Title
Serial specification during Drosophila placodal neurogenesis

Author
Hwang, Helen Jean

Publication Date
2011

Peer reviewed|Thesis/dissertation

Serial specification during *Drosophila* placodal neurogenesis

by

Helen Jean Hwang

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

Dedication

Dedicated to the friends, colleagues, and family who have supported me through graduate school and in life. Only a few are mentioned by name, but many others played a vital part.

UCSF:

Eric Rulifson, my PI and mentor. Thank you for your guidance in lab and providing Kimwipes during my many non-lab related crises. I will always have the greatest respect for you as a scientist.

Members of my thesis committee: *Didier Stainier, Ulrike Heberlein and Kaveh Ashrafi* - Thank you for championing me and taking the time to share your wisdom and expertise.

Yun Choi. Merci beaucoup for your advice as a senior BMS graduate student, accompanying me to the opera, and providing inspiration to achieve the impossible.

You Bin Lin. My dear classmate, thank you for your friendship and shared fly room conversations.

Bree Grillo-Hill. To my best labmate, thank you for being the mature one.

The awesome BMS staffers: Lisa Magargal, Monique Piazza, Kevin Luong, Nate Jew, and Caroline Rutland. Thank you for your friendliness and support over the years. You made the administrative part of graduate school easy.

Additional special thanks to other members of my extended lab family:

Brandy Alexander, Kalpana Makhijani, Michael Kissner, Li Chen, Dave Casso, Meenakshi Gaur, Melanie Bedolli, Muluye Liku, Vien Nguyen, Linda Lee, Pankaj Sahai, Kevin Schoenfelder and Raymond Ng.

FRIENDS AND FAMILY

My family, especially my parents **Shuyuan and Yatsun Hwang**, who brought me up right and gave me the opportunity for a great life and education. Thanks also to my brother Richard and my sister Annie for being supportive siblings. To my Mom, I gift the infamous three-eyed fly.

Michelle Yeung, the best landlord in the world.

Jenny Shih Wong, Ann Pu, Wenjuan Wan, all wonderful roommates with whom I will be life-long friends.

Patrick Yu, Phoebe Yang, Howard Kao, friends from way back that I re-discovered in San Francisco.

My wonderful French family, the Liziards: Guillaume, Beatrice, Guillaume (le petit), François, Paul and Emilie - your pictures have always decorated my lab desk so I could see your beautiful faces each day.

The Maruyama family - Eileen, Clark, Mitchell, and Bryan, thanks for always being such a warm and welcoming San Francisco home away from home.

And finally, to **Michael Chow**, my one constant support. Thank you for listening to my stories over and over and over again, and helping me get through it all. I couldn't have made it through without your love and saintly patience.

Acknowledgments

The text of this dissertation includes a reprint of the material as it appears in:

Hwang HJ, Rulifson E. Serial specification of diverse neuroblast identities from a neurogenic placode by notch and egfr signaling. Development. 2011 Jul;138(14):2883-93.

The coauthor listed in this publication directed and supervised the research that forms the basis for the dissertation.

Serial specification during *Drosophila* placodal neurogenesis

Helen Jean Hwang

Abstract

We used the brain insulin-producing cell (IPC) lineage and its identified neuroblast (IPC NB) as a model to understand a novel example of serial specification of neuroblast (NB) identities in the dorsomedial protocerebral neuroectoderm. The IPC NB was specified from a small, molecularly identified group of cells comprising an invaginated epithelial placode. By progressive delamination of cells, the placode generated a series of NB identities including the single IPC NB, a number of other canonical Type I NB, and a single Type II NB that generates large lineages by transient amplification of neural progenitor cells. Loss of Notch function caused all cells of the placode to form as supernumerary IPC NBs, indicating that the placode is initially a "fate equivalence group" for the IPC NB fate. Loss of Egfr function caused all placodal cells to apoptose, except for the IPC NB, indicating a requirement of Egfr signaling for specification of alternative NB identities. Indeed, de-repressed Egfr activity, in *yan* mutants, produced supernumerary Type II NBs from the placode. Loss of both Notch and Egfr function caused all placode cells to become IPC NBs and survive, indicating that commitment to NB fate nullified the requirement of Egfr activity for placode cell survival. We discuss the surprising parallels between serial specification of neural fates from this neurogenic placode and the fly retina.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter One: *General Introduction*

1.1 Diversity in the central nervous system

Despite organismal differences, the task of the central nervous system (CNS) is the same across phyla: to integrate various informational cues to coordinate the activities of the body. The CNS of bilaterian animals contains two main partitions: a truncal part, which consists of a spinal or nerve cord, and an anterior part, the brain (Altmann and Brivanlou 2001, Urbach and Technau 2008). The truncal nerve cord is composed of repetitive segmental units, while the anterior brain has a more complex segmental composition.

The CNS consists of a diverse array of neurons with distinct form and function, all of which are generated by the proliferation of a restricted number of neural progenitors during development (Doe 2008, Brand and Livesey 2011, Temple 2001). These neural progenitors are multipotent stem cells that undergo several rounds of division during the protracted period of neurogenesis. They can self-renew and are capable of producing both neurons and glia. In the developing CNS, stem cells refer to neural cells that are self-renewing, but not necessarily for an unlimited number of cell divisions (Gotz et al., 2005). Neuroepithelial cells, for example, are considered stem cells and undergo both proliferative, symmetric divisions to produce two daughter stem cells, and asymmetric divisions to produce a self-renewing daughter cell and a differentiated neuron (Gotz et al., 2005).

Neural stem cells and their progeny produce all the diverse subtypes of neurons that populate the brain. The brain has been described as the most complex system in the universe, not only in its structural makeup but also in its processing capacity (Koch and Laurent 1999). The human brain has 100 billion neurons and as many as 10,000 different

neuronal subtypes (Purves 2001). At the molecular and cellular levels, differences between individual neurons are discernible with respect to a variety of criteria, notably gene expression profiles, morphology, number of synaptic connections, and location (Laura 1998). The complexity of the human brain can be attributed, at least in part, to this remarkable neuronal heterogeneity (Koch and Laurent 1999, Muotri and Gage 2006).

The precise mechanism of how this ordered neural diversity is generated is a critical question in CNS development that is incompletely understood. Gaining insights into the mechanisms controlling the generation of NSC diversity has the potential not only to inform our understanding of basic biology, but also to impact translational research aimed at treating a range of CNS pathologies where transplantation of specific, *in vitro* differentiated cell types may mitigate lost function (Westphal and Lamszus 2011, Ravin et al. 2008).

1.2 Mechanisms of neuronal diversification in vertebrates

Diversification during neurogenesis is by necessity a tightly regulated process. Although no two individuals and thus no two brains are exactly alike, the core composite of neurons must always be present. The right number and variety of neurons need to be generated and form appropriate connections in order to form a functional brain and greater CNS. Despite the complexity of the CNS structure, neurons and glia are produced in a precise and predictable temporal order.

Neuronal diversification occurs all throughout neurogenesis, and, to start, requires the ordered spatial and temporal specification of neural stem cells. Early on in development, neural progenitors or stem cells with different lineage potentials exist (Temple 2001). Each progenitor produces a series of intermediate precursors with different temporal identities (Pearson and Doe 2004, Okano et al., 2009). The sister neurons derived from an

intermediate precursor can further diversify through binary fate decisions to produce two post-mitotic neurons with distinct fates (Jukam and Desplan 2010).

Although neuronal diversification occurs, how it is reproducibly achieved is not fully understood. Nevertheless, the generation of cellular diversity during embryonic development can be attributed to the interplay of two leading drivers: extrinsic and intrinsic influences (Purves 2001). Supporters of the lineage model emphasize the importance of intrinsic factors, while supporters of a cell interactions model focus on external factors as critical determinants in neuronal cell fate decisions. The lineage model proposes that neuronal precursors are committed to diverse fates early on, and rely principally on information intrinsic to each cell (Purves 2001). Subsequent divisions result in the proliferation of these cells, which differentiate according to their set lineage. In contrast, the cell interactions model is based on the idea that cells are descended from a multipotential precursor (Purves 2001). Diversity is primarily generated among daughter cells by extrinsic signals produced from neighboring cells and the surrounding microenvironment.

To better understand how these intrinsic and extrinsic drivers interact to drive diversification, researchers developed a real-time imaging system to track potency and fate specification from multipotent CNS precursors, with the goal of identifying discrete steps involved in producing a complex population of cells from a single cultured cell (Ravin et al., 2008). Through this effort, they found that specification occurs early in lineage trees, and precedes the appearance of fate-specific markers by several days. Initially tripotent, capable precursors capable of producing neurons, astrocytes and oligodendrocytes, rapidly lose their potency. By the fourth generation, 80% of cells are unipotent (Ravin et al., 2008).

Specification of vertebrate neural stem cells is informed by its position in the neuroectoderm. The embryonic CNS is patterned along its dorsoventral (DV) and

anteroposterior (AP) axis (Altmann and Brivanlou 2001, Urbach and Technau 2008, Wolpert 1994). This patterning occurs early on, at roughly the same time as neural induction. DV and AP patterning are accomplished by the intersection of signaling systems that establish positional information in the embryo.

Studies of the vertebrate cerebral cortex in particular have yielded powerful insight into the ordered pattern of neuronal diversification (Temple 2001, Laura 1998). In the cortex there is a tight relationship between birthdate and laminar identity. A given neural progenitor often produces an invariant neuronal lineage, with neurons acquiring different and distinct fates based on their birth-order within the lineage. For example, neurons in the developing cerebral cortex that occupy the deeper layers are made before neurons in more superficial layers (Luskin and Shatz 1985, Angevine and Sidman 1961). Birth-order dependent temporal identity is specified prior to or during the terminal mitosis of the neuron, before their morphological differentiation. Newly-derived cortical neural progenitors reliably migrate to the layer typical of its birthday even when transplanted into an environment where host neurons are destined for a different layer.

Lineage analysis studies using isolated murine cortical stem cells demonstrated that the timing of cortical neurogenesis was found to be encoded within lineages of individual stem cells (Shen et al., 2006). Key transcription factors, including Fox1, Foxa2, and Sox5 have been shown to be implicated in the sequential generation of cortical neurons (Okano et al., 2009). When transplanted, stem cells isolated from specific regions continue to generate region-appropriate progeny, implying that inherited positional information remains and is encoded in the stem cell even though its physical location may change (Temple 2001). This point is further underlined by fate-mapping experiments in mice that show fluorescent dye-labeled progenitors in the medial and lateral ganglionic eminence, when transplanted into

non-labeled host brains, yield cortical neurons and striatum plus olfactory bulb neurons, respectively (Wichterle et al., 2001). Importantly, it is not the site of transplantation but the original position that predicts the fate of the transplanted tissue. In this way, progenitors in the medial and lateral ganglionic eminence are shown to be cell-autonomously different (Wichterle et al., 2001). In culture as well, cortical neural progenitors remain capable of yielding layer-specific neurons in an appropriate order. Using an *in vitro* culture system, cortical and retinal neural progenitors generate neuronal lineages indistinguishable from those produced *in vivo* (Cayouette et al., 2003, Shen et al., 2006). This suggests that the temporal identity is largely determined by cell-intrinsic cues, although the environment may fine-tune the outcome of temporal fate specification (Shen et al., 2006, Lin and Lee 2011).

While these studies illustrate that progenitor division is driven by intrinsic programs, extracellular signals from the surrounding environment work in concert to influence the temporal pattern of neurogenesis. The relative abundance of specific subpopulations of progenitors in different regions and at different stages of development depends on spatially and temporally regulated environmental signals. Some of these signals act instructively to alter the potential of progenitor cells. In fact, stem cells of the developing nervous system exhibit different neurogenic potential over time (Qian et al., 2000). Cortical stem cells produce neurons first and then glia, with fewer neurons generated as the age of the stem cell increased.

Stem cells from different regions have different growth factor requirements. Progenitor cells may be multipotential when assessed over the course of development, but they may only be competent to manifest parts of this potential at specific times. Simple cell-cell contacts or short-range signals can also influence the fates of neural progenitors. Differences among progenitor cells help determine how they will respond to pleiotropic

environmental signals (Laura 1998). Responsiveness of the neural stem cell to growth factor signaling may change over time and explain the heterogeneity in the response of progenitor cells to specific environmental signals. When early progenitors are labeled and cultured for several hours at low density before being transplanted to older host brains, they have been shown to adopt host-appropriate cell fates (Bohner et al., 1997). If instead these labeled progenitors are pelleted before transplant, they retain their capacity to generate early fates following transplant.

1.3 Paradigms of neurogenesis in Drosophila

For almost a century, *Drosophila* has been an attractive model system for scientific studies, particularly in the fields of development, behavior, and physiology. In the last several decades, the vast genetic toolkit and abundance of resources available in *Drosophila* has powered research that has yielded tremendous insight into the molecular mechanisms underlying neurogenesis, neural fate and specification (Venken et al., 2011, Bellen et al., 2010).

The lineage trees of vertebrate CNS stem cells are remarkably similar to those of invertebrates (Brand and Livesey 2011, Shen et al., 2006, Doe and Technau 1993). In fact, studies of invertebrates and vertebrates have elucidated remarkable evolutionary conservation of many aspects of CNS development. For this reason, understanding neurogenesis in invertebrates, especially in *Drosophila*, has provided tremendous insight into mechanisms of neurogenesis in other higher organisms (Bellen et al., 2010). Many aspects of nervous system development have been elucidated in fruit flies, and have subsequently influenced approaches to neuroscience research in vertebrates. Notch signaling, which impacts virtually every aspect of neurogenesis, was first identified and characterized in

Drosophila. The *achaete-scute* complex (AS-C) of proneural genes, essential for neurogenesis across phyla, was also characterized in *Drosophila*. Identification of homeotic (Hox) genes, a set of evolutionarily conserved genes encoding homeobox-containing proteins, was accomplished following study of the *bithorax and antennapedia* complexes in *Drosophila* (Bellen et al., 2010). Hox genes have since been implicated in patterning the hindbrain, and the genetic program of the neural crest.

In comparison to the human brain with its 100 billion neurons, the *Drosophila* brain is a much simpler structure with on the order of 100,000 neurons. While diversification remains a prominent feature of the *Drosophila* brain, its simplicity compared to its vertebrate counterpart makes it an attractive model system for the study of neuronal diversification.

The *Drosophila* CNS originates from two distinct regions: the ventral neuroectoderm (vNE) which gives rise to the ventral nerve cord (VNC), and the procephalic neuroectoderm (pNE) which gives rise to the brain (Technau et al., 2006). Neural stem cells or neuroblasts (NBs) are the building blocks of the CNS (Doe 2008, Reichert 2011, Doe and Skeath 1996). Single neuroectodermal cells enlarge to become NBs, which delaminate into the interior region of the embryo, leaving neighboring neuroectodermal cells to take an epidermal fate (Hartenstein et al., 1994). Following delamination, NBs divide asymmetrically, producing a self-renewing NB plus a smaller daughter cell termed ganglion mother cell (GMC). NBs undergo several rounds of asymmetric division to generate the neuronal lineages that make up the CNS. Individual NBs can be identified based on their positions in the embryo and by molecular markers (Doe and Technau 1993, Doe 1992). The neurons produced by a given NB reside together and extend neurites through common tracks. Daughter GMCs are distinct from NBs by their smaller size, altered pattern of gene expression, and reduced

mitotic potential. In contrast to NBs, each GMC only divides once to generate two post-mitotic neurons.

To date the VNC has been the most extensively studied aspect of the *Drosophila* CNS. The VNC has emerged as a central model system for studying the molecular genetic mechanisms that control CNS development (Skeath and Thor 2003, Doe and Skeath 1996). Compared to the brain, the VNC has the advantage of being simpler and more easily accessible.

Studies of VNC development show that the generation of neural diversity is a multistep, but stereotyped process initiated by the patterning and segmentation of the neuroectoderm. Five waves of segregation from the vNE yield an invariant pattern of 30 NBs per hemisegment, the developmental unit of the VNC that refers to a bilateral half of a segment (Skeath and Thor 2003). The study of the VNC has been aided by this strict metamerism, with the same pattern of NBs produced by each segment.

As with vertebrate neural stem cells, specification of NB identity within the vNE depends on the DV and AP axial patterning systems to generate a highly regionalized vNE that has been likened to a Cartesian coordinate map (Skeath and Thor 2003, Urbach et al., 2006, Skeath 1999). The DV/AP grid subdivides the vNE into a series of neural equivalence groups in which single cells delaminate as NBs. Each NB has a unique identity identifiable by the position from which it delaminates from the neuroectoderm, its time of formation, the combination of genes it expresses, and the characteristic clone of neurons or glia produced (Skeath 1999).

At the onset of vNE neurogenesis, neighboring neuroepithelial cells that harbor a common regional identity, or map address, begin to express the proneural genes of the AS-C (Skeath et al., 1992, Martin-Bermudo et al., 1991, Skeath and Doe 1996, Skeath and Carroll

1994, Brunet and Ghysen 1999) and thus comprise equivalence groups of 5-7 cells, all of which are competent to form NBs. Once cells are competent, a lateral signal mediated by the Notch receptor and its ligand, Delta, acts through the *Enhancer of split* [*E*(*spl*)] family of proneural gene repressors to allow delamination of a single NB while specifying the remaining competent cells as epidermis (Fig. 1-1A) (Skeath et al., 1992, Lehmann et al., 1983, Technau and Campos-Ortega 1987).

The identified NBs acquire their distinct lineage properties from the factors they inherit from the regionalized vNE (Technau et al., 2006, Doe and Skeath 1996, Bhat 1999). The sequential expression of the so-called temporal identity genes, the transcription factors Hunchback, Kruppel, Pdm1, and Castor, specify the production of different neurons at different times (Fig. 1-2) (Pearson and Doe 2004, Grosskortenhaus et al., 2005). In summary, in the VNC NB fate is fixed by a combination of early embryonic patterning events and cell-cell signaling prior to delamination from the neuroectoderm.

Due to its relative complexity, the brain has not been as extensively studied as the VNC (Urbach and Technau 2004). Unlike the VNC, no overt metamerism exists in the pNE thus it has been more complicated to identify the precise pattern of brain NB identities. It is known, however, that the Notch-mediated lateral inhibition mode of NB specification in the vNE also extends to the approximately 100 identified procephalic NBs derived from the pNE, which form most of the brain (Fig. 1-1A) (Urbach and Technau 2008, Urbach et al., 2006, Urbach et al., 2003).

Recently, two distinct classes of central brain lineages, termed Type I and II, can be unambiguously identified based on the progenitor progeny generated and the combination of cell fate markers expressed. Type I lineages account for the vast majority of brain NBs - 92 of the 100 procephalic NBs per brain lobe are Type I NBs (Boone and Doe 2008). The

more prevalent Type I NB undergoes canonical asymmetric cell divisions to generate a self-renewing NB and daughter GMC that will go on to terminally divide to produce two neurons or glia. Ultimately Type I NB lineages generally contain 10-12 cells. In contrast, a rare population of recently identified Type II NBs are found to produce transit-amplifying precursors that behave more like vertebrate intermediate precursors, generating a series of neurons through limited rounds of self-renewal cell divisions during the larval stage (Fig. 1-3) (Boone and Doe 2008, Bello et al., 2008, Izergina et al., 2009, Bowman et al., 2008). There are only 8 Type II NBs per brain lobe, and they produce large lineages of up to 400 cells via an intermediate neural progenitor (INP) that retains stem-cell like properties (Boone and Doe 2008, Weng and Lee 2011). Type II NBs are not present in the VNC (Weng and Lee 2011).

Type I and II NBs can be distinguished molecularly by their differential expression of key genes (Fig. 1-3) (Doe 1992, Weng and Lee 2011). Type I NBs undergo asymmetric division during which the basal protein Prospero (Pros) exclusively segregates to the GMC by binding the scaffolding protein Miranda (Mir). Pros encodes a homeodomain transcription factor and plays a key role in specifying neuronal and glial cell types in the developing nervous system (Doe et al., 1991, Chu-Lagraff et al., 1991). Pros is expressed in NBs but is excluded from the nucleus via binding to Mir. During division of the NB, Mir is degraded, Pros is thus released from the cortex and localizes to GMC nuclei. Nuclear Pros restricts GMC potential by restricting genes that promote the NB identity, and activating genes that promote differentiation and cell cycle exit. In contrast to Type I NBs, upon division Type II NBs produce an INP that retains stem cell-like properties, and does not segregate Pros to the nucleus. Another molecular distinction between the NB types is the

presence of Asense (Ase). Type I NBs express Ase along with the NB markers Deadpan (Dpn) and Mir; in contrast Type II NBs express NB markers, but not Ase.

E(spl)-C activity largely corresponds to the pattern of proneural genes in the vNE. NBs delaminate from the pNE in a stereotyped spatiotemportal pattern which is tightly regulated to the expression of the proneural gene *Lethal of Scute* (L'sc) (Martin-Bermudo et al., 1991). In the pNE, E(spl)-C expression starts during stage 8 but expands to the L'sc-expressing regions. E(spl)-C persists longer than L'sc expression, and remains at a high level even after NBs have delaminated. Delamination of procephalic NBs ceases by late stage 11, and after this stage, L'sc disappears from the head ectoderm except for the dorsomedial protocerebrum (Pdm) and the optic lobe primordium. Expression of L'sc remains high in the Pdm until stage 14. The extended expression of both E(spl)-C and AS-C, and the shifted period of neurogenesis sets the Pdm apart from the rest of the pNE.

