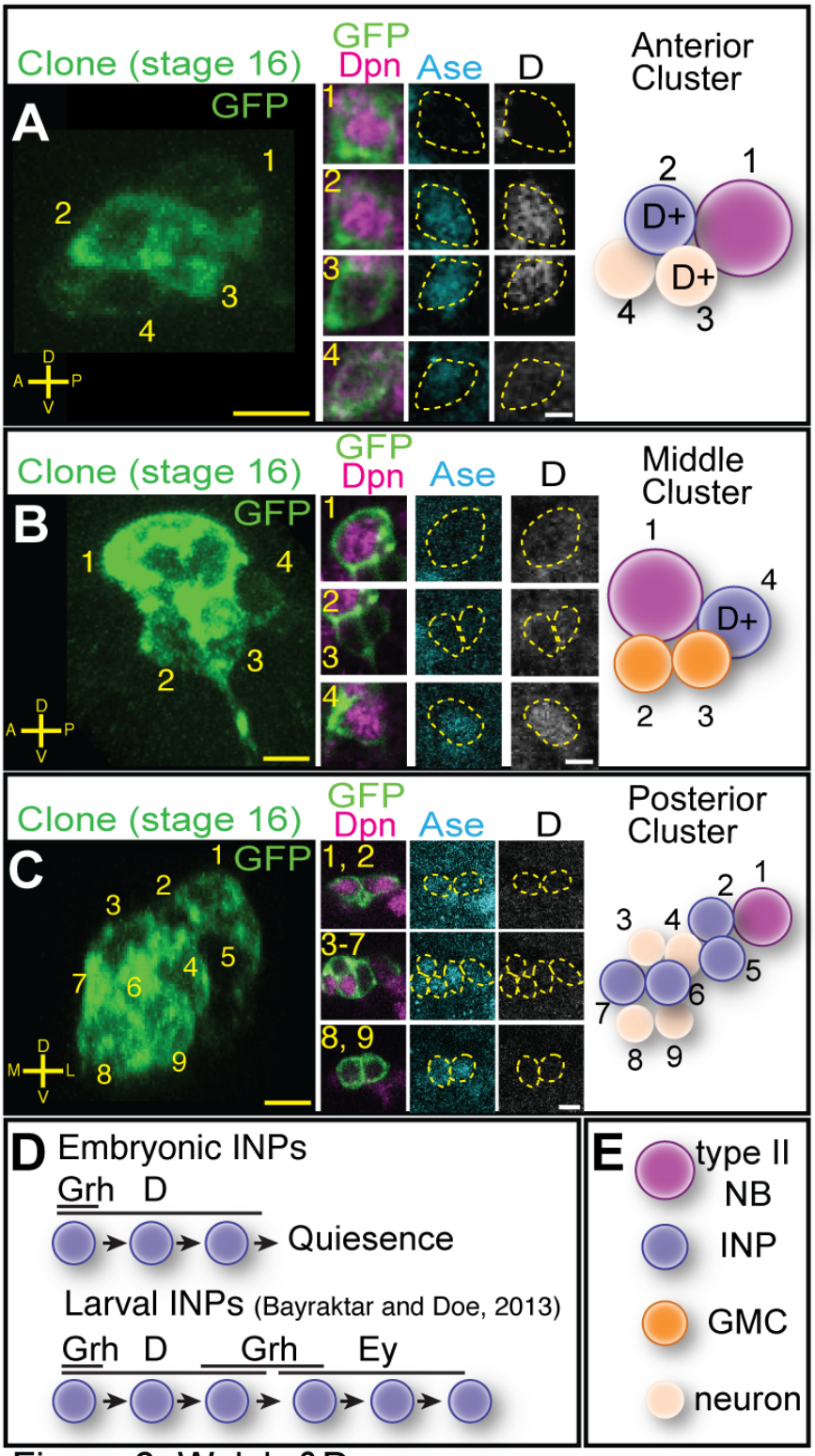


Figure 6, Walsh &Doe

Cluster	Stage	Type II NB *Dpn+ Ase-*	INP Dpn+Ase+	D+ INP *Dpn-Ase+ D+*
Anterior	14	1	1	1
Anterior	15	1	2	2
Anterior	15	1	1	1
Anterior	15	1	2	2
Anterior	16	1	1	1
Anterior	16	1	2	2
Middle	15	1	1	1
Middle	15	1	1	1
Middle	15	1	2	2
Middle	16	1	2	2
Middle	16	1	2	2
Middle	16	1	1	1
Middle	16	1	1	1

Table 2. Dichaete is expressed in embryonic INPs.
Each row represents a single neuroblast clone that is spatially separate from other clones in the embryonic brain. Stage, time of clone analysis.

Embryonic-born INPs contribute to the adult central complex.

Embryonic type II neuroblasts produce neurons with contralateral projections, where they have been proposed to pioneer the fan shaped body neuropil of the central complex (Riebli et al., 2013). To determine if embryonic-born INP progeny persist into adulthood we used the FLEX-AMP system (Bertet et al., 2014) to permanently mark embryonic INPs and their progeny and trace them into the adult brain. FLEX-AMP uses a brief inactivation of temperature-sensitive Gal80 protein (by shifting to 29^{o}C) to allow transient expression of Gal4, which induces FLP expression and the permanent expression of *actin-LexA LexAop-myr:GFP* (Fig. 7A). We crossed *R9D11-gal4* (expressed in embryonic INPs) to the FLEX-AMP stock and raised the flies at 18^{o}C

(negative control), 29°C (positive control), or with a 10 hour pulse of 29°C at late embryogenesis followed by 18°C for the rest of the fly's life ("immortalization of embryonic progeny" experiment).

We found robust labeling of >500 neurons in the positive control brains raised at 29°C, including many cell bodies innervating the protocerebral bridge, fan shaped body, ellipsoid body and noduli (Fig. 7B-H). The negative control (18°C permanently) showed labeling of just ~10 neurons that project to the dorsal part of the fan shaped body (Fig. 7G- K), which is similar to the adult pattern of R9D11 (FlyLight). We suspect the "leaky" expression at 18 °C may reflect the inefficiency of Gal80 repression in these adult neurons. Importantly, FLEX-AMP immortalization of embryonic INP progeny showed labeling of additional neurons (64 ± 4) that project to three central complex regions: the protocerebral bridge, a large portion of the fan shaped body and the ellipsoid body, but notably not the noduli (Fig. 7 L-P). Within the ellipsoid body, we observed variation in labeling. Most brains contained one to two wedge neurons (arrows in Fig. 7P') and widefield neuron innervation throughout the posterior region of the ellipsoid body (Fig. 7P", n= 12). Interestingly, a few brains contained only the wedge neurons suggesting the widefield neuron innervation may be an early-born neuron within the lineages (See Discussion) (n= 3/12, Fig. 7 Q, R). Additionally, FLEX-AMP immortalization of embryonic INP progeny identified neurons innervating the central complex accessory neuropils lateral accessory lobe (LAL) and the Gall, which were never labeled in the 18°C negative control (Fig. S1). We conclude that embryonic INPs generate progeny that persist into the adult brain, and innervate three neuropils of the central complex.

Figure 7 (next page). Embryonic INP progeny contribute to the adult central complex.
(A) The FLEX AMP memory cassette used for immortalization of embryonic INPs into the adult brain; modified from Bertet et al. 2014.
(B-P) Central complex neuropil regions from flies containing FLEX AMP memory cassette reared at different temperature regimes to permanently label neurons born within all development (29°C positive control), no stage of development (18°C negative control) or specifically during late embryogenesis (29°C pulse) stained for GFP (green) and NC82 (magenta).

(B-F) Positive controls reared at 29^{o}C from embryo to adult with over 500 (n= 4) of immortalized neurons innervating the PB, FB, EB and NO.
(G-K) Negative control adult brains of flies reared at 18^{o}C from embryo to adult showing 10 ± 5 (n=5) neurons from the adult *9D11-gal4* pattern innervating only the dorsal region of the FB.
(L-R) Experimental adult brains from flies reared for 6 hour pulse at 29^{o}C at late embryonic stages, then reared at 18^{o}C until adult (see methods); there are 64 ±4 (n=12) neurons that innervate the PB, FB, EB but not the NO.
(P'-R) Experimental adult brains with differences in innervation pattern within the EB (n=12).
(P') Single z plane from anterior region shown in (P) with innervation of two wedges within the EB (yellow arrows) seen within 12/12 brains.
(P") Single z plane from posterior region shown in (P) showing wide field neuron innervation within the EB seen within 9/12 brains.
(Q) EB with innervation of two wedges, lacking the wide field innervation (n=1).
(R) EB with innervation of one wedge, lacking the wide field innervation (n=1).
Abbreviations: PB (protocerebral bridge), FB (fan shaped body), EB (ellipsoid body), NO (noduli). Scale bar = 20μm.

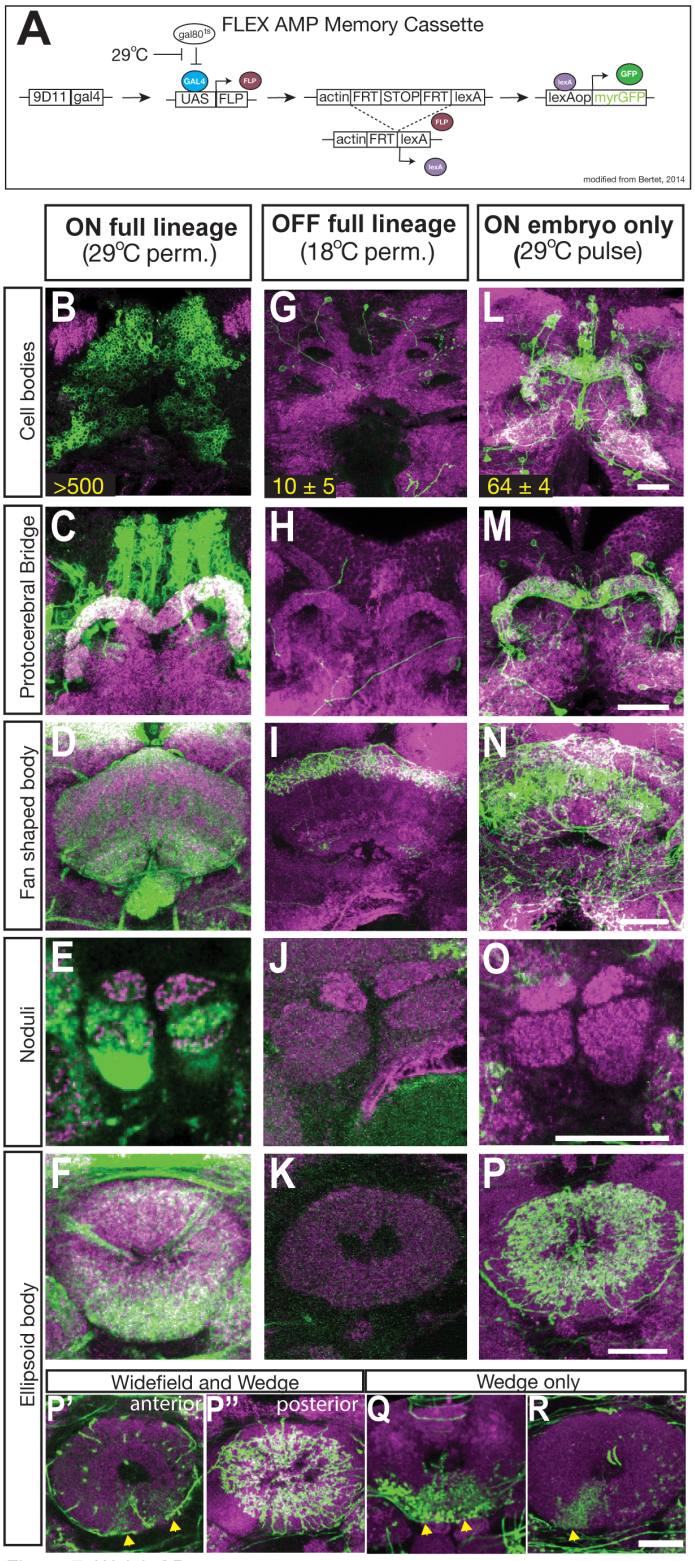


Figure 7, Walsh &Doe

Discussion

It has been difficult to link embryonic neuroblasts to their larval counterparts in the brain and thoracic segments due to the period of quiescence at the embryo-larval transition, and due to dramatic morphological changes of the CNS that occur at late embryogenesis. Recent work has revealed the embryonic origin of some larval neuroblasts: the four mushroom body neuroblasts in the central brain and about twenty neuroblasts in thoracic segments (Kunz et al., 2012; Lacin and Truman, 2016). Here we use molecular markers and clonal analysis to identify all eight known type II neuroblasts in each brain lobe and show they all form during embryogenesis, perhaps the last-born central brain neuroblasts. We were unable to individually identify each neuroblast, however, due to their tight clustering, movements of the brain lobes, and lack of markers for specific type II neuroblasts.

The single previously reported embryonic type II neuroblast formed from PntP1^{+} neuroectodermal cells with apical constrictions called a placode (Hwang and Rulifson, 2011). We have not investigated this neuroectodermal origin of type II neuroblasts in much detail, but we also observe multiple type II neuroblasts developing from PntP1^{+} neuroectoderm (data not shown). In the future, it would be interesting to determine whether all type II neuroblasts arise from PntP1^{+} neuroectoderm or from neuroectodermal placodes. Interestingly, one distinguishing molecular attribute of type II neuroblasts is PntP1, which is not detected in type I neuroblasts (Zhu et al., 2011; Xie et al., 2016). Thus, a candidate for distinguishing type I / type II neuroblast identity is EGF signaling, which can be detected in the three head placodes (de Velasco et al., 2007; Hwang and Rulifson, 2011) and is required for PntP1 expression (Gabay et al., 1996). Clearly there are more PntP1+ neuroectodermal cells than there are type II neuroblasts, however, which may require expression of an EGF negative regulator such as Argos (Rebay, 2002) to divert some of these neuroectodermal cells away from type II neuroblast specification. The earliest steps of type II neuroblast formation represent an interesting spatial patterning question for future studies.

Now that we have identified the embryonic type II neuroblasts, it is worth considering whether there are differences between embryonic and larval type II neuroblasts or their INP progeny. To date, molecular markers do not reveal any differences between embryonic and larval type II neuroblasts, with the exception that embryonic neuroblasts transiently express the temporal transcription factor Pdm (see below). Are there differences between embryonic and larval INPs? Larval INPs mature over a period of six hours and then divide four to six times with a cell cycle of about one hour (Bello et al., 2008). In contrast, embryonic INPs may have a more rapid maturation because we see $Elav^+$ neurons within $9D11^+$ INP lineages by stage 14, just 3 hours after the first type II neuroblast forms. We found that INPs undergo quiescence at the embryo-larval transition, as shown by the pools of INPs at stage 16 that do not stain for the mitotic marker pH3. The fate of these quiescent INPs – whether they resume proliferation, differentiate, or die – remains to be determined.

Neuroblasts in the embryonic VNC use the temporal transcription factor cascade Hunchback (Hb) > Krüppel > Pdm > Cas > Grh to generate neural diversity (Brody and Odenwald, 2002; Kohwi et al., 2013; Allan and Thor, 2015; Kang and Reichert, 2015; Doe, 2017). Here we show that the type II neuroblasts are among the last neuroblasts to form in the embryonic brain, and that they sequentially express only the late temporal transcription factors Pdm (in the earliest-forming neuroblast) followed by Cas and Grh (in all eight type II neuroblasts). It is unknown why most type II neuroblasts skip the early Hb > Kr > Pdm temporal transcription factors; perhaps it is due to their late time of formation, although several earlier-forming thoracic neuroblasts also skip Hb (NB3-3), Hb > Kr (NB5-5), or Hb > Kr > Pdm (NB6-1) (Cui and Doe, 1992; Tsuji et al., 2008; Benito-Sipos et al., 2010). This is another interesting spatial patterning question for the future.

Type I neuroblasts show persistent expression of the temporal transcription factors within neurons born during each window of expression (i.e. a Hb^+ neuroblast divides to produce a Hb^+ GMC which makes Hb^+ neurons). In contrast, we find that type II neuroblasts do not show persistent Cas or Grh expression in INPs born during each expression window (data not shown). Both transcription factors can be seen in INPs immediately adjacent to the parental neuroblast, but not those more distant (data not shown). This shows that Cas and Grh are down regulated in INPs rather than maintained

in the INP throughout its lineage and into all its post-mitotic neural progeny. The function of Pdm, Cas and Grh in embryonic type II neuroblasts awaits identification of specific markers for neural progeny born during each expression window.