The majority of neurogenesis in the pNE is complete by stage 11, with the exception of the Pdm. The Pdm produces several critical cell types: Type I and the rare Type II NBs as well as the neurosecretory cells (NSCs) of the *pars intercerbralis* (PI) and *pars lateralis* (PL), the core endocrine control center of *Drosophila* (de Velasco et al., 2007, Wang et al., 2007). The Pdm undergoes a different mode of neurogenesis from the vNE and majority of the pNE. In contrast to the Cartesian map paradigm for NB identity specification within a sheet of NE, additional brain NBs, including those that generate the IPCs, derive from placodes in the head midline Pdm NE, where the neuroepithelium loses its sheet-like morphology preceding neurogenesis (Fig. 1-1B). The placodal Pdm NE forms invaginated vesicles of neurogenic cells characterized by a condensation of apical membranes and expression of proneural factors of the AS-C, which then undergo neurogenesis at stages 11 through 14, immediately following neurogenesis in the remaining pNE, which occurs in stages 8 through

11. Here, the timing of NB formation results in the deposition of Pdm NBs between the outer embryonic epithelium and the pNE-derived protocerebrum (Younossi-Hartenstein et al., 1996, de Velasco et al., 2007). Unlike most of the NE, the invaginated Pdm neuroepithelium is neurogenic, such that adjacent competent cells all become NBs and not epidermis (Younossi-Hartenstein et al., 1996, de Velasco et al., 2007), a pattern that more closely parallels vertebrate CNS neurogenesis. Cells of the Pdm are internalized by a process which can best be described as mass delamination and invagination to form placodes. Compact clusters of neurogenic cell groups expressing AS-C move their nuclei basally and separate from the surface (Younossi-Hartenstein et al., 1996), but cells of a common placode retain a connection at an apical constriction point. Following their segregation, most of the dorsomedial cells become integrated as neurons and glial cells into the brain hemispheres. Some regions of the pNE are neurogenic with adjacent cells in a proneural domain taking the NB fate, but in contrast to the Pdm, they do not form invagination centers (Urbach et al., 2003).

The Pdm contains diverse NB lineage identities including Type I NBs, those that divide in an asymmetric stem cell mode to generate GMCs, which then divide symmetrically to generate neurons (Boone and Doe 2008). Pdm Type I NB identities include NBs for brain NSC lineages such as insulin-producing cells (IPCs) (Wang et al., 2007) and NBs for cholinergic neuron lineages (de Velasco et al., 2007, Wang et al., 2007). The Pdm also contains Type II NBs, which produce INPs. These NB lineages can exceed 400 cells and comprise both neurons and glia (Boone and Doe 2008, Bello et al., 2008, Izergina et al., 2009, Bowman et al., 2008, Pereanu and Hartenstein 2006, Sprecher et al., 2007).

While several genes (*tailless*, *giant*, *lethal of scute*) are critical for specification of the PI (Younossi-Hartenstein et al., 1996, de Velasco et al., 2007, Younossi-Hartenstein et al., 1997)

and epidermal growth factor receptor (Egfr) activity is essential for Pdm cell survival (de Velasco et al., 2007), it is not known how these diverse NB identities are specified within neurogenic Pdm placodes. The IPC NB and its corresponding placode provide an excellent model to interrogate the specification of the brain neuroendocrine system from a placodal neuroepithelium.

1.4 Development of insulin-producing cells in Drosophila *from a placodal neuroepithelium*

Mechanisms that allocate the fates of diverse neural stem cell lineages during development are responsible for achieving the proper function of neural circuits and are critical in patterning the neuroendocrine system that mediates homeostatic physiology. The dorsomedial placodal neuroectoderm gives rise to the neuroendocrine PI and PL in *Drosophila*, yet the mechanisms of cell fate specification in this uniquely specialized neuroepithelium are relatively unknown. We use a key element of the neuroendocrine center, the IPC lineage and its identified NB as a model to understand the novel pattern formation of the neuroendocrine system.

In both *Drosophila* and mammals the brain exerts physiological control of organ systems not only through chemical synapses of efferent and afferent pathways, but also via hormonal control through closely associated peptidergic neuroendocrine and endocrine cell compartments. Both the endocrine and NSCs of the brain-ring gland complex in flies and the hypothalamic-pituitary axis of mammals serve as key integrators of sensory stimuli and behavior with energy metabolism, reproductive functions, water balance and organismal growth. This sustained integration depends on the precise specification and maintenance of relatively small numbers of each NSC and glandular endocrine cell types; developmental

defects or disease states that alter their numbers, either up or down, can lead to catastrophic misregulation of various aspects of physiology.

Pattern formation and cell fate specification of the brain IPC progenitor occurs in the context of the developing *Drosophila* neuroendocrine system (Wang et al., 2007). The PI and PL NSCs that form the brain-ring gland complex are sparsely distributed in the larval brain as no less than seven molecularly identified cell groups that are distinguished by position, the number of cells per cluster and by the targets of their axonal processes (Siegmund and Korge 2001); the 6-8 IPCs that express *Drosophila* insulin-like peptides (Dilps) 1, 2, 3 and 5 (Rulifson et al., 2002) reside in the PI. The IPCs project axons to the *Corpora Cardiaca* (CC) and aorta (Siegmund and Korge 2001). In addition to their targets within the medial compartments of the brain, the NSC groups also target specific ring gland compartments including the neuroendocrine CC, endocrine prothoracic and *Corpora allatum* glands. Some NSC clusters also extend processes to the surface of the aorta, where secreted neuropeptide hormones are released directly into the hemolymph (Rulifson et al., 2002, Kim and Rulifson 2004).

NSCs, neurons and glia of the PI and PL are produced from both primary neural stem cells or NBs whose lineages expand during embryogenesis and secondary NBs whose lineages proliferate following embryogenesis, predominantly during the late larval stage (de Velasco et al., 2007). The 6-8 IPCs per hemisphere are the only NSCs or neurons of the PI or PL where lineage-tracing analysis has identified a progenitor; this cell is a unique Dachshund-positive (Dac+), Castor-positive (Cas+) and Chx1-positive (Chx1+) primary NB, the IPC NB (Wang et al., 2007). The IPC NB is a canonical asymmetrically dividing Type I NB, which produces 5-6 GMCs that each divide symmetrically to generate a lineage of 10-12 NSCs by the end of embryogenesis (Fig. 1-4) (Wang et al., 2007); the neuropeptide identities

of the 2-4 Dilp-negative NSCs of the lineage are not known. The remaining NSCs of the PI and PL, which do not have progenitors identified by lineage tracing, must indeed also be produced by primary NB lineages that proliferate in the embryo because the population of differentiated NSCs are readily identified in the early first instar larval brain (Park et al., 2008).

The IPC NB is one of roughly eight Chx1+ Cas+ NBs to delaminate from a medial PI cluster NE cells that are specifically Cas+. After delamination, the IPC NB and all of its progeny express Dac, whereas the other contiguous Chx1+ Cas+ NBs never do (Wang et al., 2007).

As principal insulin-producing cells, *Drosophila* insulin-producing neurons are functionally analogous to vertebrate insulin-producing pancreatic beta cells (Rulifson et al., 2002, Brogiolo et al., 2001). Genetic ablation of the brain IPCs in the fly resulted in defects that include high levels of circulating carbohydrates typical of diabetes (Rulifson et al., 2002). The IPC lineage consists of two bilaterally symmetric clusters of 6-8 neuroendocrine cells that have been traced back to a single pair of NB progenitors (Wang et al., 2007). Given that a single stem cell on each future brain lobe produces all the principal IPCs for the organism. Mechanisms must be in place to produce this singularity of cell fate. Investigating this question will surely bring insight into the molecular mechanism of neuronal diversification in the brain.

Figure 1-1. Neurogenesis in *Drosophila*. (A) In the vNE and majority of the pNE, lateral inhibition via Notch signaling restricts the NB identity to a single NB in a proneural competence group. (B) In the placodal Pdm NE, a subset of the greater pNE, proneural groups form placodes that are fully neurogenic.

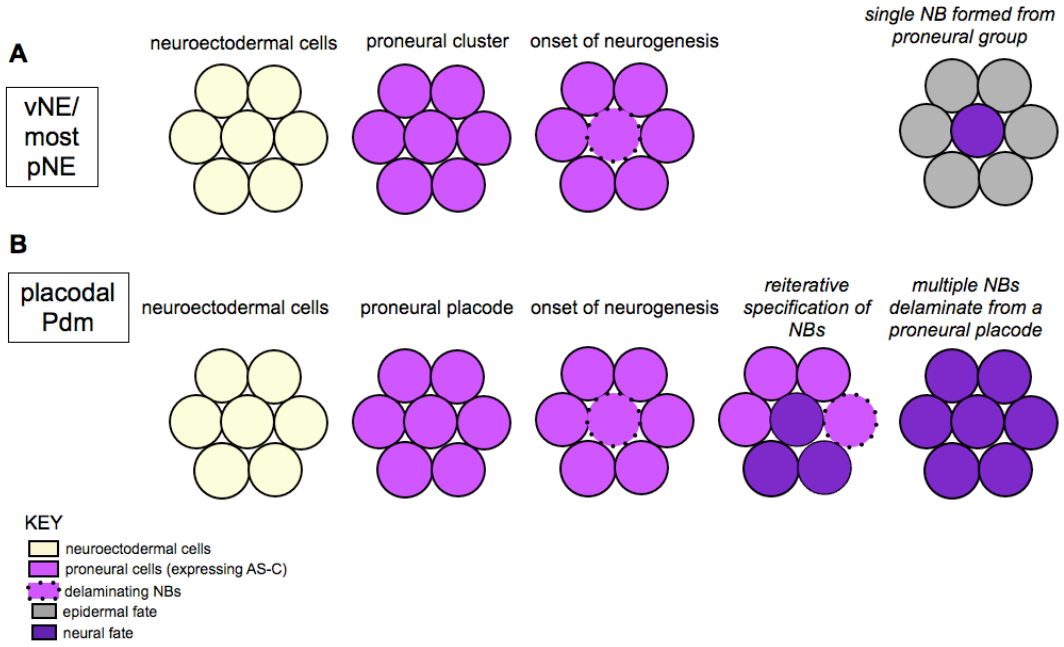


Figure 1-2. Temporal identity genes serially specify VNC NBs. NBs in the VNC sequentially express four genes *(Hunchback -> Kruppel -> pdm1 -> castor)* whose temporal regulation is essential for generating neuronal diversity.

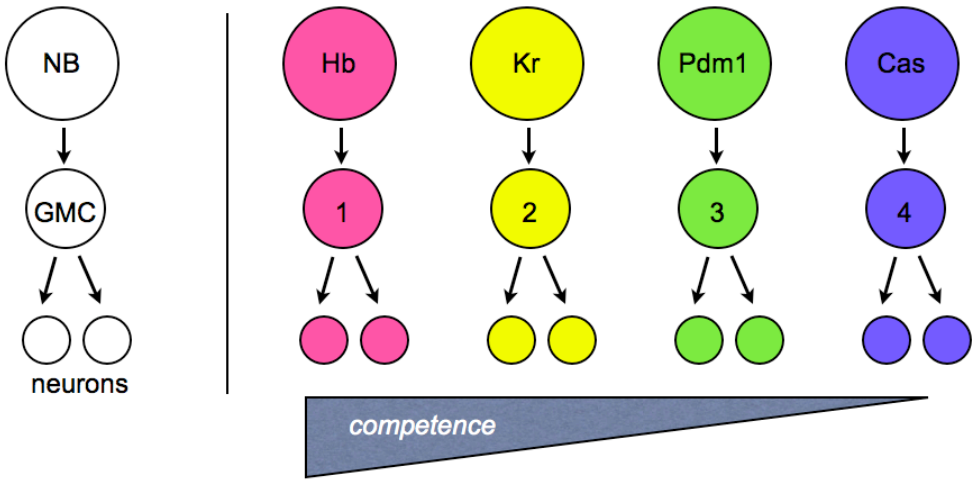


Figure 1-3. Type I and Type II NB lineage programs. (A) Type I NBs undergo canonical asymmetric division to produce a self-renewing NB and a daughter cell GMC that terminally divides to produce neurons or glia. (B) Type II NBs undergo asymmetric division to produce a self-renewing NB and an INP that retains stem cell like properties. INPs undergo multiple rounds of division, producing GMCs that terminally divide to produce neurons.

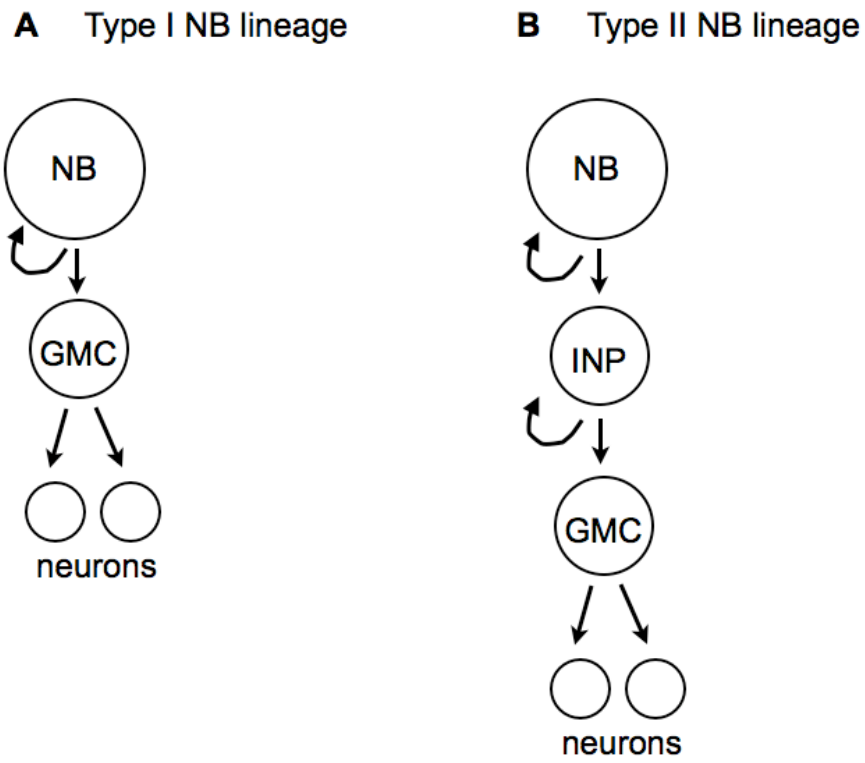
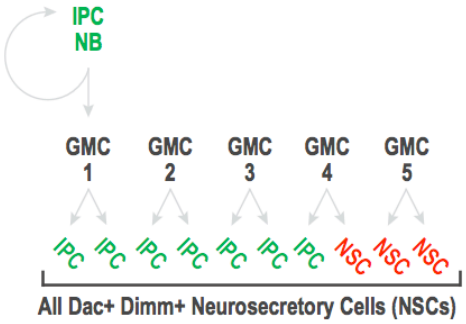


Figure 1-4. IPC NB lineage program. The IPC NB is a canonical Type I NB that undergoes asymmetric division to produce GMCs that terminally divide to form neurons. The products of the first 4-6 GMC divisions give rise to the brain IPCs. The remaining GMCs produce as yet identified NSCs.



Chapter Two: *Materials and Methods*

2.1 Fly stocks

Fly stocks were maintained at 18-25ºC on standard media. Stocks are from Bloomington Stock Center (Bloomington, Indiana) unless otherwise indicated. *Drosophila* strains included, *yw* (used as the normal control genotype), N^{ts1}, N^{55e11}, Dl^{6B}, Dl^{RF}, $Egfr^{f24}$, $Egfr^{ts1a}$, spi^{1}, $Chx1^{\Delta 23}$ (gift of T. Erclik and H. Lipshitz), hh^{21}, dpp^{h46}, cas^{24}, chn^{ECJ1} (gift of J. Modollel and S. Campuzano; (Culi et al., 2001)), $phyl^{2245}$, ttk^{1e11}, *E(spl)m8-GFP* (gift of J. Posakony; (Castro et al., 2005)), *dimm (c929)-GAL4, UAS-mCD8-GFP* (gift of R. Hewes; (Hewes et al., 2003)), sc^{B25}, *rho-lacZ* (rho^{6}) (Bier et al., 1990), rho^{7m43}, $rho^{7m}vn^{Ry}$ (Spencer et al., 1998), aop^{1}, pnt^{d88}, UAS-ΔEN (Larkin et al., 1996), UAS-rho, UAS-aop, tll- and gt-GAL4 transgenes (gift of S. Celniker); *mzVUM-GAL4, UAS-mGFP* (de Velasco et al., 2007) and *w; Act5C<stop>lacZ; UAS-flp* (Struhl et al., 1993).

2.2 Embryo fixation

Egg collections were performed on yeasted apple juice agar plates. Embryos were dechorionated in 50% bleach and transferred to 1.7 ml screw-cap tubes containing equal volumes of a 4% formaldehyde: heptane solution. Embryos were fixed by vortex-mixing at 1000 rpm with an Eppendorf MixMate for 20 minutes. Fixative was then removed and replaced by methanol, and embryos were devitellinized by vortexing. After rehydration, embryos were blocked for at least 2 hours in PBT (1xPBS, 2% BSA, 0.1% Triton X-100) with 5% normal goat serum (NGS).

2.3 Primary Antibodies

Embryos were incubated in primary antibody overnight at 4ºC. Antibodies used included mouse anti-Fas2 diluted 1:10 (mAB1D4; Developmental Studies Hybridoma Bank [DSHB]); guinea pig anti-Chx1 diluted 1:500 (gift of H. Lipshitz, University of Toronto); rabbit anti-Optix diluted 1:500 (gift of F. Pignoni, Harvard University, Boston); mouse-anti Eya diluted 1:250 (mAB10H6; DSHB); rat anti-Dsix4 diluted 1:25 (see below); chick anti-GFP diluted 1:250 (Abcam); mouse anti-Crb (mABCq4; DSHB); rat anti-Dpn diluted 1:1 (gift of C. Doe, University of Oregon, Eugene); mouse anti-Dac diluted 1:100 (mABdac2-3; DSHB); rabbit anti-Cas diluted 1:5000 (gift of W. Odenwald, National Institute of Health, Bethesda); guinea pig anti-Dimm diluted 1:200 (gift of P. Taghert, Washington University, St. Louis); rabbit anti-CC3 diluted 1:50 (Cell Signaling Technology); mouse anti-pMAPK diluted 1:10 (Sigma); guinea pig anti-Ase diluted 1:100 (gift of Y.N. Jan); rabbit anti-Mir diluted 1:1000 (gift of Y.N. Jan).

2.4 Secondary Antibodies

Secondary antibodies used (Jackson ImmunoResearch) were conjugated to Dylight 488, 546, 594 and 750 (Pierce) and Alexa Cy5 fluorescent conjugates (Invitrogen) diluted 1:1000.

2.5 Larval brain fixation

Dissect brains from larvae in PBS. Remove PBS and add 500 ml of 4% formaldehyde (made with PT, PBS + 0.3% Triton X-100) to each well to fix brains. Incubate 20 minutes up to 2 hours at 4°C. Block in PBT+ 5% NGS. Wash 3x in PBT, 10 minutes each time. Add 50-100 µl primary antibody per well. Wash 3x in PBT, 10 minutes each time. Add secondary antibody (1:300). Wash 3x in PBS before mounting.

2.6 BrdU protocol

For pulse labeling with 5-bromo-deoxyuridine (BrdU), embryos were permeabilized with octane then incubated for 2 hours in 1xPBS containing 1 mg/ml BrdU (Sullivan et al., 2000). Embryos were then fixed and blocked as described above. Prior to immunostaining, embryos were treated with DNase I (Roche) for 90 minutes in a 37ºC water bath.

2.7 Temperature-shift protocol

For temperature-shift experiments, *Egfr*^{ts} (*Egfr*^{ts1a}/*egfr*^{f24}); *Delta*^{ts} (Dl^{fB}/Dl^{RF}); and *Notch*^{ts} (N^{ts1}/N^{55e11}) embryos were reared at the permissive temperature (18ºC) and then subject to a 3 or 6-hour shift at the restrictive temperature (29ºC). Embryos were either fixed immediately following the temperature shift, or were further incubated for 6 hours at the permissive temperature. For analysis of Optix and Dimm expression, *Delta*^{ts} embryos were reared entirely at the restrictive temperature and examined at stage 17. Brains were dissected from first instar *Delta*^{ts} mutants subjected to a 4-hour temperature shift as embryos, and reared at the permissive temperature to stage L1.

2.8 Microscopy

Multiplex images were obtained using a Zeiss Axioimager Z1 equipped with Exfo-X-CITE illumination, a Photometrics HQ2 CCD camera and Semrock FISH dichroic filter sets; images were acquired in Axiovision 4.8 and figures were produced with Adobe CS4.

Chapter Three: *Placodal Neurogenesis*

Introduction

Vertebrates are distinguished from other deuterostomes by their specialized head. The vertebrate head consists of a brain encased in cartilaginous or bony skull and complex paired sense organs. Most of the evolutionary innovations of the vertebrate head originate from two embryonic tissues: the neural crest and the cranial placodes (Schlosser 2005). Although both have been studied for over a century, far more has been learned about the development of the neural crest. In comparison, the development of cranial placodes is much less understood.

The vertebrate cranial placodes are specialized areas of non-neural ectoderm, which is ectoderm located outside of neural plate and neural crest. Within placodes, cells undergo pronounced changes in cell shape via thickening, invagination or delamination to give rise to a variety of migratory non-epidermal cell types. Unlike the neural crest, they develop exclusively from the cranial ectoderm and form a hetereogeneous group of structures. These placode-derived structures contribute to different organs, including the eye, ear and olfactory epithelium, as well as the pituitary gland. First, we present an overview of vertebrate cranial placodes and their derivates. Second, we characterize the *Drosophila* anterior placode system of the Pdm as a model to study placodal neurogenesis.

3.1 The vertebrate cranial placodes and their derivatives

The cranial placodes comprise the adenohypophoseal, olfactory, lens, profundal, trigeminal, otic, lateral line, and epibranchial placodes. Each produces discrete cell types, notably a diverse array of sensory and secretory cells (Schlosser 2010, Schlosser 2006).

The adenohyphoseal placode gives rise to the adenohypohysis. Six types of endocrine secretory cells are produced in the adenohypophysis: gonadotropes (LH and FSH), thyrotropes (TSH), corticotropes (ACTH), melanotropes (MSH), lactotropes (prolactin-PRL) and somatotropes.

The olfactory placode produces the olfactory and vomeronasal epithelia with secretory cells (supporting and mucus-producing cells) and sensory cells (odorant and pheromone receptors). The olfactory placode is unique among vertebrate placodes to produce glia. NSC cells containing GnRH and other neuropeptides are also produced by the olfactory placode. They subsequently migrate into various locations in the brain where they control the release of gonadotropins (LH, FSH) from the adenohypophosis.

The lens placode gives rise to the crystalline-containing cells of the lens. The profundal and trigeminal placodes together with neural crest cells contribute sensory neurons to the ganglia of the profundal and trigeminal nerves, which relay somatosensory information (pain, temperature, and touch) from the oral cavity.

The otic placode gives rise to the entire inner ear and to the sensory neurons of the vestibulocochlear ganglion. The inner ear comprises a wide array of specialized epithelial cells, including the endolymph-producing secretory cells, supporting cells, and the axonless secondary sensory cells (hair cells). In fish and amphibians, the lateral line placodes develop both rostrally and caudally to the otic placode. They generate the receptor organs of the lateral line system as well as the sensory neurons of the lateral ganglia innervating them. Receptor organs of the lateral line are either mechanoreceptive (neuromasts) or electroreceptive (ampullary orgrans or tuberous organs) and contain secondary sensory cells lacking axons and secretory supporting cells. The epibranchial placodes develop dorsocaudal

to the pharyngeal pouches, and give rise to viscerosensory neurons that supply taste buds and other visceral sensory receptors.

Although individual placodes give rise to different cell types and structures, there are remarkable similarities between placodes. Despite producing disparate cell types, the cranial placodes share many similarities in early development (Schlosser 2005, Streit 2007). For example, all placodes form columnar epithelia next to the neural tube, contain cells that undergo epithelial-mesenchymal transition, contribute to the cranial sensory nervous system, and are neurogeneic with the exceptions of the lens and adenohyphoseal placode. Compared to the surrounding epidermal ectoderm, placodes exhibit high levels of cellular proliferation.

3.2 The placode genes

Although still under debate, classic and recent evidence support the presence of a pre-placodal region, a unique region of the head ectoderm that contains the precursors for all cranial placodes. For a true pre-placodal region to exist, it must be biased towards the development of generic placodal properties. Indeed a common ectodermal region exists that is contiguous and defined by expression of transcription factors of the Six and Eyes absent (Eya) families that are purported to promote general placodal properties, and the differentiation of ganglia and sense organs (Schlosser et al., 2008, Streit 2007). Specifically, Eya genes and members of the Six1/2 and Six4/5 subfamilies have been referred to as the placode genes. Although many transcription factors are expressed in placodes, only members of the Six1/2 and 4/5 subfamilies and their cognate cofactor Eya match precisely the location of placodal precursors (Schlosser et al., 2008). These factors are subsequently maintained in the placodal region, but lost from the ectoderm. Six proteins are

transcriptional regulators that interact with various cofactors, including Eya to modulate their activity. Placode gene mutants in mice, humans, and zebrafish share similar developmental defects affecting several placodal derivatives (Schlosser et al. 2008).

Fate maps of early embryos of zebrafish, amphibians and chicks support the existence of a continguous pre-placodal region (Schlosser 2010). These maps show that all placodes originate from a crescent-shaped pre-placodal region in the outer neural folds and immediately adjacent ectoderm. The presence of a common ectodermal thickening has been proposed as evidence of placodal bias, however there is no clear correlation between the distribution of placodes or pre-placodal ectoderm and ectodermal thickenings (Schlosser 2010). By late neurula stages, the pre-placodal region is molecularly and cellularly distinct from other ectodermal derivatives.

Six1/2, Six 4/5 and Eya genes are expressed in the crescent-shaped ectodermal domains corresponding to the pre-placodal region. Not only are they expressed in the pre-placodal domain, they continue to be transcribed in all placodes and appear to promote similar developmental processes in them, including cell proliferation, morphogenetic movements, and neuronal differentiation (Schlosser 2006).

Results

3.3 The anterior placode system in Drosophila

We investigated the anterior placode region in *Drosophila*, which has conserved expression of the Six and Eya anterior cranial placode genes (Fig. 3-1). The embryonic primordium of the PI and PL is a bilateral dorsomedial "placodal" neuroectoderm (NE), which lies immediately posterior to the clypeolabral furrow at the tip of the embryonic head (de Velasco et al., 2007), previously designated as part of the Pdm NE (Younossi-

Hartenstein et al., 1996). In stage 11, at the conclusion of primary NB delamination in the remaining pNE and vNE, the Pdm NE forms what are described as invaginated vesicles or placodes of neurogenic cells characterized by condensation of the apical membranes, which are visible in the plane of the outer epithelium as dense accumulations of the junctional protein, Crumbs (Crb); the apical constriction gives the cells a bottle-like shape and moves the cell nuclei basally under the outer epithelium (de Velasco et al., 2007, Younossi-Hartenstein et al., 1996). (diagrammed in Fig. 1B). At stage 11 of embryogenesis (Campos-Ortega and Hartenstein, 1985), placode formation is accompanied by onset of pan-placodal expression of the proneural factor L'sc, which is essential for neurogenesis (Younossi-Hartenstein et al., 1996). Additionally, several Notch-signaling target genes of the E(spl)-C family, including *m5* and *m8* are activated at this time (de Velasco et al., 2007, Tomancak et al., 2002). Notch activity persists for several hours more, which roughly spans stages 11 through 14, while NBs continue to delaminate in an orderly succession. This process proceeds until the placodal NE cells at the epithelial surface are depleted by NB formation as cells release their constricted apical adherens junction and delaminate basally into the interior (Younossi-Hartenstein et al., 1996, de Velasco et al., 2007).

The Pdm NE comprises three molecularly identified subdivisions: the *pars intercerebralis* primordium (pPI), which is demarcated by expression of the transcription factor Chx1; the *pars lateralis* primordium (pPL), which is demarcated by expression of the cell adhesion molecule Fas2; and, the pars medialis primordium (pPM) domain, which is defined by expression of the transcription factor Rx (de Velasco et al., 2007) (diagrammed in Fig. 3-2A). The PI and PL contain the NSCs that form the brain-ring gland complex (de Velasco et al., 2007, Siegmund and Korge 2001, Park et al., 2008).

At stage 11, the greater Pdm placode system comprising the pPI, pPL and pPM was demarcated by the dorsoanterior head expression of the transcription factors Dsix4 and Eya. Oddly, Eya expression is evident in all pPI cells at stage 10, but then drops in the pPIm by stage 11 (Fig. 3-2B). The Chx1-expressing (+) pPI and Fas2+ pPL were demarcated by Optix expression (Fig. 3-2C, stage 11 lateral view; Fig. 3-2D, stage 14 dorsal view) where it overlapped with Dsix4 expression (Fig. 3-2B).

To visualize the relationship of structural aspects of placode organization to gene expression subdomains we used the combination of an *E(spl)m8-GFP* reporter transgene (m8-GFP) (Castro et al., 2005) to visualize the pattern of Notch activity in placodal cell groups; the presence of an apical Crb accumulation as a marker of the placode invagination (Younossi-Hartenstein et al., 1996) and expression of Optix, Dsix4 and Eya, which were previously identified as markers of the Pdm placode domains (Wang et al., 2007). At the same stage, cellular localization of GFP from the m8-GFP transgene highlighted the boundaries of discrete epithelial vesicles just beneath the outer epithelium to reveal the structure of placodal cell groups. When the pattern of GFP labeled placodal groups was superimposed with Eya, Optix, and Chx1 expression to identify the pPI, pPL and pPM, each of the three primordia was generally seen to comprise at least two of these placodal groups

(Fig. 3-2E). Most of the vesicular GFP+ cell groups appeared to be associated with an apical epithelial constriction suggesting that these cell groups were indeed individual units, or perhaps simply refered to as a "placode". These results show that the domain of overlapping expression of the evolutionarily conserved anterior "placode genes" (Schlosser 2010, Schlosser 2006, Schlosser et al., 2008a), Dsix4, Optix and Eya demarcated a system of individual placodes (Fig. 3-2E).

During stage 11, placode formation is accompanied by the onset of pan-placodal expression of the proneural factor L'sc and activation of several Notch-signaling target genes of the E(spl)-C family. During this period the EGF signal, Spitz (Spi), Egfr activity and Ras activation maintain cell survival of placodal cells and ectopic Egfr/Ras pathway activation is sufficient to disrupt cell fate and proliferation within the placodal NE (Dumstrei et al., 1998). Notch activity persists for several hours more, spanning stages 12 through 14, during which time NBs continue to delaminate in an orderly succession until the neurogenic placodes at the epithelial surface are depleted, as all neurogenic cells have released their constricted apical adherens junction and delaminated basally into the interior (de Velasco et al., 2007, Younossi-Hartenstein et al., 1996).

While the fate map of the PI and PL from embryonic stages is well established and there are clearly a diverse array of NSC identities and neurons produced from these placodal primordia, it is not known how the diversity of NB cell fate assignments are made within the placodal NE, especially with regard to NSC fate and neuropeptide-specific identity. We use the IPC lineage and its identified NB as a model to understand the organization of patterning within this placodal NE.

3.4 The medial pars intercerebralis *primordium (pPIm) placode produces the IPC NB*

Regional molecular specification of the CNS and anterior ectodermal placodes is evolutionarily conserved between flies and mammals (de Velasco et al., 2007, Wang et al., 2007, Lichtneckert and Reichert 2008). The vertebrate head neuroendocrine system largely arises from the anterior cranial placodes (Kawamura et al., 2002, Markakis 2002, Whitlock 2005), but details of specification mechanisms operating at a single cell resolution are not known. The brain IPCs, which express several Dilps, provide an excellent model to interrogate the specification of the brain neuroendocrine system from a placodal neuroepithelium.

The IPC NB comes from a molecularly defined placode with the anterior placode system, the medial *pars intercerebralis* (pPIm) placode (Fig. 3-3A). The pPIm is molecularly identified as a cluster of NE cells that are specifically Cas+ (Cui and Doe 1992) and Chx1+, which form a morphologically distinct and coherent structure baring a single apical constriction (Fig. 3-3B).

The IPCs are the only NSCs or neurons of the PI or PL where lineage-tracing analysis has identified a progenitor (IPC NB); this cell is a unique Dac+, Cas+ and Chx1+ cell in its region (Wang et al., 2007). The IPC NB is a canonical Type I NB (Boone and Doe 2008), which divides asymmetrically to produce 5-6 GMCs that divide again symmetrically to generate a lineage of 10-12 NSCs by the end of embryogenesis; 6-8 of those NSCs are IPCs and the remainder unidentified by neuropeptide (Wang et al., 2007) (lineage diagram in Fig. 1-4).

It was recently proposed that the entire pPI comprises a single placodal structure associated with a single apical epithelial constriction (de Velasco et al., 2007). In light of our observation that the pPI contains subdomains of cells that display different gene expression

patterns, such as the Chx1+ Cas+ pPIm (Wang et al., 2007), we further investigated the relationship between the pPIm subdomain and the organization of the placodal system.

3.5 Lineage analysis of the pPIm placode

We investigated the birth order of the IPC NB from the pPIm by quadruple labeling embryos for expression of Dac, Cas, Chx1 and the NB marker, Dpn (Bier et al., 1992) to follow the position of the IPC NB with respect to the pattern of delaminating pPIm NBs. Before delamination of NBs from the pPIm begins (stage 11), Dpn is transiently elevated in the pPIm NE cells, which are still tethered to the outer epithelium at their apical constriction (Fig. 3-4A; asterisk marks position of apical constriction). Once NBs begin to delaminate, placodal Dpn expression is lost and Dpn is elevated in newly forming NBs, which reside basal to the placodal NE cells. While the IPC NB formed at the onset of stage 12, it became Dac+ approximately two hours later, at late stage 12 (Fig. 3-4B and 3-4C, white arrow). The IPC NB was stereotypically positioned at the posterior tip of a row of two or three pPIm NBs that had formed (Fig. 3-4B and 3-4C, carets), suggesting it was the first to be specified as a NB.

To confirm that the diverse NB identities in the Pdm are the products of a single placode we examined the non-IPC NBs within the pPIm for expression of Type I and Type II NB expression profiles. Type I NBs express Dpn, Mira and Ase, while Type II NBs express Dpn and Mira, but not Ase (Boone and Doe 2008, Bowman et al., 2008). The *mzVUM-GAL4* and *UAS-mCD8 GFP* (*mzVUM-GFP*) transgene combination labels the pPI (de Velasco et al., 2007) and we identified the pPIm as the Cas+ posterior region of the Chx1+ *mzVUM-GFP* domain (Fig. 3-5A). Using *mzVUM-GFP* to mark the pPIm, we found that the NBs formed by the pPIm immediately following the IPC NB, at stage 14, are also

Dpn+ Mira+ Ase+ Type I NBs (Fig. 3-5B). However, by late stage 15, the pPIm contained a single Dpn+ Mira+ Ase- Type II NB (Fig. 3-5C). We never observed more than a single Type II NB within the pPIm and it appeared at the end of pPIm neurogenesis.

To verify that this NB generates a Type II lineage, which proliferates extensively in the third instar (Boone and Doe 2008), we combined *mzVUM-GAL4* and *Act5C<stop>lacZ; UAS-flp* (Struhl et al., 1993) to permanently mark the lineages of the pPI in third instar larval brains. We found that at least two Type II NB lineages are marked (Fig. 3-5D). The example shows three widely spaced Type II NBs; most commonly there were only two dChx+ Type II NBs per hemisphere (11/12 cases examined). The presence of marked lineages in the larval PI suggests that the identified Ase- pPIm NB does indeed give rise to a Type II NB lineage (model of pPIm NB birth order is summarized in Fig. 3-5E).

3.7 The pPIm placode displays placode-autonomous development

We asked if placode cells sharing a common apical constriction comprised developmentally autonomous units that generated diverse NB identities. We examined the coordination of pPIm cell division, Notch pathway activation and Egfr pathway activation. We examined patterns of the third post-blastoderm mitosis (δ_{16} of (Foe 1989)) in the pPI by pulse labeling embryos with BrdU incorporation for 2 hours followed immediately by fixation. After BrdU labeled embryos were co-labeled for Chx1 and Cas expression we identified cells of the pPI that had undergone DNA replication in the previous 2-hour period.

We compared patterns of cell division between the Cas+ pPIm and the adjacent Cas- pPI placode at stages 10-12. In stage 10 embryos, the pPIm was labeled 83% of the time and the Cas- pPI was never labeled (0/6 cases; Fig. 3-6A). In contrast, in stage 11 embryos the

Cas+ pPIm was never labeled and the Cas- pPI was labeled 92% of the time (n=13) (Fig. 3-6B). In stage 12 embryos the pPI was quiescent (10/18 cases), or only labeled in nascent NBs that re-entered the cell cycle (8/18 cases; Fig. 3-6C). These results suggested that the individual pPI placode cell groups are clonally related, at least as a population of sibling cells. The results also show that the pPI placode cell cycles are relatively synchronous within a placode and cycle independently of other placodes, indicating that the presumptive placodes may have assigned identities before invagination and neurogenesis. Also, both pPI placode groups arrest the cell cycle before neurogenesis and NB specification occurs. We also observed from the patterns of mitoses that the pPI may comprise three placodes, however we could not identify the apical constriction for this most lateral placode (data not shown). Taken together, the results indicated that at least the pPIm placode was a structurally and functionally separate entity whose development surrounding the time of neurogenesis is regulated placode-autonomously.

We examined the temporal dynamics of the m8-GFP activity pattern in the pPIm relative to the neighboring pPI and pPL placodes. Expression of GFP revealed that Notch activity is relatively synchronous amongst cells of the pPIm, but the timing of activity in the pPIm is out of phase with neighboring placodes. In the pPIm of stage 11 embryos, m8-GFP expression is uniformly off in slightly younger embryos (Fig. 3-7A), but is uniformly on in slightly older embryos (Fig. 3-7B). Throughout the period of initial m8-GFP activation in the pPIm, m8-GFP is continually active in the lateral pPI placode group indicating that Notch signaling is independently regulated in the two neighboring placodes. We also found that briefly during stage 11, the pPIm can be found to express phosphorylated MAP Kinase (pMAPK) when the adjacent placodes do not (Fig. 3-7C), which indicated that Egfr/Ras activity, like Notch activity is at some point limited to the cells comprising the single placode.

This result suggests signaling in these pathways occurs within placodes and not between placodes, a feature of the developing ommatidia in the fly retina (Dominguez et al., 1998).

Again, using this combination of structural features and signaling activity patterns, it was clear that the Chx1+ pPI comprises at least two placodes; the Cas+ pPIm subdivision corresponds to the medial of those (Fig. 1B).

Figure 3-1. Placode gene expression across phyla. (A) Expression of Six gene family members optix, D-six4 and sine oculis in developing *Drosophila* embryos. (B) Expression of Six gene family members in developing mouse embryos. (C) Conservation of Six family gene expression across phyla.

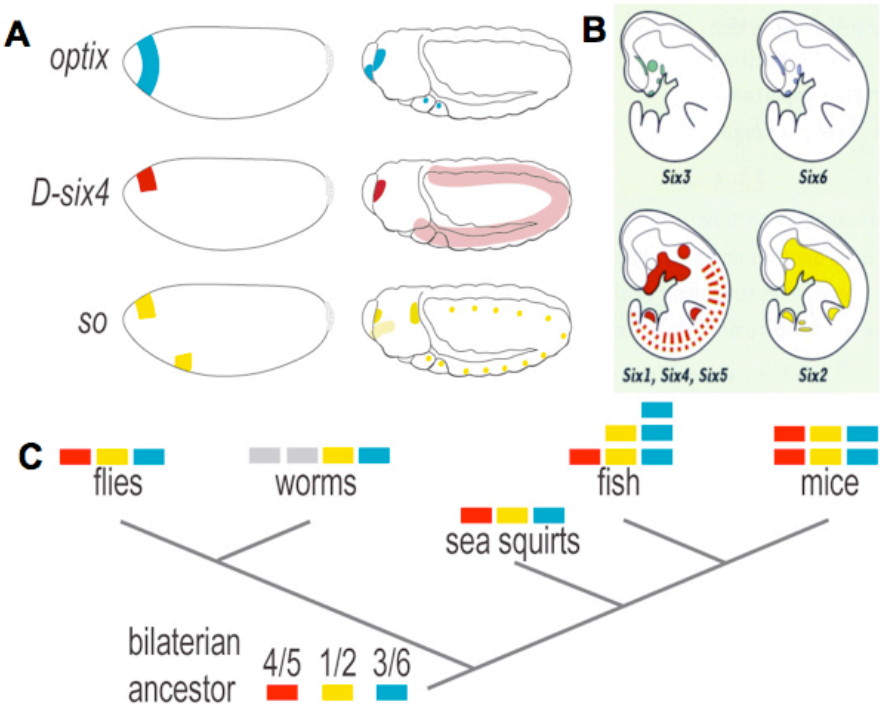


Figure 3-2. The *Drosophila* anterior placode system. (A) A color-coded regional map of the Pdm NE in dorsolateral view (colors as indicated in the legend; dorsolateral orientation is the same in subsequent figures). The Pdm NE includes the embryonic primordia of the pPI, pPL and pPM (arrowheads mark position of individual apical constrictions; clypeolabral furrow (cl)). (B) The Pdm NE consists of the pPI, pPL, and pPM and is demarcated by the intersecting expression of Eya and Dsix4 (outline). (C-D) Optix is expressed only in the proendocrine pPI and pPL at stage 11 (C) and and stage 14 (D) (outline). (E) Vesicles of *m8-GFP* expression comprise the Pdm placode system. Crb label (monochrome, right) is an overlay of three focal planes to show the position of apical constriction points across the Pdm placode system (yellow arrows). The pPIm is indicated (arrow). Scale bar: 20 μm

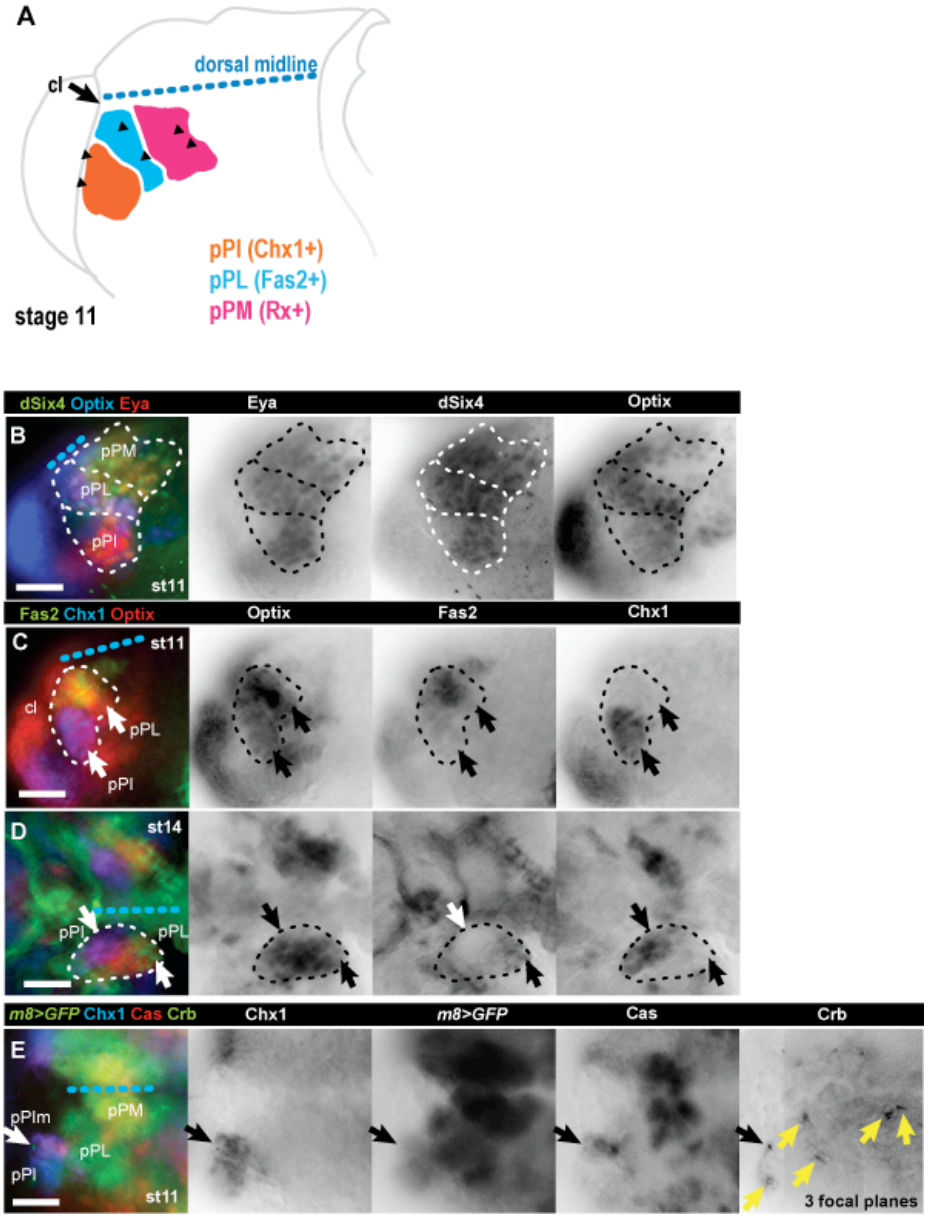
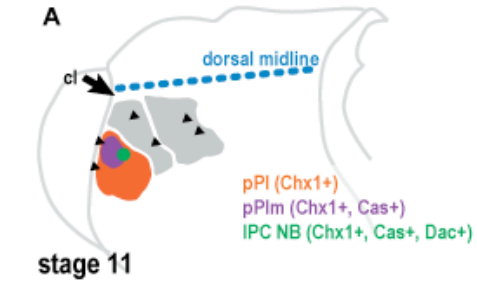