During larval neurogenesis, virtually all INPs sequentially express the temporal transcription factors Dichaete > Grh > Eyeless (Bayraktar and Doe, 2013). In contrast, embryonic INPs express only Dichaete. These data, together with the short time frame of embryogenesis, suggests that INP quiescence occurs during the Dichaete window, preventing expression of the later Grh > Ey cascade. Interestingly, INPs in the posterior cluster completely lack Dichaete, suggesting they may be using a different temporal transcription factor cascade. The posterior cluster type II neuroblasts are likely to be the DL1-DL2 type II neuroblasts, which have never been assayed for the Dichaete > Grh > Eyeless cascade in larval stages. Perhaps these two neuroblasts use a novel temporal cascade in both embryonic and larval stages.

Larval type II neuroblasts produce many intrinsic neurons of the adult central complex (Bayraktar and Doe, 2013; Ito et al., 2013; Yu et al., 2013). Here we show that embryonic INPs also produce neurons that contribute to the adult central complex. Our data show ~54 neurons (64 minus the 10 due to "leaky" expression) born from embryonic-born INPs survive to adulthood and innervate the central complex. It is likely that this is an underestimate, however, because (1) 9D11-gal4 expression is lacking from a few INPs in the embryonic brain and (2) the time to achieve sufficient FLP protein levels to achieve immortalization may miss the earliest born neurons. The variation in immortalization of the wide field ellipsoid body neuron may represent a neuron born early in the type II lineages, thus unlabeled in a subset of embryos. Additionally, some embryonic born neurons may perform important functions in the larval/pupal stages but die prior to eclosion.

Further studies will be required to understand the function of neurons born from embryonic type II lineages. It remains to be experimentally determined whether some or all embryonic progeny of type II neuroblasts (a) remain functionally immature in both the larval and adult brain, but serve as pioneer neurons to guide larval-born neurons to establish the central complex, (b) remain functionally immature in the larval brain, but differentiate and function in the adult central complex, or (c) differentiate and perform a

function in both the larval and adult CNS. It will be informative to selectively ablate embryonic-born neurons and determine the effect on the assembly of the larval or adult central complex, and their role in generating larval and adult behavior.

Materials and Methods

Fly stocks

The chromosomes and insertion sites of transgenes (if known) are shown next to genotypes. Unless indicated, lines were obtained from Bloomington stock center (FlyBase IDs shown). Enhancer gal4 lines and reporters: *P[GAL4]pnt*$^{14\text{-}94}$ (III) (gift of Jan Lab), *R9D11-gal4* (*III, attP2*), *R9D11-CD4-tdTomato* (*III, attP2*), *10XUAS-IVS-mCD8::GFP* (*III, su(Hw)attP2*) (referred to as *UAS-GFP*). hs FLPG5;;MCFO (I and III; FBst0064086). For FLEXAMP experiment, *y,w,UAS-FLP; tubGAL80ts/CyO; R9D11-gal4/TM3* and *13Xlex- Aop2-myr::GFP ; tubGAL80ts/CyO ; P{nSyb(FRT.stop)LexA.p65}*.

Immunofluorescent staining

Primary antibodies were rat anti-Dpn (1:50, Abcam; Eugene, OR, USA), guinea pig anti-Dpn (1:1000, Jim Skeath; Washington Univ.), chicken anti-GFP (1:1000, Aves Laboratories, Tigard, OR), guinea pig anti-D (1:500, John Nambu; Univ. Massachusetts, Amherst), rabbit anti-Ey (1:2500, Uwe Walldorf; Germany), rabbit anti-phospho-Histone H3 (ser 10) (1:20,000, Millipore, Temecula, CA), rabbit anti-PntP1 (1:1000, Jim Skeath; Washington Univ.), rat anti-Grh (1:1000, Stefan Thor), rabbit anti-DsRed (1:1000, Clontech Laboratories, Mountain View, CA, USA), rabbit anti-Ase (1:1000, Cheng-Yu Lee; Univ. Michigan), mouse anti-Hunchback (1:500; Abcam; Eugene, OR, USA), guinea pig anti Krüppel (1:500, Doe Lab), rat anti-Pdm2 (1:1000 Abcam; Eugene, OR, USA), guinea pig anti-Asense (1:1000; Hongyan Wang, NUS/Duke, Singapore), rabbit anti-Cas (1:1000, Ward Odenwald, distributed by the Doe lab), mouse anti-NC82 (1:200, Developmental Studies Hybridoma Bank). Secondary antibodies were from Molecular Probes (Eugene, OR, USA) or Jackson Immunoresearch (West Grove, PA, USA) used at 1:400.

Embryos were blocked overnight in 0.3% PBST (1X phosphate buffered saline with 0.3% Triton X-100) with 5% normal goat serum and 5% donkey serum (PDGS) (Vector Laboratories, Burlingame, CA, USA), followed by incubation in primary antibody overnight at 4^{o}C. Next, embryos underwent four washes 15 minutes each in PDGS, followed by a 2 hour secondary antibody incubation at 25^{o}C. After secondary, embryos were either dehydrated with ethanol and mounted in dibutyl phytalate in xylene (DPX) according to Janelia protocol (Wolff et al., 2015) or were cleared with a glycerol series: 25% for 10 minutes, 50% for ten minutes, 90% for ten minutes then into 90% glycerol with 4% n-propyl gallate overnight before imaging.

Larval brains were dissected in PBS, fixed in 4% formaldehyde in PBST for 25 min, rinsed 30 minutes PBST, and blocked in PDGS overnight at 4^{o}C. Staining as above for embryos, but after secondary were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

Adult brains were fixed in 2% formaldehyde in PBST, rinsed, and blocked in PDGS with 0.5% Triton. Brains were incubated in primary antibodies for four days at 4^{o}C, then in secondary antibodies for two days at 4 ^{o}C. Brains were mounted in DPX according to Janelia protocol.

Clones

For type II clones, *P[GAL4]pnt$^{14-94}$* (III) x *hs FLPG5;;MCFO* (I and III; FBst0064086) embryos were collected for four hours at 25^{o}C, aged four hours and heat shocked at 37^{o}C for 12 minutes, then left to develop until desired stages.

FLEX-AMP immortalization of embryonic INPs

The FLEXAMP experiment used 1- 3 day old adult females from crossing: *y,w,UAS-FLP; tubGAL80ts/CyO; R9D11-gal4/TM3* to *13Xlex- Aop2-myr::GFP ; tubGAL80ts/CyO ; P{nSyb(FRT.stop)LexA.p65}* to permanently label embryonic INPs (Bertet et al., 2014). Negative controls were raised continuously at 18^{o}C to maintain Gal80 repression; positive controls were raised continuously at 29^{o}C inactivate Gal80 and allow 9D11-gal4 expression. To "immortalize" embryonic INPs and their progeny, we exposed embryos aged 5-6 hours to 29^{o}C for ten hours to a allow *R9D11-gal4* expression

and then shifted all unhatched embryos to 18^{o}C to block *R9D11-gal4* expression during larval, pupal and adult stages.

Cell proliferation analysis

Number of proliferating INPs was calculated by dividing the number pH3 positive by the number of total INPs within each cluster of neuroblast at different stages. Each circle represents one cluster of INPs. Error bars represent standard error of the mean.

Imaging

Images were captured with a ZeissLSM700 or ZeissLSM710 confocal microscope with a z-resolution of 1.0 micron, and processed in the open source software FIJI (http://fiji.sc) and Photoshop CS5 (Adobe, San Jose, CA, USA). Figures were made in Illustrator CS5 (Adobe, San Jose, CA, USA). Three-dimensional brain reconstructions in Figs. 3 and 6 were generated using Imaris (Bitplane, Zurich, Switzerland).

Bridge to Chapter III:

In Chapter II, I characterized the presence of type II NBs within the central brain in the Drosophila embryo. Type II NBs within the embryo raises two important questions. One, what is the mechanism of type II NB formation within the early brain? The staining of the early embryonic brain with PntP1, Dpn and Ase to identify the embryonic type II NBs revealed the presence of discrete groups of NE cells positive for the type II specific marker, PntP1, that may specify type II NB identity. Two, what is the division pattern of the type II NB lineages within the embryonic brain? Experimentas using clonal analysis and molecular marker staining suggest, but can not confirm, the transit-amplifying division pattern within INPs. Both questions are best directly tested using live imaging. In Chapter III, I will address these two outstanding questions about embryonic type II NBs that necessitate live imaging within embryonic NE and central brain, the current limitations encountered and the strategies to combat these in future experiments.