Figure 3-3. The IPC NB is produced by a molecularly distinct pPIm placode. (A) A color-coded regional map of pPI placodes in dorsolateral view (colors as indicated in the legend; dorsolateral orientation is the same in subsequent figures). The pPI is subdivided into at least two placodes. The pPIm (purple) specifically expresses Chx1 and Cas. The IPC NB (green) is uniquely identified by the co-expression of Dac. (arrowheads mark position of individual apical constrictions; clypeolabral furrow (cl)). (B) *tll>mGFP* outlines cellular boundaries in the Pdm NE. Accumulated Crb expression marks apical constrictions (pPIm, arrow; adjacent Cas- pPI placode, asterisk). Scale bar: 20 μm



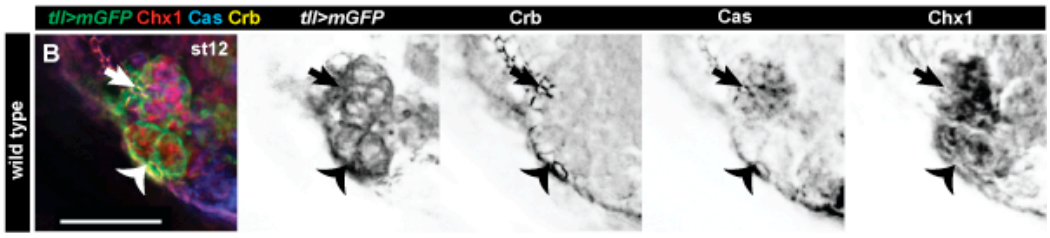


Figure 3-4. Neurogenesis from the pPlm. (A-C) Dotted lines outline the pPlm (Cas+ Chx1+). Asterisk marks the site of apical constriction. (A) Prior to the onset of neurogenesis, placodal neuroepithelial cells express transient low levels of Dpn. (B-C) High level of Dpn marks newly formed NBs. The first-born IPC NB is the posterior-most of two pPlm NBs at stage 12 (B, arrow; non-IPC NB, caret) and of three NBs at stage 13 (C, arrow; non-IPC NB, carets). Scale bar: 20 μm

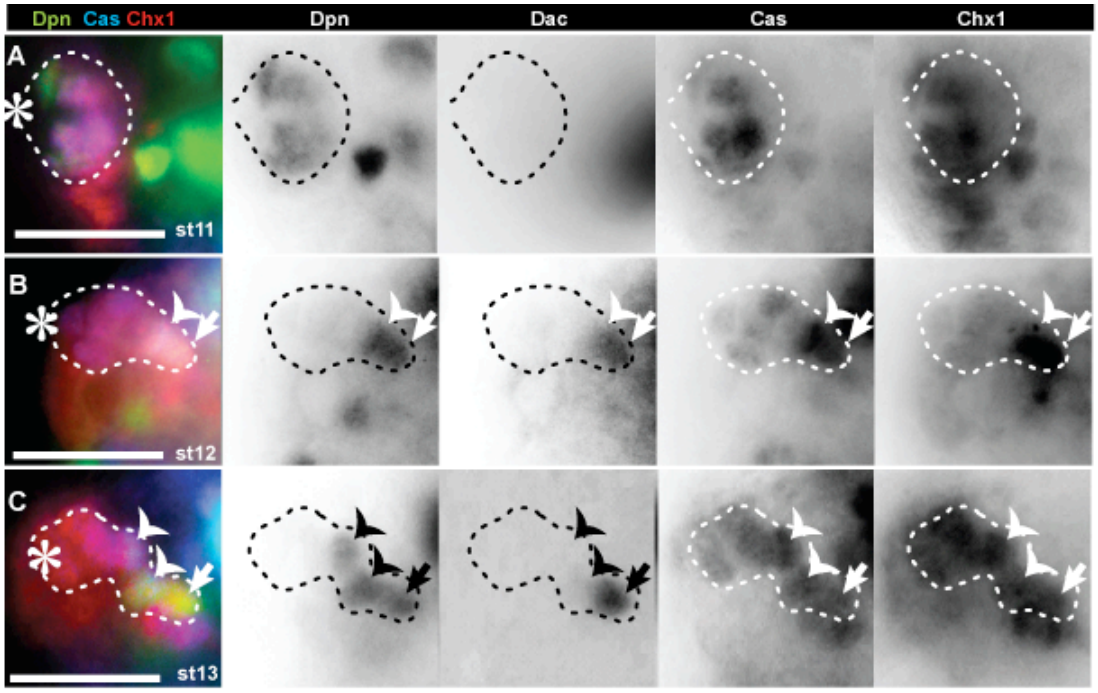


Figure 3-5. Diverse NB identities produced by the pPIm. (A-E) *mzvum-GFP* expression marks the pPI. (A) The pPIm is localized to the anterior Cas+ aspect of the pPI. (B) Four Type I NBs (Mira+Dpn+Ase+) present in the pPIm at stage 14 (arrows). (C) A single Type II NB (Mira+Dpn+Ase-) was found in the pPIm in stage 15-16 embryos. (D) Marked lineages of the pPI NE include three Type II NBs (arrows) and their respective lineages in a wandering third instar brain (wL3). (E) Summary of pPIm NB birth order. Scale bar: 20 μm

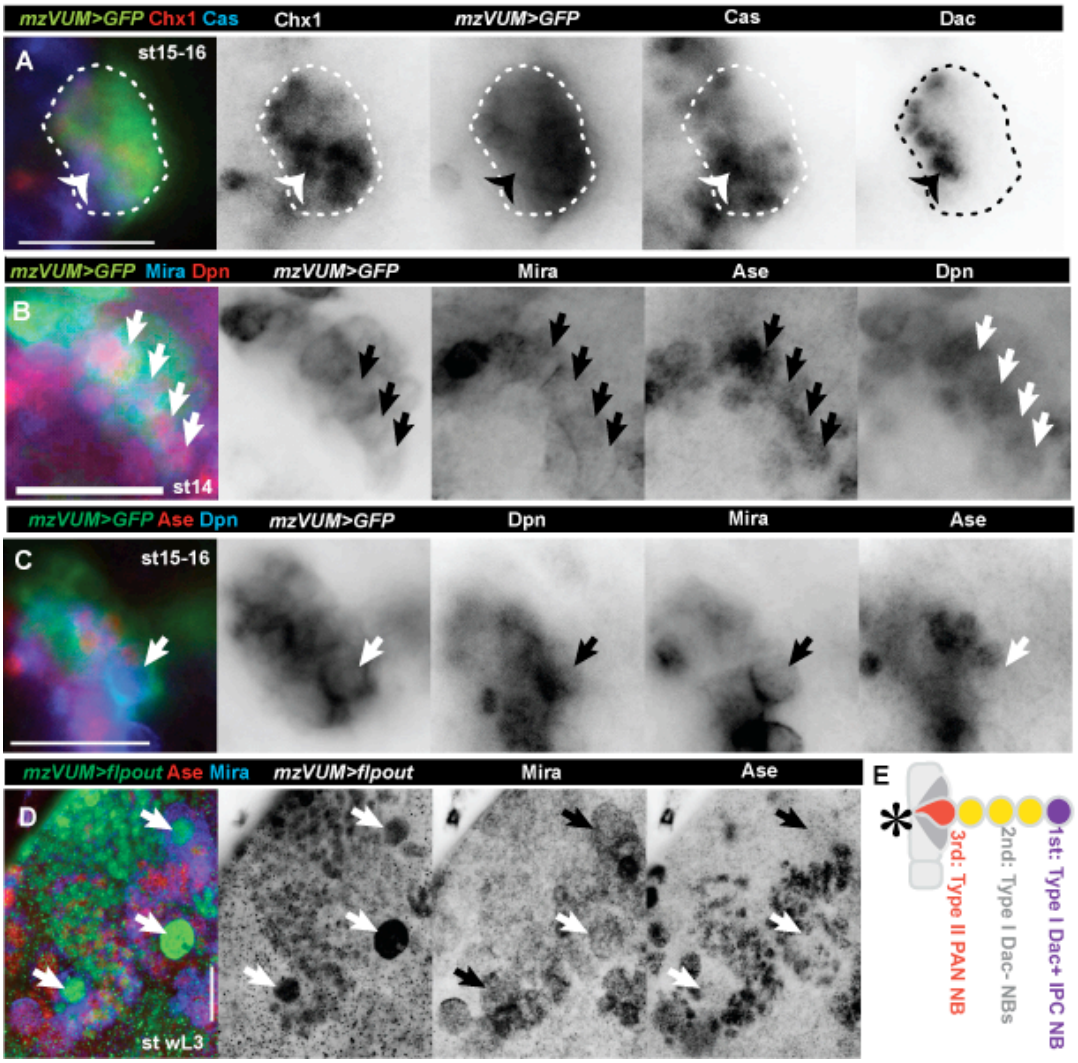


Figure 3-6. Mitoses are coordinated in placodes. (A-C) 2-hour pulse-labeling of cell cycle progression with BrdU. (A) pPIm cells are specifically labeled before stage 10-11 (arrow). (B) The adjacent Cas- pPI placode is specifically labeled before stage 11 (arrow; pPIm, asterisk; non-pPI placode, caret). (C) The pPI is mitotically quiescent before stage 12-13, with the exception of a few newly formed NBs (caret). Scale bar: 20 μm

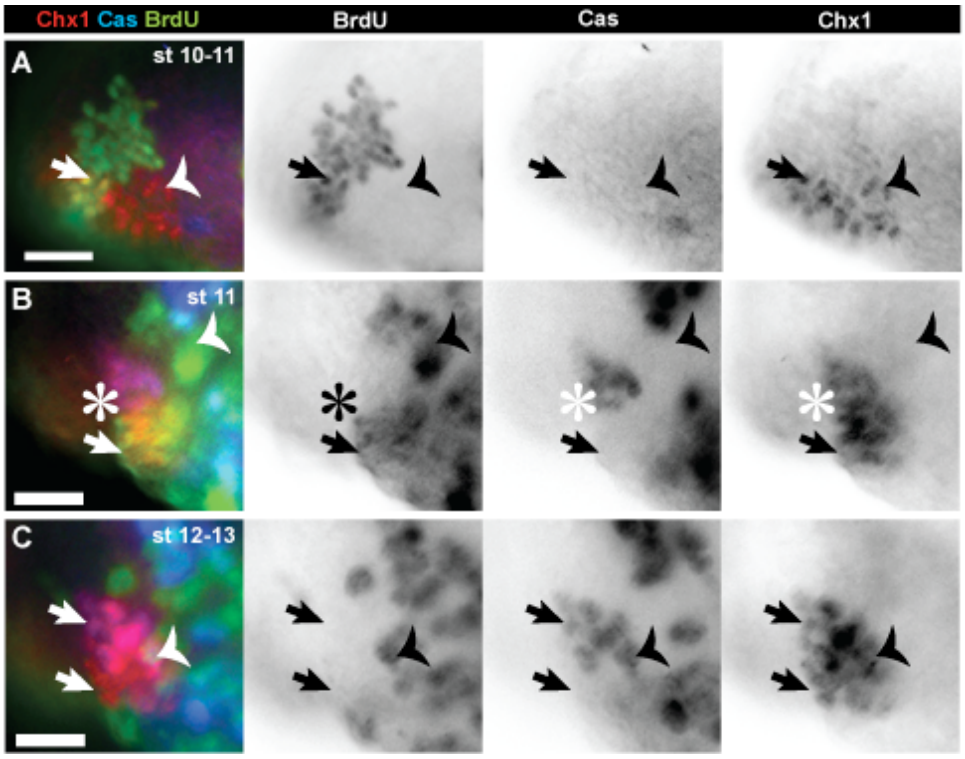
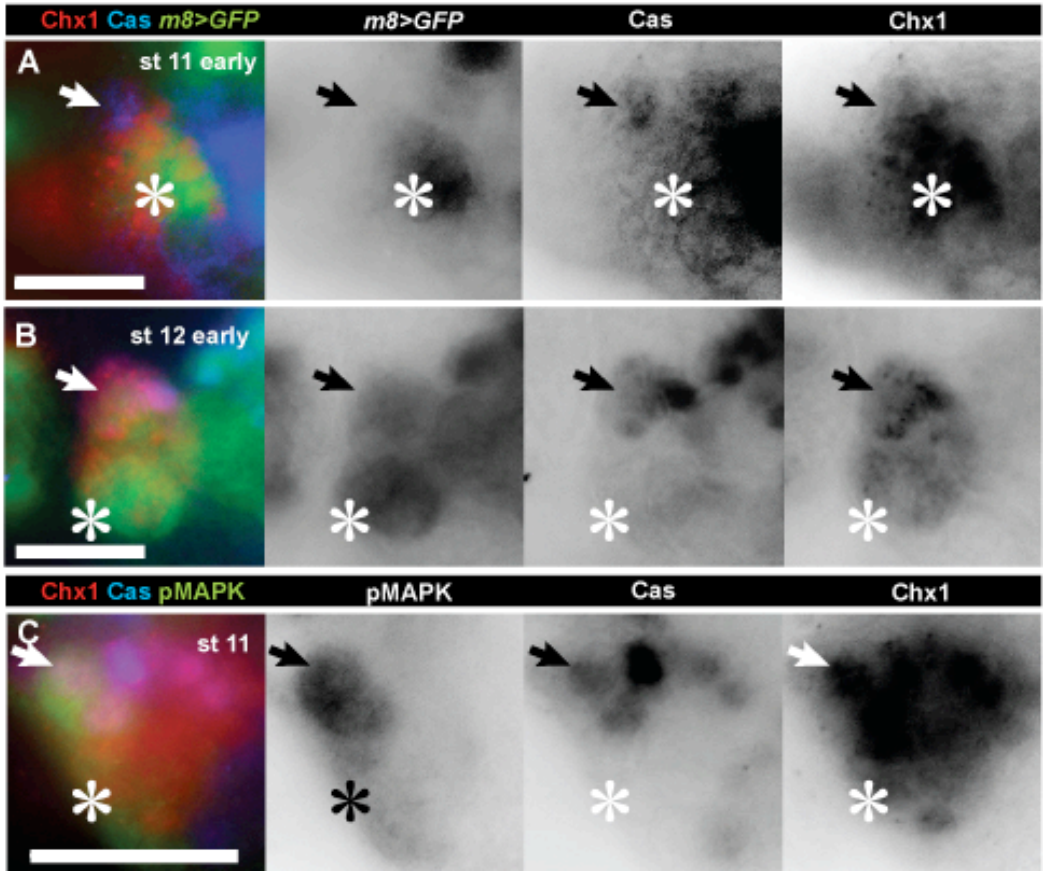


Figure 3-7. Signaling is coordinated in individual placodes. (A-C) Cas- pPl placode marked by asterisk; Cas+ pPlm marked by arrow. (A-B) m8-GFP labels cells with Notch activity. (A) Notch activity off in the pPlm, but on in adjacent pPl placode at early stage 11. (B) Notch activity in both pPl placodes at stage 12. (C) pMAPK labeling specifically in the pPlm at stage 11. Scale bar: 20 μm



Chapter Four: *The role of Notch signaling in placodal neurogenesis*

Introduction

As one of the core signaling pathways governing metazoan development, the Notch signaling pathway displays pleiotropic effects across phyla. The Notch signaling pathway is a conserved mechanism of cell-cell interaction that is implicated in binary fate decisions in nearly all tissue types (Bray 2006). Notch signaling can repress cell fates via a lateral inhibition mechanism, and also promote cell fates via inductive signaling in a variety of tissues (Lai 2004, Andersson et al., 2011, Louvi and Artavanis-Tsakonas 2006). In the development of the nervous system, Notch has been shown to have effects at many levels, including neuronal differentiation.

4.1 Notch signaling in vertebrate neurogenesis

Gain and loss of function studies in vertebrates have elucidated several roles for Notch. In particular, Notch signals affect several differentiation nodes of neural precursors (Louvi and Artavanis-Tsakonas 2006, Ramasamy and Lenka 2010, Cau et al., 2008, Shin et al., 2007, Justice and Jan 2002, Stollewerk 2002). Stem cells give rise to neuronal progenitors whose progression can be inhibited by Notch activity. Notch signals promote glial differentiation, but inhibit oligodendrocyte differentiation (Grandbarbe et al., 2003, Gaiano and Fishell 2002). Notch can also influence fully differentiated neurons, and may have an important but as yet uncharacterized role in maintaining the differentiated state (Louvi and Artavanis-Tsakonas 2006). Modulation of signal strength has been show to alter neurite

morphology (Ferrari-Toninelli et al., 2009, Bonini et al., 2011). Notch is also involved in boundary formation in the brain (Tossell et al., 2011).

4.2 The core Notch signaling pathway in Drosophila

Extensive genetic and molecular interaction studies have identified over 140 different genes that influence the Notch signaling pathway. The complex genetic circuitry of the evolutionarily conserved Notch pathway was elucidated mainly in *Drosophila* and *Caenorhabditis elegans (C. elegans)* studies. Across phyla, the number of paralogues of each element differs. For example vertebrates have four Notch receptors, while *Drosophila* has a single Notch receptor, and *C. elegans* have two. Despite this, biochemically these paralogues have been shown to be largely interchangeable, although they differ in tissue expression (Louvi and Artavanis-Tsakonas 2006, Marklund et al., 2010). We will concentrate on the core components as named in *Drosophila* since this is the model system for our studies.

Notch is a transmembrane receptor that responds to activation of the transmembrane ligands Delta or Serrate on the surface of a neighboring cell. The Serrate ligand has been shown to be dispensable for neurogenesis; Delta is the critical ligand for neurogenesis. Upon binding, the extracellular domains of the ligands and receptor interact. This leads to activation of the Notch receptor, which undergoes a series of proteolytic cleavages that release the intracellular domain of Notch (NICD). The critical cleavage depends on a presenilin-gamma-secretase complex. The NICD translocates from the cytoplasm to the nucleus and binds to the DNA-binding protein Suppressor of Hairless [Su(H)] forming the NICD-Su(H) complex, which triggers transcriptional activation of target genes.

Su(H) is the major effector of the pathway downstream of the Notch receptor, and has shown to be genetically and biochemically required for Notch signaling (Lai 2004, Doroquez and Rebay 2006, Schweisguth and Posakony 1992). Su(H) is a bifunctional transcriptional regulator, which in absence of Notch signaling acts as a transcriptional repressor. Following Notch activation, Su(H) switches to a transcriptional activator. There are many Su(H) binding sites in the genome and it is not known if they are all Notch-responsive.

While not all Notch targets have been identified, the E(spl)-C is the main class of Notch target genes and includes several basic helix-loop-helix (bHLH) transcriptional repressors (Jennings et al., 1994, Jennings et al., 1999, Heitzler et al., 1996). The seven bHLH transcripts produced are: E(spl)m8, m7, m5, m3, m[beta], m[gamma], and m[delta]. The E(spl) transcripts are expressed in multiple tissues, and none shows exclusive expression in the nervous system. A nervous system specific Notch effecter has not been identified.

4.3 Notch signaling in Drosophila *neurogenesis*

The notched wing phenotype associated with haploinsufficiency of the Notch locus was one of the earliest genetic variations observed in *Drosophila*. Classic studies by Poulson showed that homozygous loss of function mutation in the Notch locus was connected to a distinct embryonic phenotype, and these studies were instrumental in first linking the action of genes to embryonic development. Poulson showed that the Notch locus was critical for the neural-epidermal fate choice in the early embryo. Homozygous mutant Notch mutant embryos failed to segregate neural and epidermal lineages, thus all cells within proneural groups become NBs and results in a neurogenic phenotype.

Subsequent work using laser ablation studies showed that the commitment to the neural fate by a single cell in the proneural group had the consequence of inhibiting its neighbors from adopting the neural fate (Doe and Goodman 1985). The disruption of Notch-dependent cellular signaling was thus believed to cause all neighboring cells to develop into NBs, thus explaining the neurogenic phenotype observed by Poulson. Classic studies of lateral inhibition in *Drosophila* focused on NB selection in the VNC of the CNS as well as sensory organ precursor selection in the peripheral nervous system.

In the VNC, Notch mediates the binary fate decision that produces a single NB from an equipotential proneural competence group. In contrast, in the Pdm, proneural competence groups are larger and completely neurogenic, with adjacent cells delaminating as NBs until the placode is depleted of neuroepithelial cells. Previous studies described the extended period of neurogenesis in the Pdm neuroectoderm, and the corresponding extended period of Notch activity (Younossi-Hartenstein et al., 1996).

Results

4.4 Notch controls timing of placodal neurogenesis

Consistent with a requirement for Delta, we observed that Delta protein accumulated on the placodal cells and was enriched at the apical constriction of the pPIm (Fig 4-1A). In mutant embryos, we observe loss of the apical constriction point marked by Crb (4-1C) compared to wild type (4-1B). In stage 10-11 mutant embryos, most pPIm cells delaminated *en masse* and expressed a high level of Dpn, which indicated that in the absence of Notch activity near the time of onset for proneural gene expression, all placodal NE cells became NBs (9/9 cases; Fig. 4-1C); no NBs formed in similar stage controls (Fig. 4-1B). Shifted *Delta*^{ts} embryos at the same stage also showed no apical Crb accumulation (Fig. 4-1E)

when compared with controls (Fig. 4-1D), indicating that the delamination of placodal NBs was correlated with the loss of the apical adherens junction with the outer epithelium.

4.5 Notch is the only NSC from the pPIm

Our previous lineage tracing experiments showed that the IPC NB cell fate is assigned to only one cell of the pPIm placode, while others showed that PI NB lineages in general give rise to many small cholinergic neurons in the larval brain as well as sparsely distributed NSCs (de Velasco et al., 2007). Within the PI and PL there are approximately twenty-six large NSCs that all express the gene dimmed (dimm), a determinant of NSC differentiation, and are identified by their position and expression of particular neuropeptides (Park et al., 2008). Dimm is expressed in NSCs that are not fully differentiated and thus do not yet express neuropeptides (Hewes et al., 2003), which makes it possible to follow NSC fate in the embryo before IPCs express Dilps.

We followed Dimm expression in stage 17 embryos with a *dimm-GAL4* enhancer trap driving membrane bound GFP (dimm-GFP, (Park et al., 2008)), which recapitulates Dimm expression in the brain, and labeled the IPC NB lineage by its expression of Dac and Chx1 (Fig. 4-2A). We found that the entire PI, labeled by Chx1 expression, contains two groups of Dac and dimm-GFP expressing cells, the larger and posterior of the two being the IPC NB lineage as previously shown by clonal analysis (Wang et al., 2007). Thus in a stage 17 embryo, the IPC NB lineage was entirely comprised of NSCs and these were the only NSCs to arise from the pPIm. We labeled the IPC NB lineage by Dac expression in the first instar larval brain and found that at this stage, the IPC NB lineage remained the only NSC group in close vicinity (Fig. 4-2B).

4.6 The pPIm placode is an equivalence group for the IPC NB fate

Our observations that the IPC NB was the first fate specified from the pPIm and that activation of Notch pathway targets temporally coincided with IPC NB specification led us to examine the role of the Notch pathway in fate specification. We observed that a well-characterized zygotic Notch null hemizygote male embryo (N^{55e11}/Y) (Rulifson and Blair 1995), which received two doses of maternal Notch mRNA contribution from the compound balancer chromosome, gave the most severe loss of function phenotype where the pPIm could still be recognized. At late stage 12, the time of normal Dac+ IPC NB appearance, most if not all Cas+ Chx1+ pPIm cells expressed high Dpn and Dac, suggesting they had mass delaminated as supernumerary IPC NBs (4/5 cases; Fig. 4-3A). The ectopic IPC NBs were judged to come from the pPIm because they occupied the same position as normal, they were the same number of Cas+ Chx1+ NBs as are found in the normal pPIm, and they remained as a contiguous group. To further test the temporal requirements for Notch signaling we used temperature up-shift experiments with temperature sensitive (*ts*) genotypes of *Delta* (Dl^{RF}/Dl^{9B} = $Delta^{ts}$) (Parks et al., 2006) and *Notch* (N^{ts1}/N^{55e11} = $Notch^{ts}$) (Heitzler and Simpson 1991).

We examined the specification of IPC NB fate in $Delta^{ts}$ embryos at stages 10 through 17 following a 3-hour shift from the permissive temperature of 18ºC to the restrictive temperature of 29ºC. Quadruple labeling for Dpn, Dac, Cas and Chx1 expression was used to follow cells with the IPC NB identity. Following upshifts ending at stage 12-13, the time of normal IPC NB appearance, most if not all pPIm cells in up-shifted $Delta^{ts}$ (Fig. 4-3B) and $Notch^{ts}$ (6/8 cases; Fig. 4-3C) embryos expressed high Dpn and Dac, phenotypes that matched the Notch null hemizygotes. Controls for the effect of temperature shift were normal in $Notch^{ts}$/+ embryos (5/5 cases; Fig. 4-3C).

4.7 Critical period for Notch

When later stage *Delta*^{ts} embryos were scored for the presence of supernumerary IPC NBs and their lineages, we found that only stage 12 through stage 14 had supernumerary IPC NB lineages following the 3-hour shift, while stage 15 and later embryos were no different than controls. No ectopic Dac+ IPC NBs lineages were observed earlier than late stage 12 (Fig. 4-4). 3-hour (180 minute) upshifts after 680 minutes of development produced no supernumerary IPC NBs, hence the end of a competence period for pPIm cells to take the IPC NB fate occurs at 500 minutes of development, the end of stage 12, which is the time that the IPC NB first expresses Dac.

We found that in both *Notch*^{ts} and *Delta*^{ts} embryos at early stage 12 or younger, before the time of normal IPC NB appearance, there was no precocious differentiation of the Dac+ IPC NB. While neurogenesis occurs earlier in mutants, and results in mass delamination from the placode in St 10-11, the competence period to form an IPC NB in normal embryos ended near the time that the IPC NB normally first appears. Following the end of this IPC NB competence period, Notch activity and neurogenesis in the pPIm placode continued to persisted for at least three hours after this point, through the end of stage 14 (de Velasco et al., 2007).

4.8 Supernumerary IPC NBs produce functional IPCs

In order to further test the fate equivalence of the induced supernumerary IPC NBs, we examined their potential to proliferate and differentiate as fully-fledged IPCs. *Delta*^{ts} embryos were shifted for 3 hours to restrictive temperature to induce supernumerary IPC NBs and then shifted back to permissive temperature and allowed to develop to stage 16.

The Cas+ Chx1+ pPIm of stage 16 embryos was entirely comprised of supernumerary Dac+ cells (3/3 cases; Fig. 4-5B compared with the control in Fig. 4-5A).

The volume of the Dac expressing cell cluster, while difficult to quantify, was roughly six to eight-fold greater in size than in control embryos, as would be expected if the cluster arose from the proliferation of 6-8 IPC NB lineages. *Delta*^{ts} first instar larval brains harboring supernumerary IPC NB lineages induced by 4-hour temperature shifts, delivered at stage 11-12, formed large clusters of supernumerary Dac and Dilp2 expressing NSCs that had larger than normal fascicles of cell processes extended in the normal IPC projection pattern (Fig. 4-5D; 13/13 cases compared with 0/25 in normal control brains Fig. 4-5C). Together, these results suggest that the pPIm placode is a group of roughly eight neuroepithelial cells, each possessing the equivalent developmental potential to become an IPC NB at the time that the IPC NB is normally specified.

4.9 Sustained activated Notch delays but is not required for IPC NB formation

Notch affects proliferation and delamination in the vNE. In embryos expressing truncated Notch cytoplastmic domain (hsp70-N^{intra}), vNE cells go into mitotis prematurely, and fail to delaminate (Hartenstein et al., 1994). The neurogenic phenotype of Notch mutant embryos becomes manifest in late stage 10 embryos. Most cells of the vNE postpone their second mitosis, round up while still at the surface and express NB specific markers.

We analyzed the impact of increased transgenic Notch activity on IPC NB specification using a *giant* (*gt*) enhancer-GAL4 fusion transgene and the *UAS-Notch*^{act} intracellular fragment transgene (*UAS ΔEN*; (Larkin et al., 1996)), which activates the pathway in the Pdm NE. In contrast to Notch loss of function causing early NB formation,

gain of Notch activity caused a delay in the specification of the IPC NB from late stage 12 (Fig. 4-6A; Dac+ IPC NB not present in 5/5 cases) to stage 14 (Fig. 4-6B; Dac+ IPC NB present in 9/21 cases). Although the IPC NB was ultimately specified, perhaps due to a drop in transgene activity, the result suggests that the competence period for the IPC can be extended by at least 2 hours when Notch activity is maintained at a high level in the placode. The result is then also consistent with a role for IPC NB specification in closing the competence period in the pPIm.

4.10 Notch: pan-placodal restriction of the NSC fate

Given the pPIm placode is only one of several that comprise the primordium of the PI and PL neuroendocrine center, we asked if all pPI and pPL placodes might also be equivalence groups that produce a single NSC lineage NB and several non-neurosecretory lineage NBs. To preliminarily address this possibility, we examined the expression of Dimm in the Optix+ pPI and pPL in late stage 17 *Delta*^{ts} embryos reared entirely at the restrictive temperature. In the absence of Notch activity, the pPI and pPL appeared expanded in size with a majority of ectopic Optix+ cells expressing Dimm (4/4 cases; Fig. 4-7B compared with normal control in Fig. 4-7A).

We suspect the increased size of the Optix+ domain was due to re-specification of late proliferating primary and secondary NBs to early proliferating NSC NB primary lineages. Many of the ectopic Dimm cells within the pPI and pPL also expressed Dac, similar to the IPC NB lineages. The mass conversion of cell fates to NSCs was largely restricted to the pPI and pPL with the exception of the neuroendocrine CC cells, which were also dramatically increased in number (Fig. 4-7B, caret).

We had proposed earlier that the CC cells are produced from a NB lineage that arises adjacent to the pPIm, in the same field of Eya and Dsix4 expression, though a placode for the CC NB has not been identified, in part because of the rapid migration of differentiating CC cells away from the placodal NE (Wang et al., 2007).

Figure 4-1. Notch signaling regulates the timing of NB delamination from the pPIm. (A) Delta expression in wild type embryos accumulates at the apical constriction of the pPIm (arrow). (B-C) Labeling of pPI apical constrictions by Crb at stage 12 in heterozygous controls (B, arrows) and in *Delta*^{ts} after 3-hour temperature shift (C, carets). (D-E) Stage 10-11 *Delta*^{ts} embryo (E) following a 3-hour temperature upshift shows early delamination of pPIm NE cells compared with the control (D). Scale bar: 20 μm

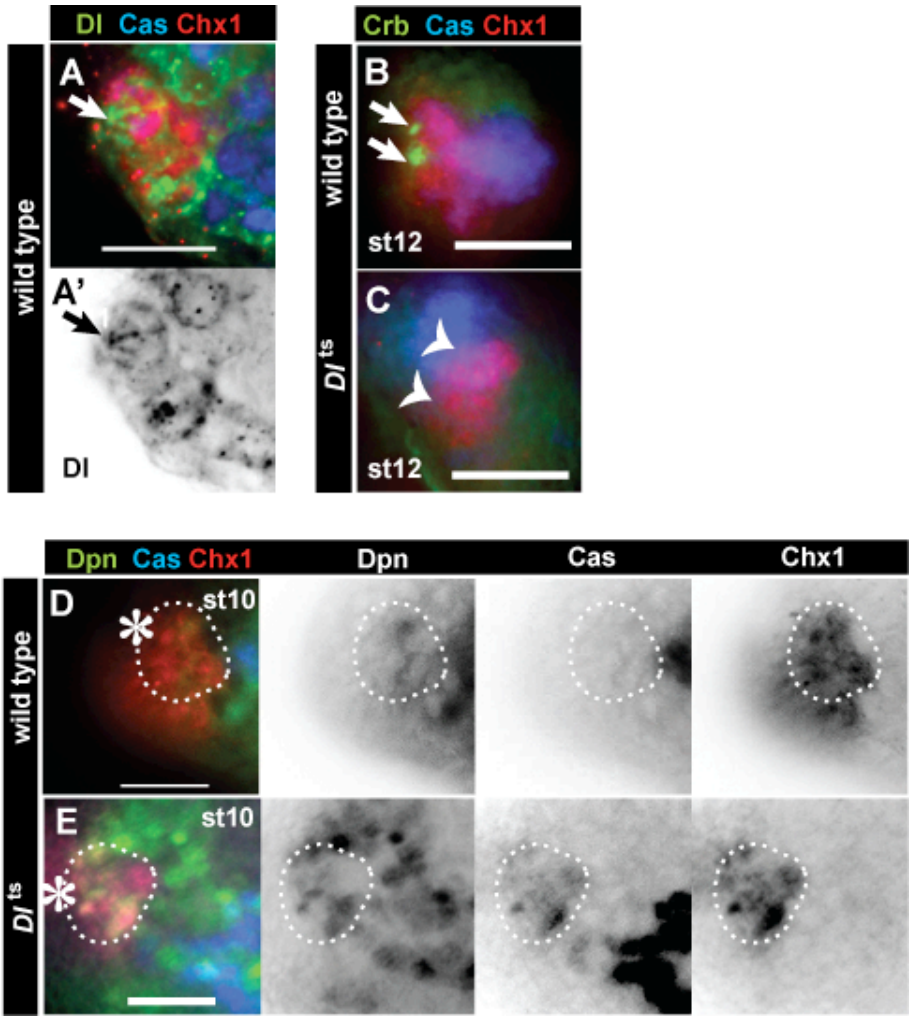


Figure 4-2. The IPC NB lineage is the only pPIm to produce NSCs. (A) Two Dac+ cell groups (arrow and caret) produced by the pPI are Dimm+ NSCs. The posterior group is the IPC NB lineage (arrow). (B) In a first instar larval brain the Dilp2+ IPC NB lineage continues to express Dimm, whereas immediately neighboring PI cells do not (carets). Scale bar: 20 μm.

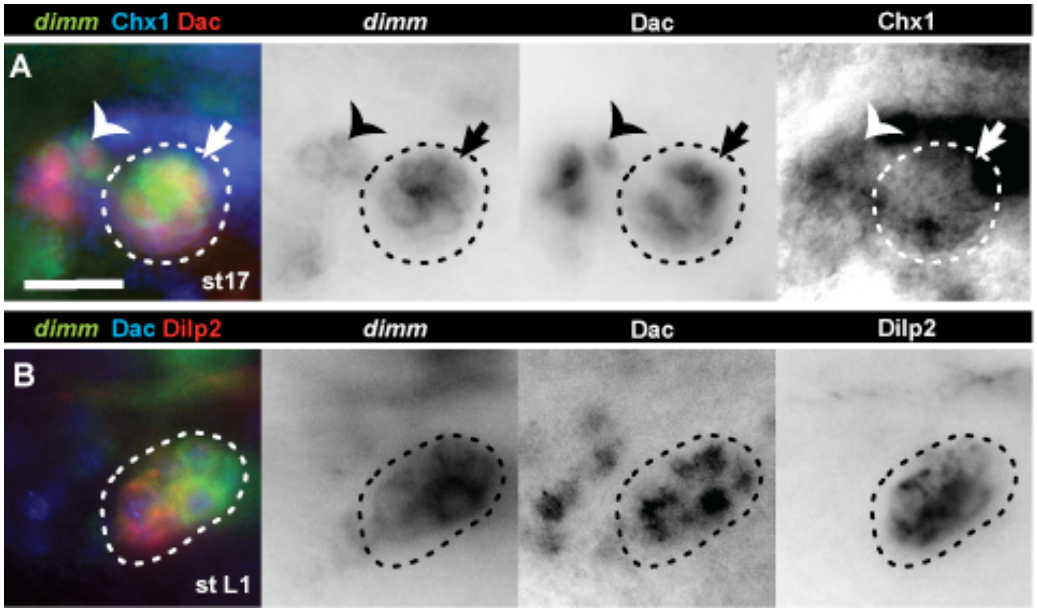


Figure 4-3. The role of Notch signaling in IPC NB specification. (A-C) Supernumerary IPC NBs within the pPIm at stage 13 (dashed line outlines the pPIm): (A) N^{55e11}/Y hemizygote; (B) *Delta*^{ts} after 3-hour temperature shift; (C) *Notch*^{ts} after 3-hour temperature shift. (D) Heterozygous control after 3-hour temperature shift. Scale bar: 20 μm

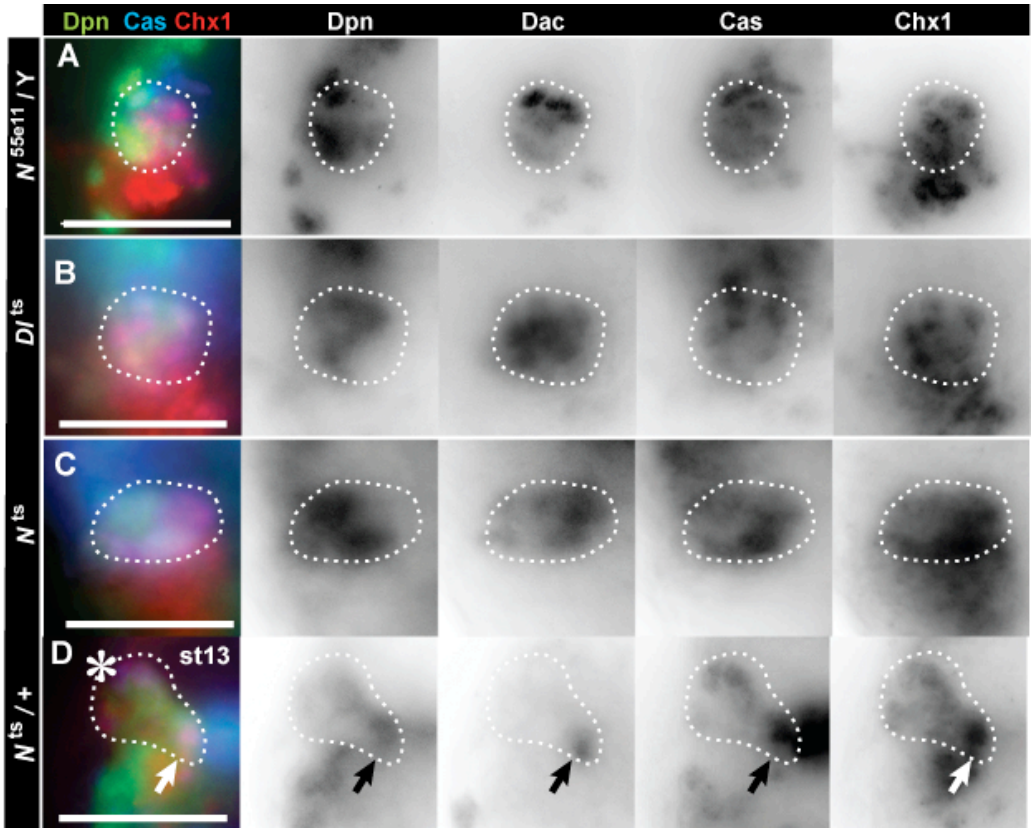


Figure 4-4. Critical period for Notch activity brackets normal IPC NB formation. Stage by stage quantification of ectopic IPC lineages in *Delta*^{ts} embryos subjected to 3-hour temperature shift.

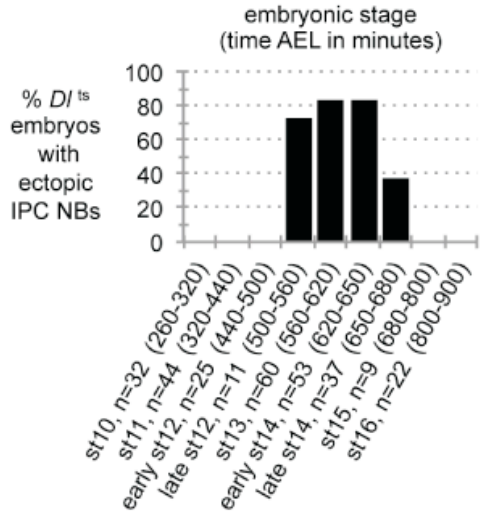


Figure 4-5. Supernumerary IPC NBs form functional IPCs. (A-B) The IPC lineage (pPIm is outlined) in stage 16 heterozygous control (A) and *Delta*^{ts} embryos subject to a 3 hour upshift followed by a return to permissive temperature for further development (B). Ectopic IPC NBs in *Delta*^{ts} mutants proliferated to produce an expanded IPC lineage. (H-I) IPCs in heterozygous control and *Delta*^{ts} first instar larval brains following 4-hour temperature shift during competence period. *Corpora cardiaca* marked by carets. (H) Control brains with 6-8 Dac+ Dilp2+ IPCs per hemisphere. (I) *Delta*^{ts} brains with supernumerary Dac+ Dilp2+ IPCs. Scale bar: 20 μm

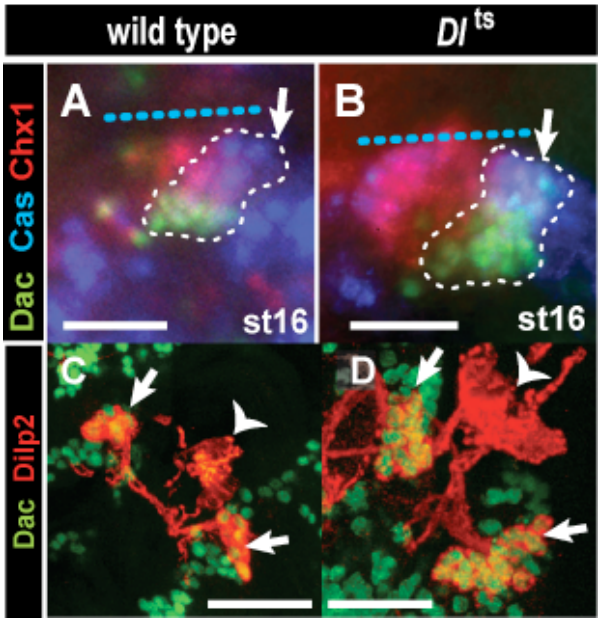


Figure 4-6. Constitutive Notch activity delays specification of the IPC NB. (A-B) *giant-GAL4* driving *UAS-N*^{act} results in a delay of IPC NB formation. The IPC NB is not yet present in stage 13 (A, arrow), but is specified by stage 14-15 (B, arrow). Blue dashed lines mark head midline. Scale bar: 20 μm