CHAPTER III

LIVE IMAGING OF DROSOPHILA EMBRYOS: NEUROECTODERM AND TYPE II LINEAGES

Live imaging can provide insight into cellular and molecular level dynamics unanswerable using only fixed samples. Recent advances in techniques have allowed researchers to answer outstanding topics in developmental biology such as cellular migration, transcription factor expression during border formation within developing embryos, nascent RNA transcription, chromatin dynamics and axonal pathfinding (Holloway and Spirov, 2017; Khairy et al., 2015; Lee et al., 2016; Muramoto et al., 2012; Pantazis and Supatto, 2014; Sardo et al., 2017). Understanding of embryonic type II NBs would benefit from live imaging. I have showed via molecular markers for cell identity and gal4-expression, asymmetric cell division protein expression and clonal analysis that type II NBs are producing transit-amplifying within the embryo, however the cell division pattern would be answered most definitively with live imaging of embryonic type II NBs. Additionally, the exact mechanism of formation of embryonic type II NBs from the NE layer remains mysterious from only fixed sample data.

Embryonic Type II Formation from the Neuroectoderm

Embryonic and larval type II NBs are defined as Dpn^{+}, $Asense^{-}$ and $PntP1^{+}$. The mechanism by which type II NBs form from the NE cell layer is still unknown. To determine when PntP1 is first expressed, we assayed for PntP1 expression in the procephalic neuroectoderm at the time of type II neuroblast formation. We identified three domains of PntP1 expression visible in a dorsal view of the late stage 11

procephalic neuroectoderm: strong expression in an anterior and a posterior domain, and weaker expression in a middle domain (Figure 1A, yellow lines). When viewed from a lateral perspective, we reliably detected a single type II neuroblast internal to the middle domain (Figure 1A' white box, top inset) and often a pair of type II neuroblasts located internal to the anterior domain (Figure 1A' white box, bottom inset). During stage 12, the anterior and middle domains shift to form an almost continuous domain running anterior-posterior adjacent to the dorsal midline (Figure 1B; white arrowheads); this domain produced three more type II neuroblasts to bring the total to six type II neuroblasts per brain lobe. We do not observe any type II neuroblasts associated with the posterior PntP1 domain; this domain shifts ventrally and gives rise to the optic lobe anlagen and Bolwig's organ and is not considered further (Green et al., 1993). During the late stage 12/ stage 13 border, a seventh type II NB forms within the existing pattern of type II NBs along the dorsal midline (Figure 1C). Due to its position, we were unable to definitely link it to the middle or anterior PntP1 domains. By stage 14, there is only a small patch of PntP1^{+} neuroectoderm located just anterior to the previously formed type II neuroblasts; a single type II neuroblast is located just internal to this domain (Figure 1D). Finally, by stage 16 there is no PntP1+ neuroectoderm and no additional type II neuroblasts will develop (Figure 1E). At this stage the eight type II neuroblasts are arranged in three clusters, similar to their position in the larval brain. We conclude that all type II neuroblasts develop from PntP1^{+} neuroectoderm.

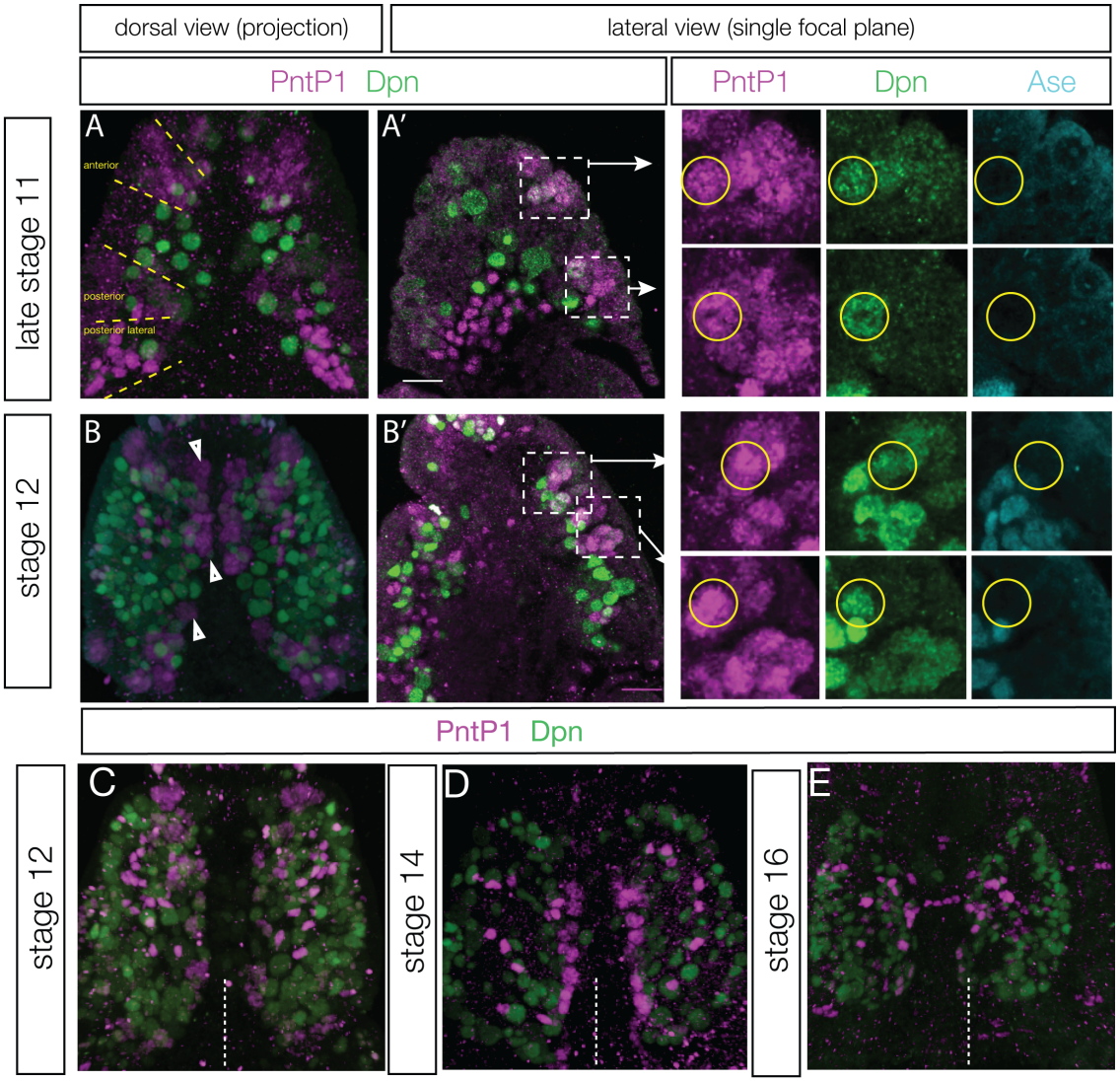


Figure 1. Embryonic type II neuroblasts arise from PointedP1-positive neuroectoderm.
(A) Type II NBs form internal to PntP1 positive neuroectoderm (NE) beginning at late stage 11. Maximum intensity projection of dorsal view of the three stage 11 PntP1 NE expression domains: anterior, middle and posterior (yellow dashed lines). (A') Lateral view of single focal plane of embryo in (A) with views of type II NBs internal to PntP1 positive NE (white boxes). The anterior PntP1 NE domain (box 1) contains two PntP1+, Dpn+, Ase- type II NBs (yellow circles) just internal to the NE (cyan dashed line). The posterior domain (box 2) contains one type II NB (yellow circle) internal to the NE (cyan dashed line). Midline, white dashed bar. Scale bar = 10μm.
(B) Dynamic expression of PntP1 in neuroectoderm and type II neuroblasts. Dorsal views of maximum intensity projections of PntP1 expression in the neuroectoderm (magenta; yellow dashed outlines) and neuroblasts (circled); Dpn (green) marks all neuroblasts. Scale bar = 20μm for B', 5 μm for insets.
(C-E) PntP1 (magenta) neuroectoderm expression showing Dpn (green) at stage 12 (C), stage 14 (D), stage 16 (E). Dashed white line represents midline.

The embryonic central brain PntP1 expression appears in clusters of NE cells along the dorsal midline with a distinct apically constricted shape (Figure 2A, stage 11, Figure 2B, stage 12). Placodes have traditionally been a term reserved for higher organisms, and the term "invaginations" has been proposed to be more appropriate for these groups of cells in the central brain. As there has been work previously published on these NE clusters along the midline of the developing central brain, I will use this terminology (de Velasco et al., 2007; Hwang and Rulifson, 2011). Previous work on embryonic central brain placodes has shown a single placode along the anterior midline gives rise to five type I NBs before generating one type II NB around stage 15 (Hwang and Rulifson, 2011). It was shown that both Notch and the EGF signaling pathways contribute to maintaining this placode and specifying the type II NB; however, PntP1 expression was not documented. It remains to be determined if the additional seven type II NBs come directly from PntP1 placodal NE, as the PntP1 staining suggests.

Using fixed samples to determine if all type II neuroblasts develop from such placodes, we assayed the morphology of the PntP1^{+} neuroectodermal cells by using a Pnt-gal4 line (R45D11-gal4) to drive the membrane marker myristoylated GFP (myr:GFP). R45D11-gal4 is expressed within PntP1 positive NE, some of the type II NBs, and a few type I NBs. It is also expressed within larval-born type II neurons that contribute to both the central complex primordium and adult neuropils (Riebli et al., 2013). We observed that PntP1^{+} neuroectodermal cells form placodes of neuroectodermal cells with apical constrictions (Figure 2A, inset top row), and just internal are the type II neuroblasts (Figure 2A, inset bottom row, yellow circle). Although we observed low

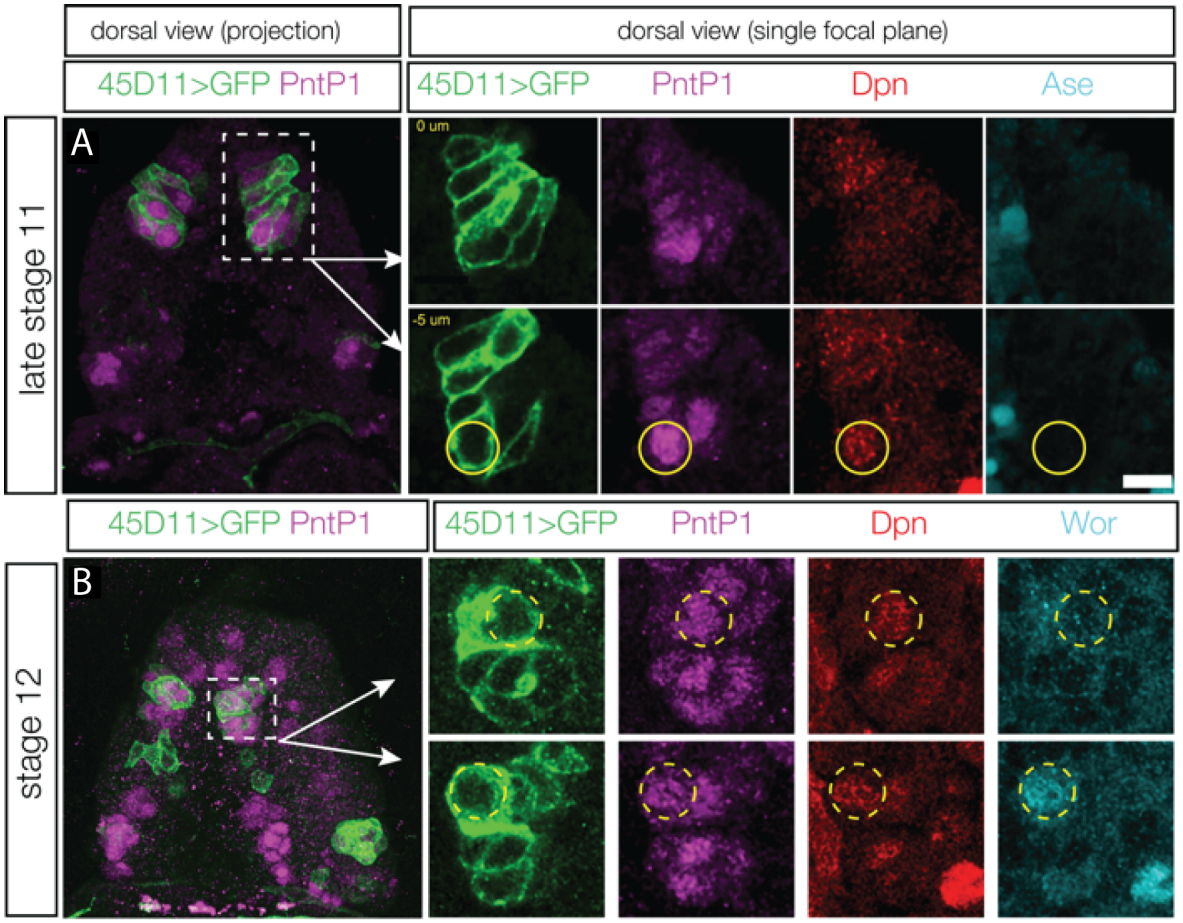


Figure 2: Embryonic PointedP1-positive neuroectoderm has placodal morphology
(A) *R45D11-gal4 UAS-myr:GFP* expression within anterior PntP1 neuroectodermal domain at late stage 11. Two focal planes are show, one more apical (inset, top) to visualize apical constrictions and one 5µm more internal (inset, bottom) to show the PntP1+ Dpn+ Ase- type II neuroblast associated with the placode (circled).

(B) *R45D11-gal4 UAS-myr:GFP* expression within anterior PntP1 neuroectodermal domain during stage 12. Two focal planes are show, one more apical (inset, top) to visualize apical constrictions and one more internal (inset, bottom) to show the two PntP1+ Dpn+ Ase- type II neuroblasts associated with the placode (circled). Scale bar = 5µm.

levels of Dpn in the neuroectodermal placode (Figure 2A, insets), only the type II neuroblasts expressed both high levels of Dpn and the definitive neuroblast marker, the transcription factor Worniu (data not shown). We assayed all eight type II neuroblasts to determine whether all of them delaminated from a placode, and found that the first type II

neuroblast to form developed from PntP1 neuroectoderm that did not have placode morphology. In contrast, the five more anterior type II neuroblasts developed from PntP1 neuroectoderm with placodal morphology (similar to what is shown in Figure 2A-B). We were unable to definitively score for placode origins of the final two type II neuroblasts to form. Thus, we conclude that some but not all of the type II neuroblasts develop from neuroectoderm with placode morphology. It is likely that type I neuroblasts are also born from these placodes (Hwang and Rulifson, 2011).

Although the fixed samples suggest the type II NBs upregulate Dpn and delaminate from these PntP1^{+} placodes, the data remain circumstantial. We attempted live imaging to address the question of how type II NBs form from apically constricted clusters of PntP1^{+} NE. We hypothesized two possibilities for the origin of type II lineages in the embryo. One, type II NBs begin generating progeny only after delamination from the placode, or two, that the type II NBs divide to produce progeny while still within the NE layer. To visualize both the NE cells and the forming type II NBs in embryos, the combination of *45D11-gal4* and a marker for INP progeny, 9D11-tdTomato fusion, were used. Unfortunately, attempts to track the fate of the individual placode cells, type II NBs and 9D11+ INP progeny were unsuccessful. Table 1 lists the markers used in identifying type II lineages within the embryo. After multiple imaging attempts, the data support the hypothesis that the type II NBs delaminate from the NE layer prior to generating any progeny. It remains uncertain if type I NBs were also generated from these placodes as the ability to track the fate of cells leaving the placode was difficult. With the movement of the head involution combined with the internal movement of cells within the lineages,

tracking cells was not possible. A major problem with long-term live imaging is the need to maintain a confident track on cells of interest.