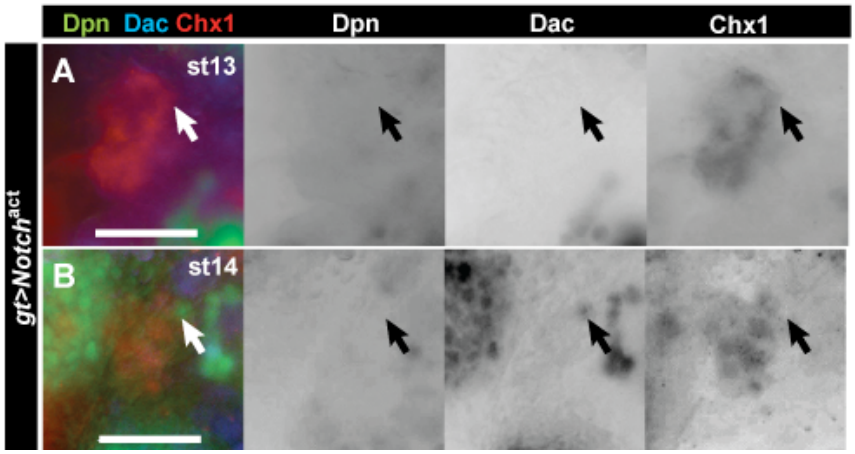
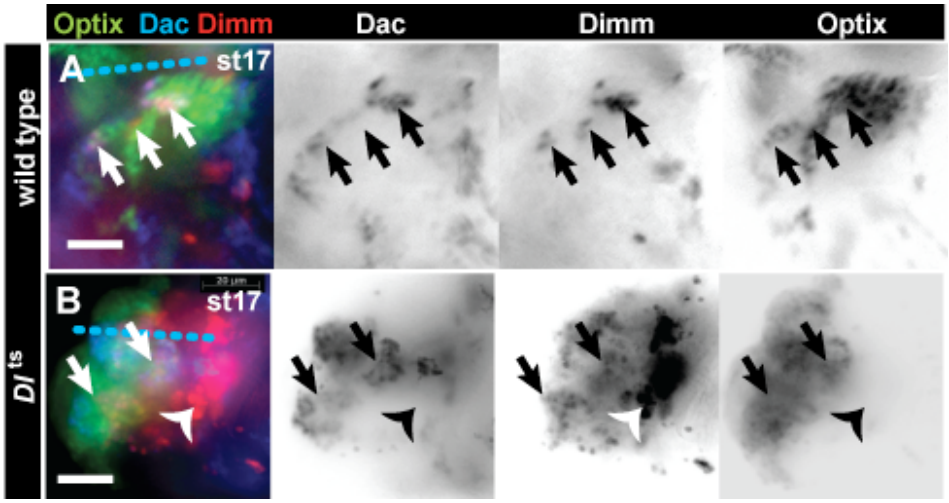


Figure 4-7. The role of Notch signaling in IPC NB specification. (A-B) Optix+ PI/PL neuroendocrine compartment in stage 17 embryos (NSC, arrows). In *Delta*^{ts} embryos, the vast majority of the pPI and pPL differentiated as NSCs (A) compared with heterozygote controls (B), which showed very few Dimm+ NSC lineages in the proendocrine pPI and pPL, Scale bar: 20 μm



Chapter Five: *The role of Egfr signaling in placodal neurogenesis*

Introduction

The Egfr pathway, like the Notch signaling pathway, is one of a few fundamental and evolutionarily conserved mechanisms in metazoans employed in development. The Egfr pathway is used repeatedly in development to direct cell fate choices, cell division, and cell survival (Shilo 2003). We will begin with an overview of Egfr function and structure in vertebrate development, continue with an analysis of Drosophila Egfr signaling and its role in development, and conclude with our elucidation of the role of Egfr signaling in placodal survival and cell fate specification.

5.1 Egfr signaling in vertebrates

In humans, Egfr belongs to a family of four receptor tyrosine kinases: the ErbB receptors, which include ErbB1 (Egfr) (Ullrich et al., 1984), ErbB2 (Neu, or HER-2) (Bargmann et al., 1986, Semba et al., 1985, Fukushige et al., 1986), ErbB3 (HER-3) (Kraus et al., 1989, Plowman et al., 1990) and ErbB4 (HER-4) (Plowman et al., 1993). All ErbB receptors are single-pass transmembrane proteins bound and activated by a variety of growth factors of the EGF family. Binding of ligand to receptor induces receptor dimerization and consequent transphosporylation of tyrosines that is the principal trigger for activation of the pathway. Complexity of the pathway in vertebrates is granted by the numerous ErbB ligands, which have different affinity and binding preferences for different homo-and heterodimeric combinations formed by the four receptors (Lemmon and Schlessinger 1994).

Disregulation of the ErbB family has been implicated in many human cancers (Britsch 2007, Callahan and Hurvitz 2011, Normanno et al., 1994). Hyperactivity of the pathway is associated with malignant transformation and progression of multiple tumour types. ErbB is amplified in 84% of squamous cell carcinomas and 40% of glioblastomas (Salomon et al., 1995). ErbB2 is amplified in adenocarcinomas of breast, stomach and ovary (Slamon et al., 1989). ErbB3 is mainly associated with breast carcinomas (Kraus et al., 1989).

5.2 The core Egfr pathway in Drosophila

The *Drosophila* genome encodes a single locus for the Egfr family. The *Drosophila* homologue is known by multiple names: faint little balls, Torpedo, *Drosophila* EGFR (DER). Signal tranduction through Egfr requires activation in the form of small protein ligands. Egfr is known to act with four activating ligands: the Neuregulin Vein and the TGF-alpha ligands Spitz, Keren, and Gurken. Via activation of these multiple ligands, Egfr signaling controls a variety of key developmental processes in a variety of tissues (Shilo 2003, Shilo 2005).

Vein (Vn) is a Neuregulin-class secreted ligand with a weak activation capacity, and is utilized in tissues that require low Egfr activation (Schnepp et al., 1996, Wessells et al., 1999). Developmentally, Vn is known to be involved in embryogenesis for the patterning of the ventral ectoderm and specification of muscle precursors (Golembo et al., 1999, Yarnitzky et al., 1998). Vn also has multiple roles in wing development (Simcox 1997) and neurogenesis (zur Lage et al., 2004, Lage et al., 1997, Klaes et al., 1994). In the eye Vn is not strictly required, but it may stimulate Egfr signaling in the contexts of cell proliferation, survival, and possibly differentiation (Spencer et al., 1998).

Spitz (Spi) is the primary Egfr ligand and is responsible for activation of the Egfr pathway in most tissues during *Drosophila* development. Spi, as well as the other TGF-alpha ligands Keren and Gurken, is generated as a transmembrane precursor (Urban et al., 2002). Spi undergoes a special processing step, as it must be cleaved to produce an active form known as secreted spitz (Tsruya et al., 2002). Rhomboid (Rho) and Star are accessory proteins involved in Egfr activation via Spi processing. Rho is an intramembrane serine protease whose activity is required for Spi cleavage (Urban 2006). Star is also a transmembrane protein involved in processing Spi, and activating the other TGF-alpha ligands Keren and Gurken (Urban et al., 2002, Reich and Shilo 2002).

The repressive ligand, argos, is a novel secreted protein with a cystein-rich region similar to an EGF-repeat. Argos inhibits Egfr signaling at the ligand-receptor level, and acts by sequestering activating ligand away from the Egfr receptor (Doroquez and Rebay 2006).

Egfr is a receptor tyrosine kinase (RTK) and thus feeds into the Ras/Raf/MAPK cascade to effect transcriptional changes (Doroquez and Rebay 2006). Upon activation of the receptor, a membrane localized protein complex forms that recruits and activcates Ras, a cytoplasmic proto-oncogene. Ras cycles between active GTP-bound isoforms and inactive GDP-bound isoforms. Activation of Ras leads to activation of a cascade of protein kinases, the Raf/MEK/MAPK cascade. The final step in the cascade results in activation of pMAPK to a diphosporylated form (dpMAPK), which translocates to the nucleus upon activation. This step is critical in affecting output from the Egfr pathway. Once in the nucleus dpMAPK phosphorylates specific target proteins.

Two *Drosophila* Ets transcription factors of interest for our studies are Pointed (Pnt) and Yan/Anterior open (Aop). Both contain an ETS DNA-binding domain and compete for access to promoter regions of common downstream transcriptional targets. Pnt exists in

two isoforms PntP1 and PntP2. It is positively regulated by Egfr activation and becomes a potent activator (Klaes et al., 1994). Yan is a negative regulator that is degrade and exported from the nucleus following activation of the pathway. Aop/yan has been implicated in cell division and differentiation (Rogge et al., 1995).

5.3 Egfr signaling in Drosophila *cell fate and survival*

Egfr signaling plays a critical role in *Drosophila* development and is involved in oogenesis, wing vein determination, and determination of cell fate in the ventral ectoderm (Shilo 2005). During larval stages Egfr is also required in development of the eye and wing.

In the Pdm, Egfr signaling is required for both differentiation and maintenance of neural progenitors along the dorsal midline (Dumstrei et al., 1998). Egfr signaling has also been shown to have a key role in cell survival by downregulating expression of head involutions defective (Hid) (Kurada and White 1998, Bergmann et al., 1998). Regulation of cell number by MAPK has also been shown (Bergmann et al., 2002). Interestingly, the expression of hid mRNA is placodal, although the protein is not expressed at a high level there (Grether et al., 1995). We will investigate the role of Egfr in cell differentiation and cell survival in placode of the Pdm.

Results

5.2 Egfr signaling promotes survival of non-neurosecretory pPIm lineages

Throughout Pdm neurogenesis the EGF/TGF-α homolog Spi, Egfr activity and Ras activation maintain survival of placodal cells and ectopic Egfr/Ras pathway activation is sufficient to disrupt cell fate and proliferation within the placodal NE (Dumstrei et al., 1998, Rogge et al., 1995). Loss of Egfr activity results in an increase in apoptosis throughout the

anterior placode system beginning at stage 12, with the pPI and pPL being severely reduced in size as shown by loss of Chx1+ and Fas2+ cells (de Velasco et al., 2007, Park et al., 2008, Dumstrei et al., 1998).

We examined whether, in addition to promoting cell survival, Egfr activity was essential for specification of the IPC NB or other NB identities in the pPIm. We first examined homozygous embryos of the well-characterized *Egfr*^{f24} null allele (Clifford and Schupbach 1989). Surprisingly, we found at stage 14 that a single IPC NB was specified as normal, however there was reduction of the pPI to only a few cells overall (14/14 cases; Fig. 5-1A), which we suspect were the first NBs formed from adjacent pPI placodes. We compared the *Egfr*^{f24} mutant phenotype to another allelic combination, *Egfr*^{ts1a}/*Egfr*^{f24} (*Egfr*^{ts}), which is null function at 29ºC (Kumar et al., 1998). In up-shift studies that paralleled those performed with *Notch* and *Delta* alleles, we examined *Egfr*^{ts} embryos immediately following a 6-hour shift from the permissive temperature of 18ºC to the restrictive temperature of 29ºC. We found that up-shifted stage 12-13 embryos had the same phenotype as *Egfr*^{f24} mutants (6/9 cases compared with 0/5 cases of the control; Fig. 5-1B). However, embryos shifted at later stages had progressively less loss of pPI cells the later the shift occurred; at stage 17, there was no noticeable defect in the size of the pPI (0/5 as with 0/5 cases of the control; Fig. 5-1C). These temperature shift results indicated that commitment of pPIm cells to NB fate abrogates the requirement for Egfr-dependent survival. Consistent with this view, pMAPK levels were higher in pPIm placodal NE cells than in NBs (Fig. 5-1D).

Parallel results were seen again with *spi*^{1} homozygous mutant embryos, where the IPC NB is specified but the remaining pPIm NE cells are absent (Fig. 5-2A). We examined whether the loss of pPIm cells and NBs was due to apoptosis based on previous observations (de Velasco et al., 2007, Dumstrei et al., 1998). In early stage 11 *spi*^{1} embryos,

the Chx1+ pPI was normal in size and showed no evidence of cell death (Fig. 5-2C; compare to control in 5-2B). However, in late stage 11 *spi*^{1} embryos we observed many Chx1+ cells with elevated cleaved Caspase-3 (CC3) and an overall reduction in the size of the pPI (Fig. 5-2E; compare to control in 5-2D). Control embryos also contained apoptotic cells but they were not Chx1+; they were likely scavenging hemocytes that had phagocytosed apoptic cells (arrows). Homozygous mutant *spi*^{1} embryos phenocopied the egfr mutants, with a loss of pPIm neuroepithelial cells. The IPC NB lineage is specified normally. To quantify the loss of cells seen in the spi and egfr mutants, we assayed the size of the pPI in egfr, spi mutants, and also mutants of other key Egfr pathway components. The pPI size was quantitated as the mean number of Dac- Chx1+ cells per hemisphere (mean ± SEM, n). The pPI was reduced from normal size (14.6 ± 0.75, n=5; Fig. 5-1D wt st14) in the following mutants: *spi*^{1} (1.87 ± 0.35, n=15; Fig. 5-2A); *Egfr*^{f24} (2.38 ± 0.59, n=16; Fig. 5-1A); *rho*^{7m43} (*rhomboid*, which encodes the protease essential for activation of Spi signaling activity (Urban et al., 2002) (2.93 ± 0.29, n=15; Fig. 5-3A); *rho*^{7m} *vn*^{ry} , a combined loss of Rho and the Rho-independent Egfr ligand of the neuregulin type, Vein (Schnepp et al., 1996) (2.14 ± 0.51, n=7; Fig. 5-3B). This suggested that Spi was the ligand principally responsible for promoting Egfr-dependent non-IPC NB pPIm cell survival.

Given the IPC NB was specified normally in Egfr pathway mutants, we also investigated whether EGFR activity was essential for the morphogenesis of placode formation. We observed that *spi*^{1} embryos made a pPIm apical constriction during stage 11 as normal (Fig. 5-3C). We observed similar results with *Egfr*^{f24} homozygous embryos (not shown).

Egfr-dependent pMAPK activity leads to activation the ETS-domain transcriptional activators, Pointed1 (Pnt1) and Pnt2. We examined homozygous embryos with both *pnt1* and

pnt2 genes deleted ($pnt^{\Delta 88}$; (Klaes et al., 1994)) and found that by stage 14 there was no significant loss of cells from the pPIm and the IPC NB lineage was specified normally (14/14 cases; Fig. 5-4A), which indicated that the cell survival signal was not principally transduced by *pnt1/2*. Thus, the suppression of cell death may come from an upstream pathway component such as MAPK or by an unidentified parallel pathway. Pnt activity is repressed by the ETS factor Yan (Aop), which is removed from the target gene enhancer and exported out of the nucleus following phosphorylation by pMAPK, which then allows Pnt1/2 to activate transcription (Doroquez and Rebay 2006).

5.3 Egfr is implicated in fate choice of secondary NB fates

We examined the phenotype of *yan* homozygous mutants (aop^{1}). Loss of yan function leads to hyperplasia of the Pdm (Rogge et al., 1995), yet we found that the IPC NB was normally specified (5/5 cases; Fig. 5-4B). Interestingly, we found that in stage 15 to 17 embryos, loss of Yan led to specification of supernumerary Dpn+ Ase- Type II NBs in the pPI (5/6 cases; Fig. 5-4C). This suggested that Egfr signaling might promote the Type II fate at the expense of non-IPC Type I NBs within the pPIm. Alternatively, remaining pPIm cells that would otherwise apoptose, were directed to the Type II fate. Either way, the requirement for Yan implicates Egfr activity in pPIm cell fate specification, beyond mere regulation of cell survival, which did not require Pnt1/2 function.

Figure 5-1. Egfr activity is essential in pPlm NE for non-IPC NB survival. (A) pPlm in *Egfr*^{f24} embryo at stage 14 is reduced in size, but the Dac+ IPC lineage is present. (B-C) *Egfr*^{ts} after 6-hour temperature upshift. Stage 12 embryos with a reduced pPlm (B); stage 17 embryos with a normal sized pPlm. (D) pMAPK is elevated in placodal pPlm NE cells (caret), but off in NBs (arrow). Scale bar: 20 μm

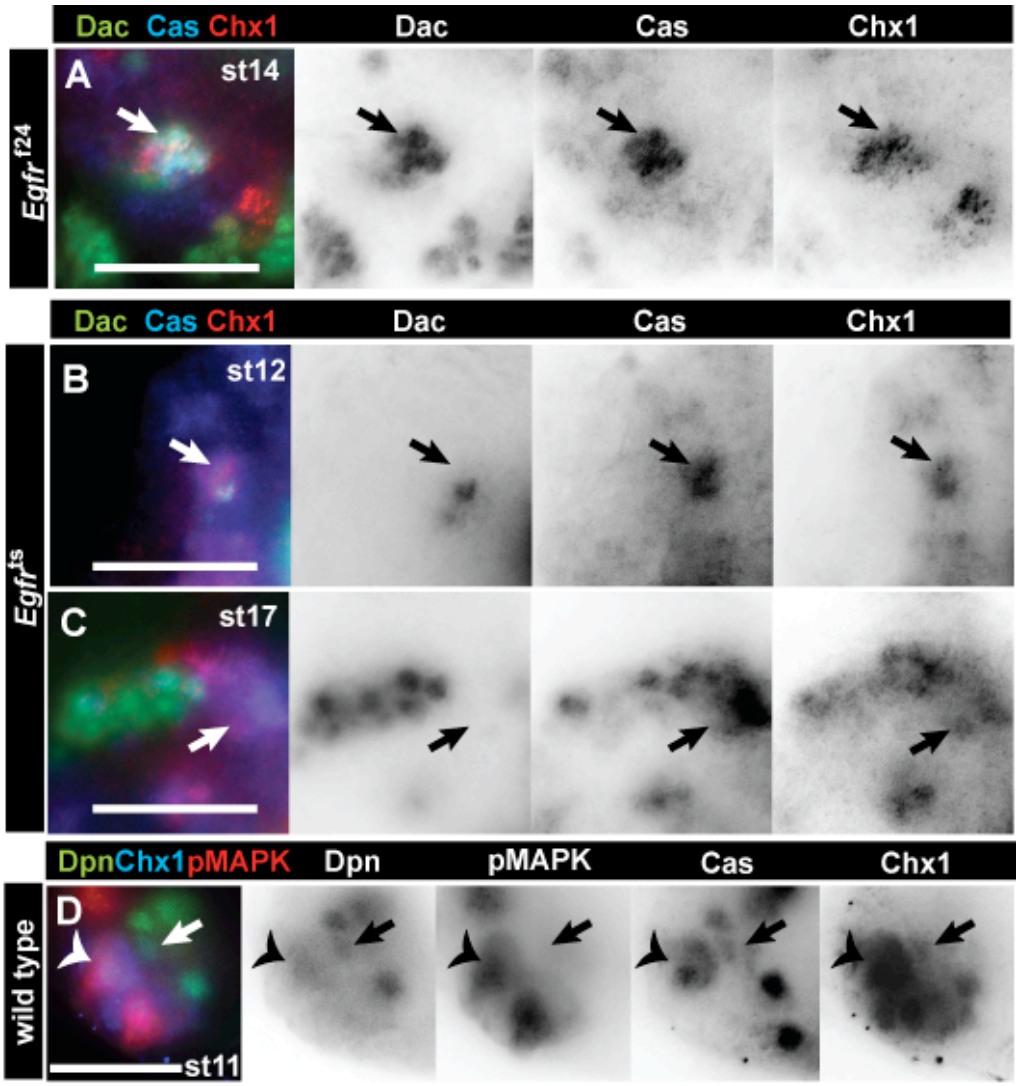


Figure 5-2. Spi mediates Egfr-dependent cell survival of the pPlm. (A) *spi*[1] mutants show a comparable phenotype to the *Egfr*[f24] mutants. (B-C) Absence of cell death (CC3+) in the pPl (arrows) of early stage 11 embryos in both *spi*[1] mutants and control. The pPl is normal in size in both (B) the heterozygote control and (C) *spi*[1] embryos. (D-E) Cell death in heterozygous control and *spi*[1] embryos labeled by anti-CC3. pPlm do not express CC3 in controls (D, arrows mark Chx1- hemocytes adjacent to the pPl), but do label in *spi*[1] embryos (E, arrow). Scale bar: 20 μm

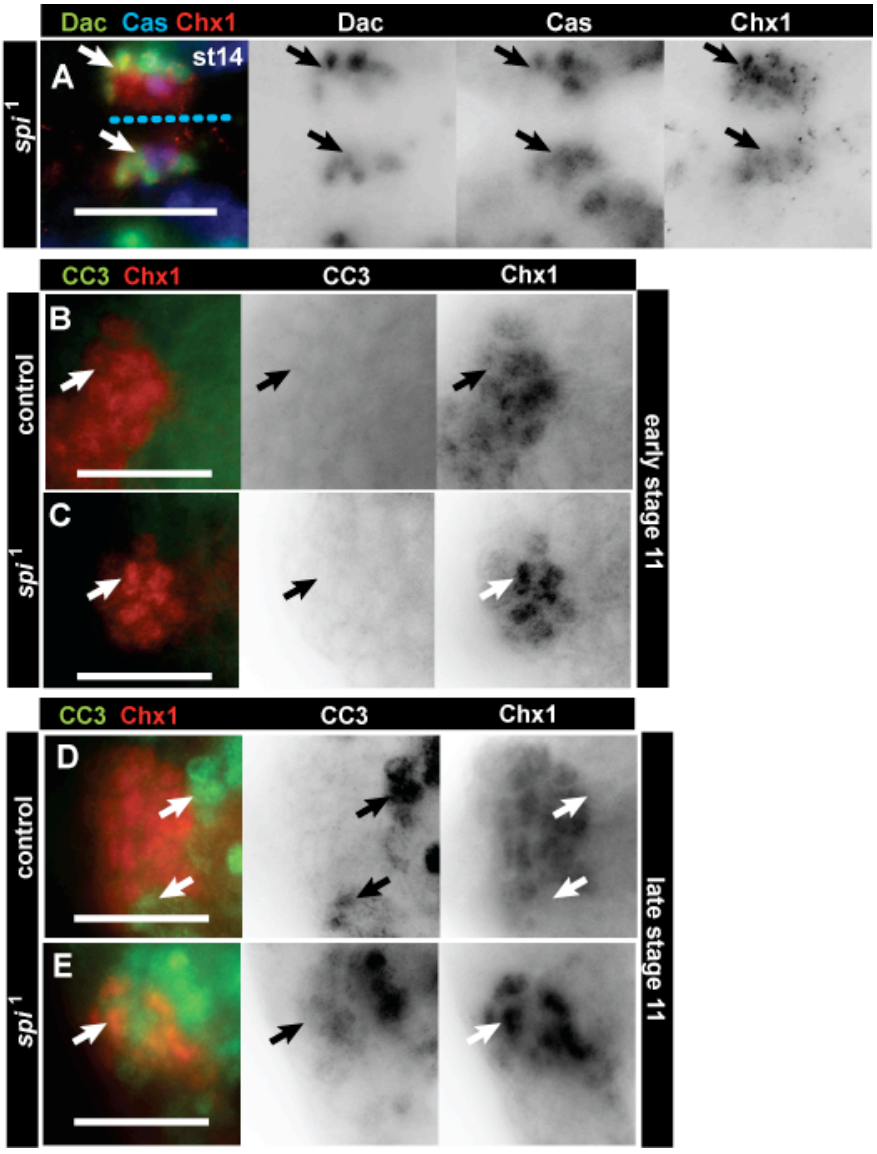


Figure 5-3. Other Egfr pathway components display similar phenotypes. (A-B) Stage 14 *rho*^{7m43} (A) and *rho*^{7m}*vn*^{Ry} (B) embryos had reduced pPIs (arrows), except for the Dac+ IPC NB lineage. (C) *spi*^{1} mutants still form an apical constriction by stage 11 (Crb accumulation). Blue dashed lines mark head midline. Scale bar: 20 μm