Gal4 Lines Used to Identify Embryonic Type II Neuroblasts			
Experiment	Marker	Cell Type	Issue
Placodal Origin	45D11-gal4	NE, type II NB and progeny, type I NB and progeny, hemocytes	Too many cells to use to uniquely ID type II progeny
Type II division pattern	Pnt^{14-94}-gal4	Type II NB and lineages, few glia	Does not begin expressing until stage 14, onset varies between lineages
Placodal Origin/ Type II division pattern	9D11-tdtomato fusion	Type II INPs, Type I lineages (early), optic lobe (late)	Not specific until stage 14
Placodal Origin/ Type II division pattern	9D11-gal4 (II)	Type II INPs, Type I lineages, many other cells	Not specific at any stage
Placodal Origin/ Type II division pattern	9D11-gal4(III)	Type II INPs	Does not come on in INPs until stage 15 in a subset of lineages

Table 1. Gal4 Lines Used to Identify Embryonic Type II Neuroblasts

Embryonic Type II division profile

Type II NBs are defined by their molecular marker profile and their ability to produce transit-amplifying progeny, intermediate neural progenitors (INPs) (Bello et al., 2006; Boone and Doe, 2008; Bowman et al., 2008). In Chapter 2, multiple lines of data were presented in support of the existence of embryonic type II NBs: 1) molecular profile of PntP1+ Dpn+ Ase+ (Chapter 2 Figure 1), 2) clonal data showing the type II NB produce progeny that produce multiple INPs (Chapter 2, Figure 2), and 3) molecular

staining for asymmetric cell division proteins within the INPs (Chapter 2, Figure 3). These data strongly suggest that embryonic type II NBs exhibit a type II division pattern, but only live imaging of the divisions provide the necessary irrefutable evidence to show embryonic type II NB progeny divide at least twice.

Live imaging of embryonic type II NBs was attempted repeatedly using multiple type II NB molecular markers, including gal4 lines but remained unsuccessful. Table 2 details the markers used for live imaging. The main issues preventing the data collection include lack of specific live-imaging markers for the cells, inaccessibility of the location of the lineages of interest within the developing central brain region of the Drosophila embryo, and the time required for tracking to confidently identify members of the lineage. Many gal4 lines express within embryonic type II lineages in fixed samples, but most are too weak to visualize in live imaging. Antibody staining can amplify the signal of a gal4 line in fixed samples, but the weak signal of many gal4 lines presented as a problem during live imaging.

Additionally, live imaging requires a specific gal4 line as live imaging does not allow molecular profiling to compliment the gal4 lines used. Many gal4 lines expressed within type II NB lineages had off-targets that were not spatially segregated (Table 1). Furthermore, the dorsal location of type II NBs at the time of formation was beneficial for identifying the NBs in live imaging; however, later brain morphogenesis movements in the posterior and ventral direction precluded lineage analysis for two reasons. One, the location of the type II lineages within the center of the brain of the type II NB lineages resulted in only a few later born lineages amenable to live imaging. Two, the imaging depth necessary to track cells within the lineage long enough to assign identity was

unachievable due to loss of signal clarity. For example, to identify a cell as a ganglion mother cell (GMC) progeny of an INP requires the tracking of this cell for more than two cell cycles to ensure it only divides once more to generate two progeny cells. This was difficult to achieve.

Markers used for Live Imaging		
Marker	Pro	Con
Histone fusion	-Does not rely on a driver line to visualize cells of interest (ie marker will not miss early cells or fade in later born cells) easy to track divisions	-Non-specific, and can make tracking difficult to link parent and daughter cells ("sea of green")
Uas-Histone fusion	Specific to cells of interest, facilitates accurate tracking, long lived histone protein may not lose signal after driver line expression ceases	Requires a driver line
Uas-membrane fluorescent protein	Facilitates tracking parent and daughter cells, easy identification of lineages, can visualize neuronal axon tracts	Difficult to track cells during mitosis, relies on specific driver line
UAS-nuclear fluorescent protein	Facilitates tracking parent and daughter cells within lineages	Difficult to track cells during mitosis due to diffuse signal, relies on specific driver line
Global nuclear fluorescent protein	Does not rely on a driver line, maybe useful in combination with driver line with uas-Histone	can make tracking difficult to link parent and daughter cells ("sea of green")

Table 2. Markers used for Live Imaging

Microscope Options for Live Imaging

Advancements in microscope options, fluorescent proteins and genetic tools have allowed probing into real-time developmental events in vivo previously inaccessible due to technical constraints. For the remainder of the Chapter, I will address the options,

technical considerations and hurdles involved in live imaging in vivo Drosophila embryonic neuroectoderm dynamics and type II NB divisions.

Spinning disc (SD) laser confocal microscopy is a standard in live imaging experiments. The design allows for diffuse illumination of the specimen through a spinning disc with ~1000 pinholes that reduce laser toxicity on the sample. The low laser broad illumination makes this microscope ideal for long term imaging experiments. Additionally, the spinning disc presents a clear signal to noise ratio, yet this signal can often be weak with weak signal from the sample. Weak signal necessitates a longer exposure time and reduces the usefulness of the spinning disc in some live imaging applications (Rebollo et al., 2014). Additionally problematic for whole mount embryo imaging, the decrease in signal clarity with increasing z depth can prevent cell tracking deep within the embryo. Furthermore for some mounting methods, the inverted objective can be problematic.

Laser point scanning confocal microscope (LSC) uses a laser to spot scan across the sample and acquires an image point by point (Rebollo et al., 2014). Embryos were imaged using three LSC microscopes from Zeiss: LSM 700, LSM 710 and LSM 800. Laser scanning confocal microscopy has many drawbacks, and is not ideal for long-term live imaging. The high laser necessary to image can harm the sample and cause death in as short as one hour. Also, the slower acquisition time can be confounding when imaging a large z area, taking up to three minutes to image 80 um causing the top and bottom of the sample to be at different time points for each time point scanned.

In a collaboration with the Keller Lab at Janelia, who has successfully live imaged Drosophila embryogenesis in its entirety using a lightsheet microscope (LS), we

attempted to image the developing embryonic central brain. Lightsheet microscopy moves the sample through a plane of light, illuminating the sample with thin sections of light. As only a small part of the sample is illuminated at any time point, there is little photobleaching or toxicity to the same making this technique ideal for long term live imaging. A major drawback of light sheet microscopy that prevents its usefulness is that the clarity at larger depths decreases (Rebollo et al., 2014). The lack of transparency within Drosophila embryo exacerbates this penetrance issue. Even with a sparse gal4 pattern driving a nuclear marker paired with a global histone fusion (Pnt-gal4, Uas-nuclear red stinger, Histone2A RFP) the computer software at Janelia was unable to track the cells due to loss of the signal at greater depths.

Table 3 details the experiments attempted in lab and on which microscopes. The only microscope not attempted is a two-photon microscope (TP). Multiphoton microscopes are ideal for live image as they allow for excellent signal to noise ratio, lack of toxicity, and great depth clarity. Multiphoton microscopes illuminate the sample with two or more low power lasers that excite fluorophores only where they meet, and thus the only region where laser strength is sufficient for excitation (Rebollo et al., 2014). Due to lack of availability, whole-mount embryos where not imaged using multiphoton microscopes. However, the problems encountered in live imaging of embryonic type II NB lineages may be solved if imaged with a two photon microscope.

Genotypes Live Imaged on Microscopes				
Genotype	Microscope			
	LSC	SD	LS	TP
45D11-gal4; Uas-membrane::GFP; 9D11-membranetdTomato		x		
9D11-tdtomato; His-2A::GFP	x	x		
Pnt-gal4; Uas-nuclear::red-stinger,	x	x		
Pnt-gal4; Uas-membrane::GFP; 9D11-tdtomato	x	x		
9D11-gal4; UAS-membrane::GFP		x		
Pnt-gal4, Uas-His-2A::RFP, His-2A::GFP		x	x	

Table 3. Genotypes Imaged on Microscopes

Technical Considerations for Live Imaging

Important to confirming cellular division pattern is the ability to accurately identify cells, identify the division, and confidently track cells within the lineages for long periods of time. Perhaps most important is accurate identification of the cells of interest. When using a gal4 line, the live imaging signal is weaker than in fixed samples due to amplification of single in immunohistochemistry. It is also important to note, a delay occurs in visualizing positive cells in live vs. fixed samples. Furthermore, off targets within a gal4 line in live imaging are more difficult to deal with due to lack of other markers for identity or landmarks.