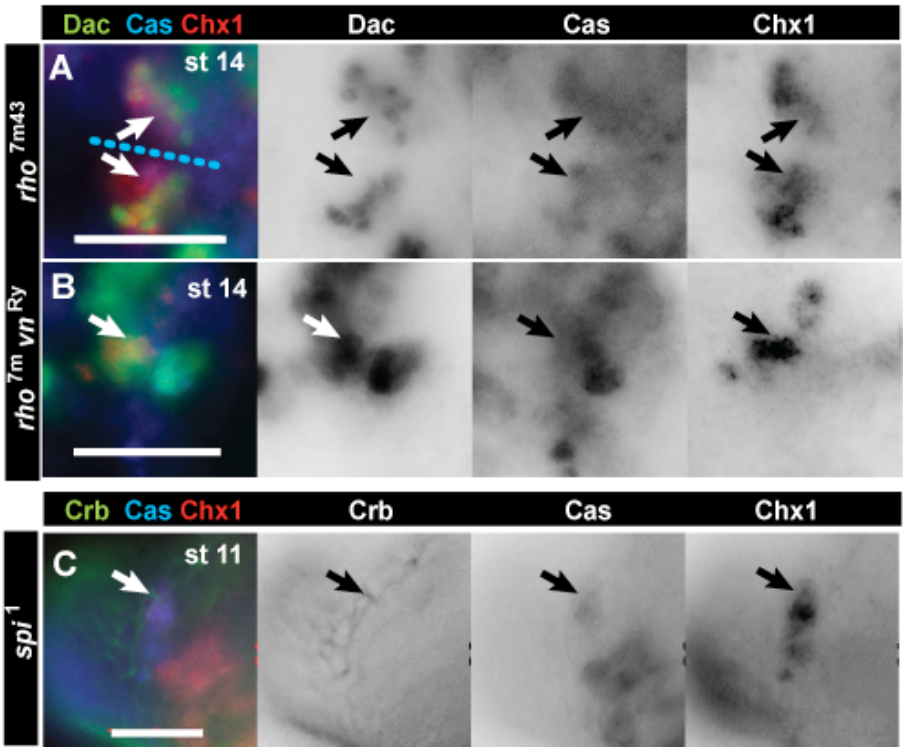
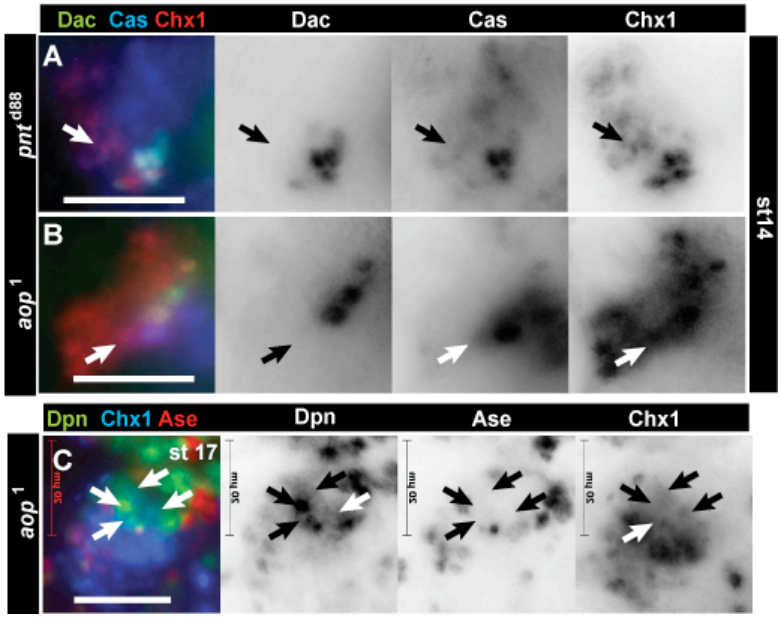


Figure 5-4. Pnt and Aop not involved in IPC NB specification, but may be required for Type II NBs. (A) Stage 14 *pnt*^{d88} embryos with a normal sized pPI (arrow). (B) stage 14 *aop*^{1} mutants specified the IPC NB lineage and the pPI was not reduced in size. (C) *aop*^{1} mutants produce extra Chx1+ pPI Dpn+ Ase- Type II NBs (arrows). Blue dashed lines mark head midline. Scale bar: 20 μm



Chapter Six: *Investigation of Notch/Egfr cross-talk in IPC NB cell fate determination*

Introduction

The pleiotropic activity of Notch, Egfr and other signaling pathways cannot be merely explained by the existence of different sets of effecter target genes. The temporal and spatial mechanisms that coordinate the function of these pathways must impart specificity to achieve a diverse array of cellular events (Sundaram 2005, Doroquez and Rebay 2006). In order to produce such a varied effect in development, it must necessarily involve synergy or interaction with other signaling pathways. Thus, signaling pathways cannot be considered in isolation. Rather, a more realistic picture of their function should take into account how these pathways interface with specific interactions that the cell integrates and integrates in a spatially and temporally appropriate manner.

6.1 Cross-talk between Notch and Egfr in development

There are many instances in development where Notch and Egfr cross-talk influence cell fate. In *Drosophila* the Egfr and Notch signaling pathways interact antagonistically or cooperatively over specific time and space (Doroquez and Rebay 2006). Mutual antagononism is the most common relationship, although there are multiple instances in development in which the two pathways cooperate to potentiate each other's signaling activity.

In the *Drosophila* eye much has been elucidated about the interaction of the Notch and Egfr pathways. Signaling paradigms have emerged from these studies, particularly during

Drosophila eye development (Doroquez et al., 2007, Cagan and Ready 1989, Freeman 1996). Egfr and Notch function cooperatively to promote the formation of the morphogenetic furrow (Kumar and Moses 2001). Egfr may play a parallel role with Notch lateral inhibition to focus R8 to a single cell within proneural clusters (Kumar et al., 1998, Frankfort and Mardon 2002, Hsiung and Moses 2002). Egfr and Notch are required for cell cycle regulation during eye formation. The Egfr pathway works in concert with Notch and other signaling pathways as well as a suite of pre-patterned transcriptional regulators to mediate specification of cell types in the developing disc. Cone cell development is dependent on inductive signaling through both Notch and Egfr (Fu and Noll 1997). They are also involved in regulating programmed cell death and the development of pigment cells.

Results

6.2 Cross-regulation of Notch and Egfr pathways in the pPIm

Our observation that the IPC NB was the only pPIm cell to survive led us to question whether the commitment to the IPC NB, or simply a NB fate alone, was sufficient to prevent pPIm cells from ever becoming dependent upon Egfr activity for survival. We tested this by analyzing double homozygous mutants for both Spi and Delta (spi^{1}; Dl^{RF}), a strong hypomorphic allele combination at 29°C. Simultaneous loss of both Egfr and Notch activity lead to a Notch phenotype with supernumerary IPC NBs produced at the expense of other pPIm cells (15/20 cases; Fig. 6-1A). In no example, where Egfr pathway activity alone was lost, did we observe a neurogenic effect, where NBs abnormally formed. Hence, we concluded that Notch signaling does not critically depend on Egfr activity to be maintained.

We then tested whether Notch activity was required to activate Spi/Egfr signaling. We found that *Notch* null hemizygote embryos were still able to activate Rho, as assayed by

the *rho* transcription reporter, *rho-lacZ* (Bier et al., 1990) (Fig. 6-1B). Additionally, in *Notch* null hemizygote embryos we also detected pMAPK in the pPIm (Fig. 6-1C). Conversely, the m8-GFP reporter of Notch activity was expressed in the pPI of $Egfr^{f24}$ mutant embryos (Fig. 6-1D). Thus, there was no clear evidence of interdependency between the two pathways, or for cross-talk that was essential for correct IPC NB specification.

6.3 Potential mediators of Notch and Egfr activity

We also examined mutants for *phyllopod* (phyl) and *charlatan* (chn), two factors essential for cell fate specification in the *Drosophila* eye and peripheral nervous system where they mediate cross talk between Notch and Egfr (Escudero et al., 2005, Dickson et al., 1995, Tsuda et al., 2006, Nagaraj and Banerjee 2009, Pi et al., 2004). Phyl acts to promote Notch activity and Chn, a fly homolog of the NSRF/REST repressor of neural fate, acts to repress *Delta* transcription (Doroquez and Rebay 2006). Interestingly, both *phyl* and *chn* are specifically expressed during the competence period in all Pdm placodes (Tomancak et al., 2002), though we did not resolve overlapping expression at the single-cell level. We found that both $phyl^{P245}$ (Pi et al., 2004) and chn^{ECJ1} (Escudero et al., 2005) homozygous null embryos specified the IPC NB normally and there were no observable defects in cell survival or neurogenesis that would indicate significant deviation in Egfr or Notch activity (8/8 cases for *phyl* and 10/10 cases for *chn*; Fig. 6-2B and Fig. 6-2C; wild type Fig. 6-2A). Moreover, Phyl, an ubiquitin ligase adaptor, is essential for Notch-mediated induction of photoreceptor R7 fate where it targets the transcriptional repressor, Tramtrack (Ttk) for degradation. Loss of Ttk activity relieves Delta from repression and leads to Notch activation (Tsuda et al., 2006, Tsuda et al., 2002). We further observed the same lack of IPC NB defects in homozygous null ttk^{1e11} embryos (Xiong and Montell 1993) (8/8 cases; Fig. 6-2D). Together,

the results indicated that Egfr and Notch need not cross talk though Phyl, Ttk or Chn to specify the IPC NB identity.

Figure 6-1. Notch and EGFR in the pPlm are not mutually dependent. (A) Stage 13 *spi*[1]; *Dl*[RF] double mutants with a normal sized pPlm, supernumerary IPC NBs (arrow). (B-C) EGFR activity in the pPlm (arrows) persists despite loss of Notch in *N*[55e11]/Y hemizygotes. (B) Expression of *rho-lacZ* reporter, a reporter of Spi/EGFR activity. (C) pMAPK expression. (D) The *m8-GFP* reporter of Notch activation was activated in the pPl of *Egfr*[f24] mutant embryos. Scale bar: 20 μm

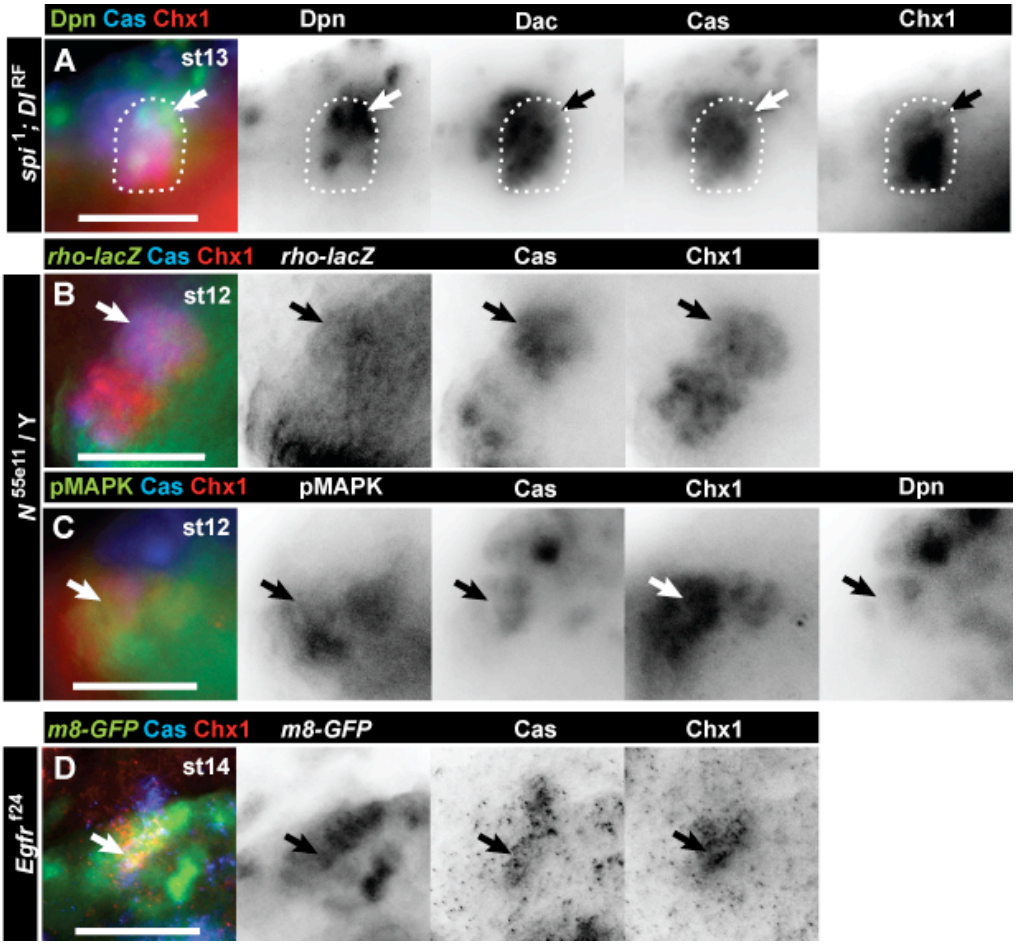
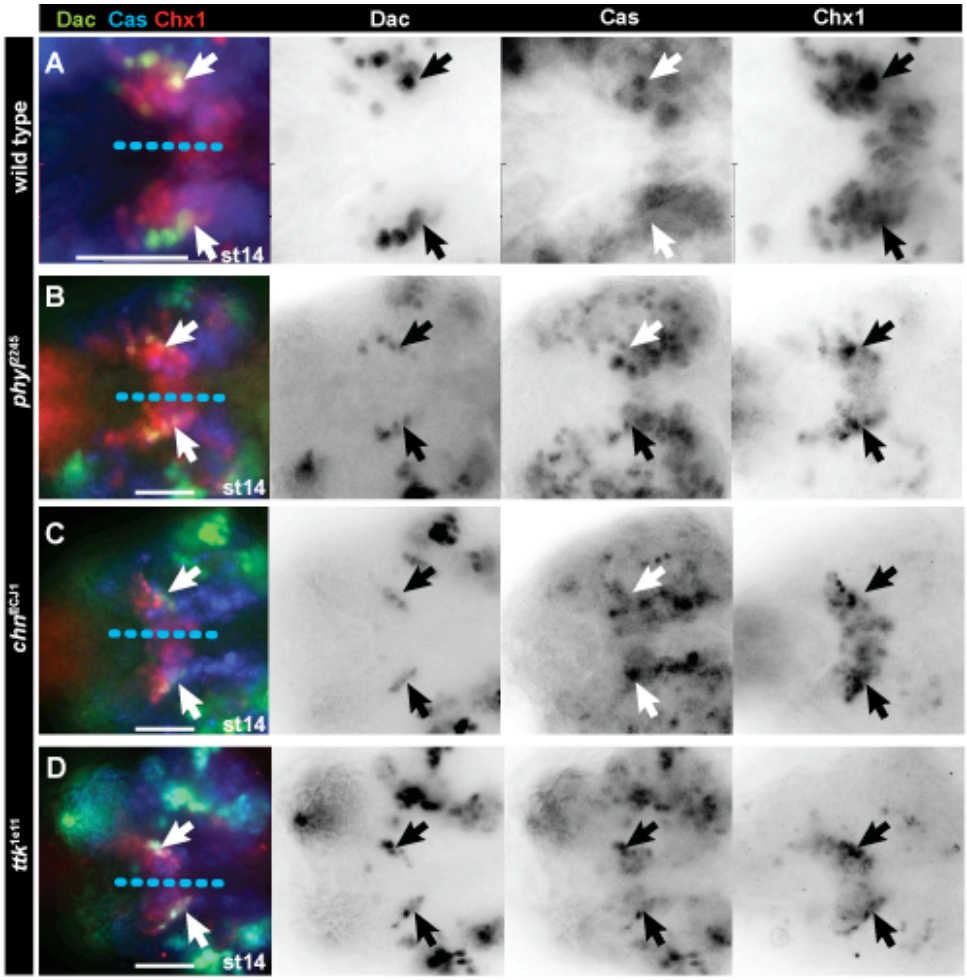


Figure 6-2. Phyllopod, Charlatan and Tramtrack are not required for specification of the IPC NB or survival of the Pdm NE. Blue dashed lines indicate head midline. (A-C) Stage 14 mutant embryos specify the IPC NB (arrows). Genotypes as follows: (A) wild type; (B) *phyl*^{2245}; (C) *chn*^{ECJ1} and (D) *ttk*^{1e11}. Scale bar: 20 μm



Chapter Seven: *Regional specification in the Pdm*

Introduction

How are different regions of the CNS patterned to generate the diversity of cell types? Regional specification in the brain and spinal cord play an important part in the final distribution of cell fates in the correct spatial orientation. Embryonic patterning begins with the etablisment of the A/V and D/V axes which are coordinated by a concentration gradient or secreted morphogenetic molecules, including Hedgehog (Hh) and Decapentaplegic (Dpp) signaling. Signals from morphogen molecules are translated into transcription factor codes for regional specification, which delineate different proteins. These in turn regulate different phases of neuronal development to generate different cell types in each brain region. The presence of prepattern factors that compartmentalize the brain and predispose regions to certain fates prior to specification has been proposed as a mechanism to achieve regional specification.

Aspects of regional specification are evolutionarily conserved for the central nervous system and anterior ectodermal placodes (Wang et al., 2007, Lichtneckert and Reichert 2008), both contributors to the head neuroendocrine system (Kawamura et al., 2002, Markakis 2002, Whitlock 2005). Details of specification mechanisms operating at a single cell resolution, however, are scant. We have previously discussed the idea of a common pre-placodal region, that is a contiguous stretch of the cranial ectoderm that is biased toward generation of placodal precursors. In this section we will explore the possibility that the pre-placodal region is further pre-patterned to support the development of distinct cell fates. We

examined the case of the pPIm and the neurosecretory IPC NB fate. To accomplish this we examined the transcription factors and signaling molecules that influence development of the Pdm and examined their role in the pPIm and in IPC NB specification.

Results

7.1 Regional specification of a prepattern in the Drosophila *anterior placode system*

Our results support a model where the pPIm cells are the only cells of the Pdm NE that harbor the developmental potential to produce IPC lineages. We hypothesized that the IPC NB lineage identity could be conferred by the combinatorial activity of transcription factors and growth factor signals whose regulatory activities intersect within the pPIm to define a unique regulatory state, or "prepattern" for the placode equivalence group. By virtue of their intersecting expression in the pPIm, Chx1 and Cas were obvious candidates for prepattern factors. We examined *Chx1*$^{\Delta 23}$ homozygous null mutant embryos that expressed a N-terminal fragment of the Chx1 protein localized to the cytoplasm, which permitted us to follow the fate of the Chx1+ cells (Erclik et al., 2008). We observed that the pPIm was specified but the IPC NB was not specified (10/10 cases; Fig. 7-1B with control in Fig. 7-1A). The IPCs were also absent from the PI of *Chx1*$^{\Delta 23}$ mutant first instar larval brains (Fig. 7-1D; compare to control in Fig. 7-1C). Furthermore, no Dimm+ NSCs formed at the position of the bilateral 10-12 Dac+ IPC NB lineages in *Chx1*$^{\Delta 23}$ mutants (5/5 cases; Fig. 7-1D; compare to control in Fig. 7-1C). Together, these results indicate that Chx1 is essential for specification of the IPC NB identity and for development of any Dimm+ NSCs from the pPIm. Curiously, the Dac+ NSC lineage from the adjacent PI placode, which was positioned at the anterior tip of the Chx1+ domain, was specified in *Chx1*$^{\Delta 23}$ mutants (Fig. 7-1B, carets), suggesting that the role of Chx1 as a prepattern determinant may be essential

only for the pPIm placode. In contrast, *cas*[24] homozygous null mutant embryos (Cui and Doe 1992) specified the IPC NB normally (7/7 cases; SFig. 7-1E). Thus, while Cas a definitive marker of the pPIm, it was not an essential regulator of IPC NB identity.