To accurately track a cell, it is necessary to walk the line between labeling too many cells- as in tracking one cell in a sea of positive cells- or too few cells, where the marker is not expressed throughout the lineage. To achieve cell specificity and track division, it is best to use a gal4 line to drive histone. However, the fusion of a fluorescent protein with a histone protein work well, and do not "soak up" GAL4 protein from binding other UAS domains, thus keeping any marker or RNAi gene fully expressed within the cells of interest. However, the histone fusions can confound the ability to track cells as every cell in the sample will be positive for the marker. Additionally, histone markers have drawbacks. If more than one cell divides within close proximity that are both expressing the histone marker, it makes it difficult to assign progeny cells to the correct parent cell. An alternative to histone markers to track cells is a membrane marker. These are helpful to use because the outline of both the parent and daughter cell are identifiable after mitosis, but presents a problem signal become diffuse to absent at mitosis.

Additionally, for live imaging it is important to ensure 1) the health of the sample and 2) to ensure conditions mimic the native cellular environment. Wholemount live imaging of an embryonic brain in vivo or to dissociate the embryonic cells and image the division in vitro both have their own benefits and drawbacks. For wholemount imaging, mounting the embryo on a poly-lysine coating glass bottom dish for use on inverted microscope was found to be the best to maintain embryo health and ease of mounting. Live imaging wholemount in vivo cells reduces any cell cycle or migration artifacts, yet the clarity of image acquisition decreases with depth of the imaging. In vitro live imaging requires a specific gal4 driver to identify the cells, as all spatial organization of the

embryo is lost with dissociation. Currently for embryonic type II NBs a specific enough driver line does not exist to merit in vitro imaging feasible. Sometimes it is helpful to pair live imaging with fixed and stained samples with markers to confirm gal4 line expression, which proves difficult when attempting to stain a single Drosophila embryo but can be useful with in vitro imaging.

Additionally, signal clarity declines at greater imaging depths- an important consideration when long tracks are required to declare cells have terminally divided. Unfortunately, the type II NBs location in the middle of the embryo for most of the embryonic timeframe complicates tracking multiple cell divisions over time difficult. Another problem with long term cell tracking is the intense twitching movements of late stage embryos which prevents accuracy of tracking. Image acquisition time is important as sometimes cells can move or divide in between frames, and also before the scan is finished in the z axis. Additionally, oversampling or long scans can result in photobleaching of fluorophores or artifacts of laser toxicity, shifting cell cycle timing, protein localization or even cell death.

Another option to "human eye" tracking is computer cell tracking such as in the Imaris or MatLab platforms. Tracking with any computer software has been unsuccessful for a few reasons. One, the current algorithms have difficulty maintain a trace during mitosis due to diffuse signal during division and nuclear envelope break down. Two, the few attempts with computer tracking have not resulted in a lineage tree with branches for cellular divisions. Rather, the algorithm generates linear outcomes when it begins new tracks for each pair of cells from one division. Further development of the programs should improve cellular tracking. Histone markers produced the best outcomes with

computer tracking algorithms, as there is an increase, rather than a loss or weakening, of signal during mitosis. One must balance all these consideration to achieve successful and interpretable live imaging of healthy Drosophila embryos.

CHAPTER IV

Conclusion

In this dissertation, I show in Chapter 2 that embryonic type II NBs 1) form directly with a type II identity in the embryonic brain, 2) produce transit amplifying INP progeny, 3) enter quiescence in the late embryonic brain, 4) express the known temporal patterning cascade of Hb > Kr > Pdm > Cas > Grh, and 5) contribute neuronal progeny that diffusely innervate multiple neuropils of the adult central complex. Furthermore, I report that the INP progeny of embryonic type II lineages express only the first of the known larval INP temporal patterning factors, Dichaete, and enter quiescence at the end of embryogenesis. Chapter 3, details embryonic type II NBs formation from PntP1 positive neuroectoderm during mid-embryogenesis. Furthermore, I raise central questions concerning Drosophila embryonic type II lineages left unanswered with conventional fixed embryo analyses. Finally, I elaborate upon the challenges of live imaging within the central brain of wholemount Drosophila embryos and describe improvements needed for successful *in vivo* imaging for future experiments. Below, I address the next directions for this topic implicated by the results presented in this dissertation.

Specification of type II identity within the embryo

All eight type II NBs form in each brain lobe during embryogenesis instead of forming from a type I > type II switch prior to larval stages. How is the type II identity restricted to a fixed number within the developing brain? Prior work has shown an isoform of the transcription factor Pointed, PntP1, can maintain type II identity within larval type II NBs by repressing Ase expression within the NB and promoting production

of INP progeny (Zhu et al., 2011). I have shown PntP1 to be expressed within embryonic type II NBs. My work has shown restricted expression of PntP1 within clusters of NE cells located directly superficial to the nascent type II NBs, implicating PntP1 in specifying type II identity. However, previous work has described the presence of three head neuroectodermal placodes, each expressing PointedP1, and at least one of these placodes generates both type I and type II neuroblasts; this result indicates that expression of PointedP1 alone in the neuroectoderm is not sufficient to specify type II neuroblast identity (de Velasco et al., 2007; Hwang and Rulifson, 2011). There may be an additional factor promoting type II neuroblasts that is transiently expressed during the phase of type II neuroblast production, or a factor blocking type II neuroblast identity transiently expressed during the phase of type I neuroblast production. A candidate for distinguishing type I / type II neuroblast identity is EGF signaling, which can be detected in the three head placodes (de Velasco et al., 2007; Hwang and Rulifson, 2011). For example, EGF signaling could promote type II neuroblast identity, whereas transient expression of the EGF negative regulator Argos could allow for type I neuroblast specification (Rebay, 2002).

Diversity within the type II NB population

The data presented here distinguish between type I and type II NB identity but do not address individual characteristics of NBs within the type II population. Based on positional information in late embryonic stages, I assign provisional larval identities to embryonic type II NBs. Confirmation of these identities requires genetic immortalization using specific driver lines, embryonically induced clones, or unique molecular profiles.

As the diversity within the type II NB population, i.e. DM1 vs. DM 5, most likely arises during initial specification within the embryonic NE, this supports the possibility of unique molecular signatures. This suggests type II NBs may be uniquely identified with combinations of spatial factors as has been done for the embryonic type I NBs in the VNC and CB (Doe, 1992; Urbach and Technau, 2004). We have attempted to link the embryonic type II NBs to the existing spatial factor map for the CB, but were unsuccessful due to the late timing of the type II NB formation. Information on spatial factor specification of individual type II NBs could provide insights into their unique lineages, as well provide candidates for the ongoing search for specific gal4 driver lines for individual type II NBs (Manning et al., 2012). Furthermore, spatial factor code would assist in tracking individual NB during embryogenesis, a task that has proven difficult with the dynamic involution of the developing brain.

As it is known that individual type II larval lineages contribute to distinct regions within central complex neuropils, it raises the question if a similar embryonic origin contributes to similarities in progeny function (Riebli et al., 2013; Wang et al., 2014). As most type II NBs form from NE placodes and some placodes make multiple type II NBs, are there similarities between NBs formed from the same group of PntP1+ NE placode cells? Does a shared NE origin and spatial factor expression code contribute shared features to the identities, functions or locations of their neuronal and glial progeny? Would manipulating spatial factor expression within embryonic type II NBs change the axonal targeting of their progeny in predictable ways? Having a map of type II NB formation within the embryo will contribute to answering these questions.

Differences between embryonic and larval INPs

INPs have not previously been identified in the embryo, which raises the question if differences exist between embryonic and larval INPs. Larval INPs have a roughly six hour maturation period before the first division. The short timeframe of embryogenesis suggest transit-amplifying embryonic INPs have an abbreviated cell cycle. Live imaging experiments are needed to assign cell cycle times to embryonic INPs. Furthermore, live imaging would clarify remaining questions about embryonic INP quiescence. Larval INPs complete their four to six divisions within the larval brain, whereas at least some embryonic INPs enter quiescence. Embryonic INPs likely enter quiescence at late embryonic stages as 1) they are present but not dividing, and 2) they are identifiable at early larval stages (Chapter 2, Figure 4); however it remains unknown if embryonic INPs enter a true "quiescence" similar to type I and type II NBs. Alternatively, they may differentiate or die prior to resuming the cell cycle in larval stages.

Larval INPs express the D > Grh > Ey temporal factor cascade, whereas embryonic INPs express a truncated form of only the D window. If the embryonic INPs exit quiescence in larval stages, do they resume the temporal sequence in the Grh window? Larval INPs have a six hour maturation phase before their first division, whereas embryonic INPs do not appear to have this maturation requirement. Additionally, all larval INPs inherit Grh protein from the Grh+ type II NB (Bayraktar and Doe, 2013). In the embryo, INPs are only Grh+ in late embryonic stages when the type II NB is in the Grh window. Notably, during the Grh expression window both NB and INPs appear close to entering quiescence as most Grh+ type II NBs are quite small; although the NB and INPs divide at least once within the Grh window. Does Grh serve a function

within the INP to regulate the cell cycle timing of maturation? Do Grh negative embryonic INPs in the early and mid embryo skip the maturation period seen in larval INPs as a consequence of lacking Grh? Misexpression experiments within embryo and larval INPs are needed to address this question.

Integration of temporal factors in embryonic NBs and INPs

Embryonic type II NB express the known type I NB temporal cascade. Progeny of type I NBs divide only once but maintain expression of inherited temporal factors into the neurons for a short time (Hirono et al.; Isshiki et al., 2001). It is suspected that neuronal temporal factor expression causes epigenetic changes within the progeny to permanently maintain the genetic landscape and removes the need for continued temporal factor expression (Hirono et al.). Type II INP progeny divide multiple times and express a separate temporal factor cascade. Maintenance of NB temporal factors into INP progeny has not been assayed in larval lineages, probably because the expression windows of the identified larval NB temporal factors is quite long. Identification of the well-studied embryonic Hb > Kr > Pdm > Cas > Grh temporal cascade within embryonic type II NBs, with its accompanying extensive genetic toolkit, allows investigating of NB temporal within INPs. Is the NB temporal factor maintained over multiple divisions of an embryonic INP? Furthermore, individual manipulation of NB and INP temporal factors would reveal the role of the individual temporal factor cascades.

Function of the embryonic-born neurons born within the embryo

I have identified a population of ~ 64 embryonic-born neurons from type II lineages that survive into the adult brain to innervate a subset of central complex neuropils. This raises many interesting questions. Does this represent the entire population of embryonic-born neurons from type II lineages? Do the neurons born within the embryo have a functional role in central complex function? Do any embryonic-born type II neurons play a scaffolding or pioneering role? I will address the specifics of these questions below.

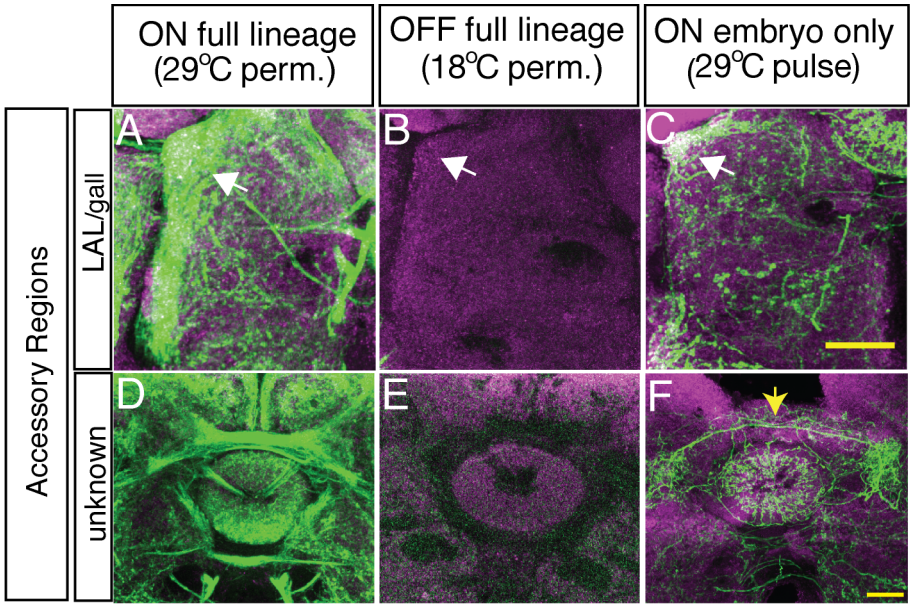
Do embryonic type II lineages produce only the ~60 neurons from the immortalization experiment? A report analyzing electron microscopy images of early larval brains estimated the neuronal contribution of presumed embryonic type II lineages to be ~125 neurons per lobe based on position of the lineages (V. Hartenstein, personal communication), suggesting ~60 neurons to be an underestimation. The driver used to immortalize the neuronal progeny, *9D11-gal4 (III)*, misses some of the early progeny-accounting for some underestimation of type II neuron numbers. Furthermore, there are two considerations with the genetic strategy used here to immortalize the type II embryonic-born neurons. First, the driver, *nsyb-gal4 (III)*, used for immortalization expresses only within mature neurons; thus prohibiting identifying any neurons that may serve an early role without fully differentiating or those that may die before adult stages. Assaying larval or pupal stages or using an *actin-gal4* driver instead of a differentiated neuron driver would allow for visualization of neurons before differentiation and at earlier developmental stages. Two, the neuronal specific gal4 driver prevents visualizing glia, which could be important contribution of embryonic type II lineages.

Using a *9D11-tdTomato* fusion to visualize type II INPs and progeny within the embryo reveals the presence of neurons with axonal outgrowth by stage 14 (8 hours into embryogenesis, 3 hours after the first type II forms), suggesting lineages have a faster cell cycle than in the larva. Live imaging is necessary to quantify embryonic type II NB and INP cell cycles. If the cell cycles are similar to larval stages, it may be possible that the type II NBs begin divisions while still part of the placode. There is precedent for this type of neurogenesis in other organisms. Interestingly, neurogenesis within the spider *Cupiennius salei* contains a cell layer with invaginated clusters of neural stem cells that directly make neurons without delamination from the clusters. Spider neural stem cells, that all reside within the NE layer, divide to produce progeny that differentiate directly into neurons (Stollewerk et al., 2001). Could the type II NBs begin producing progeny prior to delamination from the NE placode cell layer? The PntP1+ NE placodes form quite early in development of the central brain (five hours). The placode molecular profile is PntP1+ Ase- and weakly positive for Dpn similar to type II NBs only with weaker Dpn staining. This raises a possibility that molecular marker staining for Dpn, PntP1 and Ase misjudges the timeframe of type II NB formation. Future live imaging experiments addressed in Chapter 3 would test this hypothesis.

At least 60 neurons born from embryonic INPs survive to populate the adult central complex. Aside from the total number of neurons produced from embryonic type II neurogenesis, it is of interest to understand the function of the neurons. What is the function of this population of ~60 central complex neurons? Analysis of central complex-mediated behaviors in adult flies in the background of an ablation of all embryonic *9D11-gal4 (III)*-positive neurons would reveal their function. Do the flies exhibit any

behavioral deficits in central complex mediated functions, such as flight, spatial memory or geotaxis? Additionally, if immortalization in the adult brain marks only a subpopulation of embryonic-born type II progeny, it is possible that some of the neurons that die prior to adulthood serve a pioneering role for the central complex. Does the central complex form correctly if the scaffolding neurons are ablated? Removing all embryonic-born INPs would be informational in understanding how an intricate adult brain structure with multiple interconnected nueropils form over development.

Understanding the entirety of a neural stem cell lineage is crucial to revealing its complete role in neurogenesis. The finding that type II NBs have an embryonic period of proliferation brings with it many new questions about NB division pattern specification, diversification, progeny generation and contribution to the forming brain. Embryonic type II NB lineages can contribute to a more thorough picture of neurogenesis.



Supplementary Figure 1, Walsh &Doe

Supplemental Figure 1. Embryonic INP progeny contribute to the adult Lateral Accessory Lobe (LAL) and Gall neuropils.
(A-F) Staining of central complex accessory regions in FLEX AMP positive control (A, D), negative control (B, E) and embryo-only (C, F) groups. The LAL and gall (white arrows in top panel) are strongly innervated in positive control (A), negative in control (B), and diffusely innervated in embryonic labeled brain (note strong density within gall, arrow) (C). An unknown region adjacent to ellipsoid body is densely innervated in the positive control (D), absent in negative control (E), and innervated sparsely in the embryonic-only brain (F). Note the commissural axons within the pattern in (F, yellow arrow). Scale bar = 20 μm.

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