7.3 Regional specification of a prepattern in the Pdm: Growth factor signals

In addition to intrinsic prepattern factors, we tested whether growth factor signals previously implicated in the patterning of the PI and head midline epidermis were essential for regional specification of the pPIm and IPC NB identity. The TGF-β signal Dpp is secreted at the dorsal midline of the head and is essential for specification of the head midline epidermis (Chang et al., 2001). In the absence of Dpp, the brain hemispheres and the pPI, which are normally separated by epidermis, are fused at the dorsal midline and the pPI is expanded (de Velasco et al., 2007). When we examined severe loss of function *dpp*[h46] homozygous mutant embryos (Irish and Gelbart 1987) that reached stage 14 and initiated head involution, we found that both the pPIm and the single IPC NB were specified. Even in the absence of Dpp, the IPC NB had proliferated to form a small group of Dac+ Cas+ Chx1+ cells, however these IPC NB lineages formed at the dorsal midline in what appeared to be an immediately adjacent bilateral pair of lineages (5/5 cases; Fig. 7-2A). Hh signaling is also essential for specification of the dorsal midline epidermis and its loss causes a reduction in the size of the brain (Chang et al., 2001). We found that like the *dpp* mutant phenotype, *hh*[21] homozygous null mutant embryos (De Velasco et al., 2004) specified the pPIm and the single IPC NB (6/6 embryos; Fig. 7-2B). These results indicate that Dpp and Hh signaling were not essential for regional specification of the pPIm prepattern.

Figure 7-1. The pPlm prepattern. Blue dashed lines mark the head midline. Arrows identify the IPC lineage. (A-D) At stage 14, the Dac+ IPC NB lineage is not specified in *Chx1*^{A23} mutants (B, arrows), compared with the heterozygote control (A, arrows); the Chx1 protein is truncated at the C-terminal end, but is still recognized by N-terminal specific antibody, which labels the pPI. (C-D) First instar larval brain with Dac+ Dilp2+ IPCs were not formed in *Chx1*^{A23} mutants (D, arrows), but were formed in a heterozygous control (C, arrows). (E) The IPC NB lineage (arrows) is specified and proliferates in *cas*^{24} at stage 15. Scale bar: 20 μm

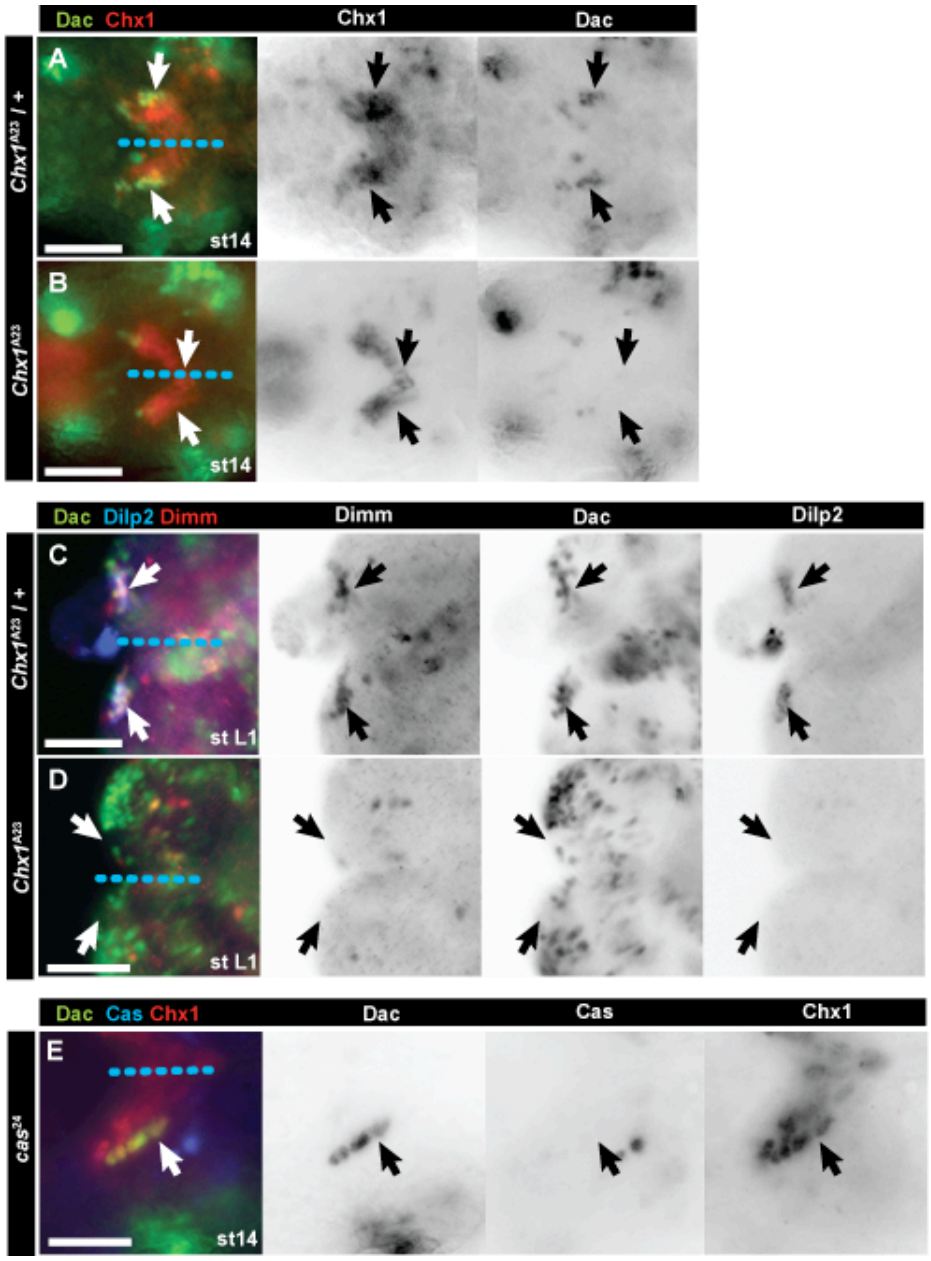
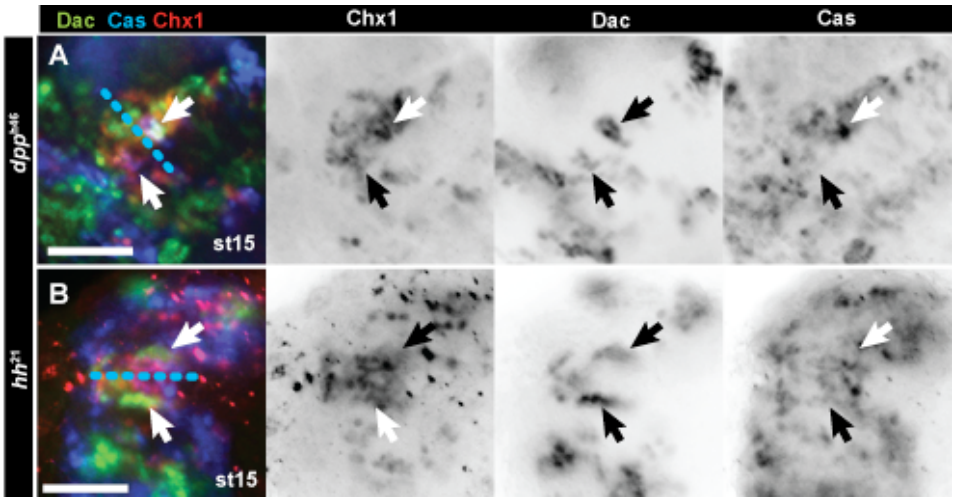


Figure 7-2. The pPlm prepattern:signaling. Blue dashed lines mark the head midline. Arrows identify the IPC lineage. The IPC NB lineage (arrows) is specified and proliferates in the following genotypes at stage 15 (A) *dpp*^{h46} and (B) *hh*^{21}. Scale bar: 20 μm



Chapter Eight: *Conclusions*

8.1 Summary of results

We investigated the role of Notch and Egfr signaling in specification of the IPC NB and other NB identities from a molecularly identified 8-cell Pdm placode corresponding to the pPIm. The embryo produces only one IPC NB per brain hemisphere, which generates an exclusively NSC lineage comprising the entire set of 6-8 IPCs in addition to several other unidentified NSCs (Wang et al., 2007). We find that the 8-cell pPIm produces diverse NB identities including the IPC NB, several Type I NBs for small cholinergic neurons and a single Type II, which are formed in that sequence, respectively. In the absence of Notch signaling all pPIm cells delaminate as IPC NBs, indicating that the pPIm begins neurogenesis as a fate equivalence group for IPC NB identity. In the absence of Egfr signaling all cells of the pPIm, except the IPC NB, are lost to cell death indicating that Egfr activity maintains cell survival in the remaining placode cells, which then allows for the specification of later-specified NB identities. In contrast, the absence of both Notch and Egfr activity allows all pPIm cells to survive but they acquire the IPC NB fate suggesting that the IPC NB identity, or NB fate in general, releases cells from the requirement of Egfr activity for survival. Furthermore, we find that loss of yan/aop function, which represents de-repression of Egfr/pMAPK target genes, causes an over-production of Type II NBs suggesting that regulation of the Egfr signal is also required for specifying later-born Pdm NBs properly (mutant phenotypes summarized in Fig. 8-1).

8.2 Key steps of placode development

Our observations thus provide a framework for understanding several key features of placodal neurogenesis in the Pdm; the steps in placode development are summarized as follows. (Diagrammed in Fig. 8-2). The Pdm NE placode, comprising roughly 8 cells, with its underlying gene regulatory network, appears to be highly specialized to serial specify a range of distinct neural stem cell identities, beginning from an initial state of equivalent developmental potential. In the case of the pPIm, the initial state of competence is to form IPC NBs. The first indication that the pPIm had acquired prepattern identity was the synchronized round of cell division we observed before placode morphogenesis; this synchronicity implied there was a coordinated development of equivalent cells from the 4-cell pPIm stage. After the prepattern cell expansion to the 8-cell stage, the cells entered a cell cycle arrest and formed a neurogenic placode; the pPIm then initiated a lengthy proneural competence period as the various NB identities were produced.

Our mutant and temperature-shift analysis of Notch signaling suggested that a window of competence for the IPC NB fate exists from the time the pPIm expanded to eight cells and acquired proneural competence until the time that IPC NB fate was normally specified (the time that it became Dac+). At roughly this point, the pPIm became dependent on Spi/Egfr activity to promote survival of NE cells not yet specified as NBs. This Egfr-dependent specification period then extended through stage 15, while alternative NB identities were specified. Neurogenesis then ended with specification of the last NB identity, which was the single Type II NB fate.

8.3 Parallels to photoreceptor R8 specification

Among the well-studied examples of *Drosophila* neurogenesis, perhaps the most intriguing parallels are found between the process of serial fate specification in the placodal

pPIm and in the developing facets of *Drosophila* retina, particularly between specification of the IPC NB and the specification of the R8 photoreceptor within each developing ommatidium (Kramer and Cagan 1994, Basler and Hafen 1991) (Summarized in Fig. 8-3B). The R8 photoreceptor is the first of a series of photoreceptor and cone cell fates to be specified by progressive recruitment to an apically constricted cluster of twelve cells (Frankfort and Mardon 2002, Hsiung and Moses 2002). Each R8 cell, the ommatidial founder, is specified from a proneural R8-fate equivalence group generated by the activity of bHLH factor, Atonal (Ato) (Jarman et al., 1994), and is singled-out by Notch mediated lateral inhibition (Cagan and Ready 1989). While parallel with respect to specification from a proneural fate-equivalence group, the pPIm NE required activity of the AS-C for IPC NB specification while Ato was not essential (data not shown). In contrast to R8 specification from a proneural group, the photoreceptor R1-7 and the cone cell fates are locally recruited within an ommatidium through inductive and serial Notch and Egfr/RTK signaling from R8 (Frankfort and Mardon 2002). Analogous to the pPIm and IPC NB specification, Egfr activity is not essential for proneural competence and specification of R8, but is essential for survival of all photoreceptor precursors, except for R8 (Dominguez et al., 1998). Egfr-mediated cell survival in the developing retina requires that the pathway activates MAPK by phosphorylation and that pMAPK in turn phosphorylates the pro-apoptotic factor Hid (Bergmann et al., 1998, Kumar et al., 1998).

With normal Egfr signaling, phosphorylated Hid is targeted for degradation, which permits survival of the developing ommatidium (Bergmann et al., 1998); cell survival is also promoted by the activity of Pnt1/2, which represses Hid expression (Kurada and White 1998). It was previously reported that hid mRNA accumulates in a pan-placodal Pdm NE

pattern in stage 12-13 embryos (Grether et al., 1995, Tomancak et al., 2002), yet we found that Pnt1/2 was not obviously essential for survival to stage 14 (Fig. 5-4A). This suggests that in contrast to the retina, most of the anti-apoptotic activity of Egfr signaling was relatively independent of Pnt1/2; hence, it may act primarily through the action of pMAPK on turnover of Hid. This hypothesis will need to be more fully tested in future studies.

8.4 Notch and Egfr function in the pPIm placode

While there are many examples of systems where Notch and Egfr either antagonize each other's activity or cooperate to promote cell fate decisions (Doroquez and Rebay 2006, Sundaram 2005), we found no evidence of a mutual dependence between Notch and Egfr activity states; either pathway became active in the absence of the other's activity. However, from our experiments we could not definitively rule out all cross talk between the pathways. Hence, the specification of the IPC NB and the R8 photoreceptor do not appear to rely on cross talk between Notch and Egfr pathways because they do not strictly require Egfr activity. In contrast, other instances of neurogenesis in *Drosophila*, such as in the optic lobe (Yasugi et al., 2010) and notum macrochaete (Culi et al., 2001, Escudero et al., 2005) depend on Egfr activity to promote the neurogenesis by activating AS-C genes. In these contexts the Egfr dependent proneural state is antagonized by Notch activity. In the pPIm proneural region, it remained unclear from our experiments if EGFR is essential for neurogenesis subsequent to the IPC NB because the pPIm cells are lost with the loss of Egfr activity. However, it remained a possibility that Egfr activity was required to help extend the long Pdm NE proneural period (Dumstrei et al., 1998). Indeed, there is a potential parallel to neurogenesis of the abdominal chordotonal precursors, which do form in the absence of Egfr activity but then signal back to the epithelium via Spi to activate *ato* and extend

neurogenesis, thereby recruiting additional chordotonal precursors (zur Lage et al., 2004, zur Lage and Jarman 1999, Lage et al., 1997).

In conclusion, the parallels between serial neural fate specification in the Pdm placode and in eye development raise the interesting possibility that some aspects of the two underlying gene regulatory networks may have points of overlap. If so, it is intriguing to consider whether distinct regions of proneural epithelia that express the "placode genes" were derived in evolution from a common ancestral neuroepithelial patterning circuit that was capable of serial specification. If this were the case, the implication would be that this mode of neurogenesis, where diverse neuronal or neural stem cell identities are generated through local interactions in prepatterned cell groups, is more widely distributed across animal phylogeny and vertebrate species. Hence, a deeper understanding of such a neural stem cell diversification mechanism will certainly aid efforts to control differentiation of specific neural progenitor fates *in vitro*.

Figure 8-1. Summary of mutant phenotypes. Phenotypes are as labeled with the events of normal development aligned in the top row.

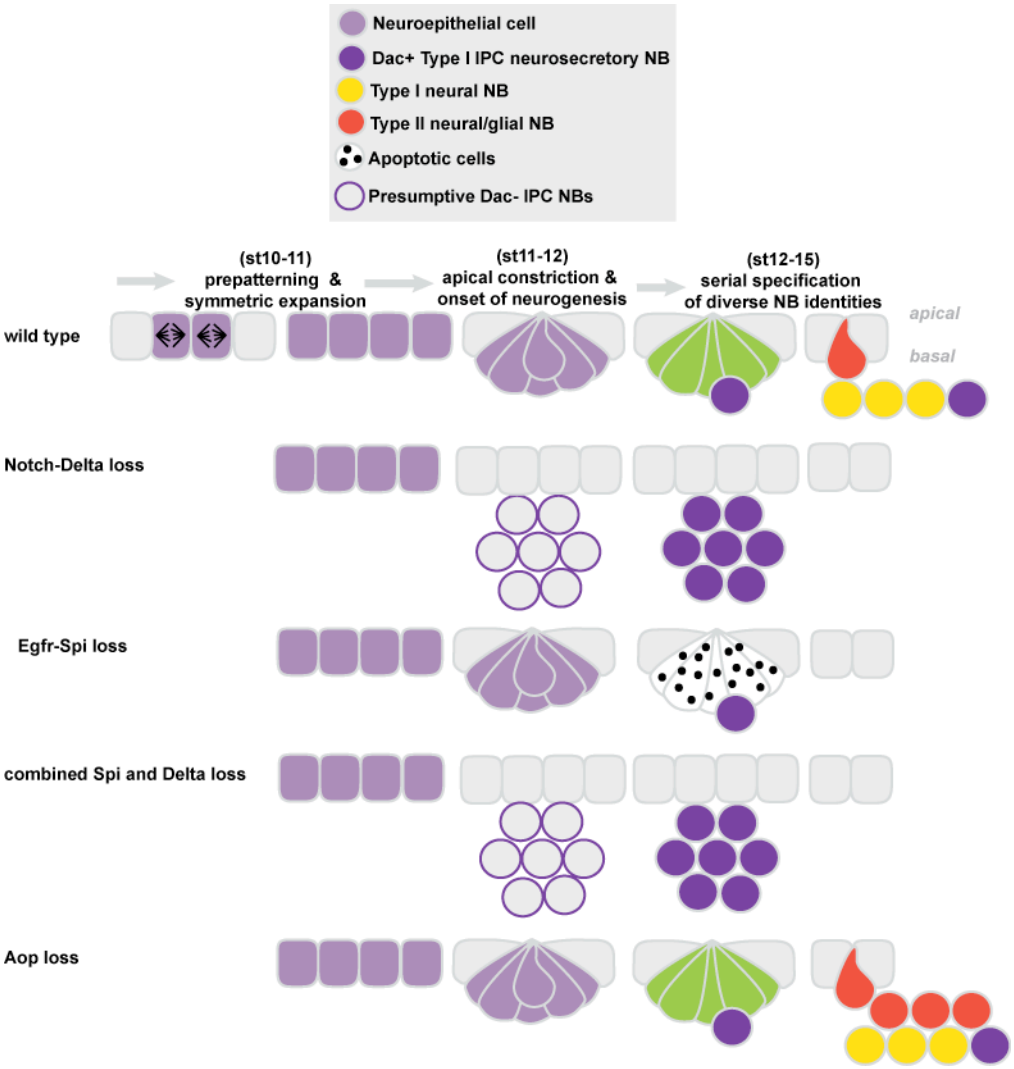


Figure 8-2. Model for serial specification of NB identities from the pPlm. Temporal progression of major developmental events in the pPlm is displayed from left to right, with corresponding stages indicated (see Discussion for details). Various cell types are color-coded (see legend). The blue bar designates the extended period of proneural competence seen in the Pdm NE. The purple bar designates the period during which pPlm cells are competent to take the IPC NB fate. The green arrow designates the period following IPC NB specification where the pPlm NE is dependent on Egfr activity for survival.

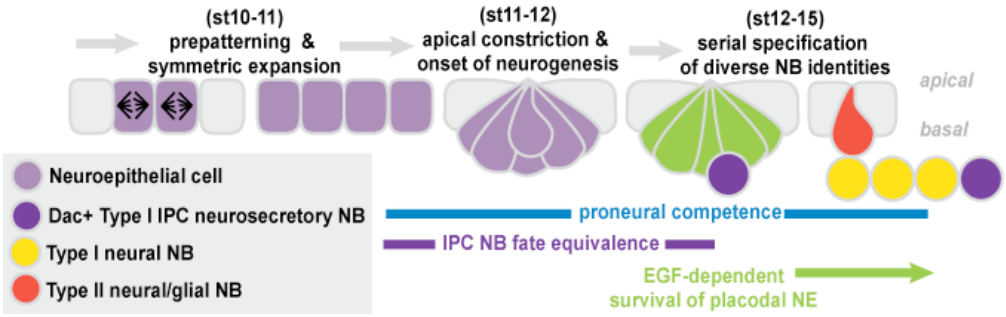
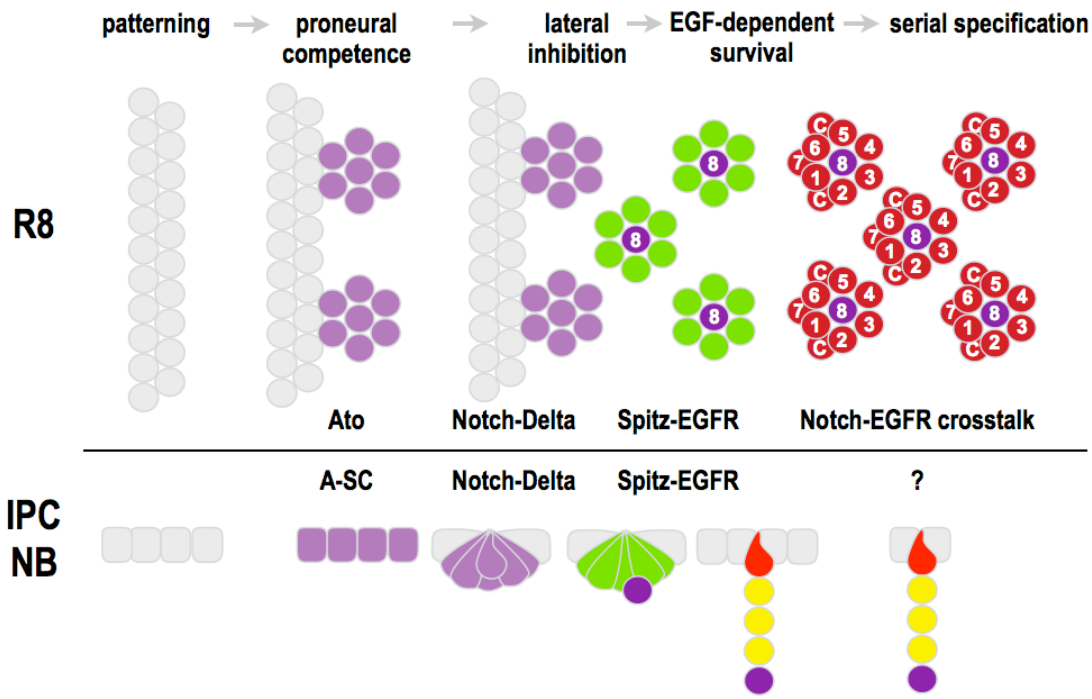


Figure 8-3. Parallels between R8 and IPC NB specification. Parallels between R8 and IPC NB specification.



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