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Developmental Cell
Review

Programmed Cell Death in Neurodevelopment

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Programmed cell death (PCD) is an evolutionarily conserved contributor to nervous system development. In the vertebrate peripheral nervous system, PCD is the basis of the neurotrophic theory, whereby cell death results from a surplus of neurons relative to target and competition for neurotrophic factors. In addition to stochastic cell death, PCD can be intrinsically determined by cell lineage or position and timing in both invertebrate and vertebrate central nervous systems. The underlying PCD molecular mechanisms include intrinsic transcription factor cascades and regulators of competence/susceptibility to cell death. Here, we provide a framework for understanding neural PCD from its regulation to its functions.

Cell death is inevitable, just as death of the individual organism, and is a central element of the multicellular society underlying metazoan life (Raff, 1992). During development and homeostasis, cells die physiologically and spontaneously. This phenomenon has been termed programmed cell death (PCD) or naturally occurring cell death. PCD was first described in the middle of the 19th century, the dawn of cell biology (Maghsoudi et al., 2012). The physiological significance of cell death in development has been systematically researched for over 60 years (Glücksmann, 1951). The most prominent and best characterized example of the involvement of cell death in development is described by the neurotrophic factor hypothesis, which is based on extensive findings in the peripheral nervous system (PNS) in which neurons are overproduced and their survival depends on competition for limited amounts of survival-promoting factors produced in target tissues (Cowan, 2001; Levi-Montalcini, 1987; Oppenheim, 1991; Purves et al., 1988; Raff, 1992). This mechanism enables quantitative matching of neurons with their targets.

Studies of cell death were accelerated in the late of 20th century by the findings that the PCD is genetically determined, and that the machineries for PCD are evolutionary conserved in all metazoan organisms (Maghsoudi et al., 2012). Recent progress in modern experimental techniques, including precise genetic manipulation, lineage tracing, and real-time monitoring have allowed researchers to address the mechanisms, triggers, and biological significance of PCD. Many excellent comprehensive reviews have described essential roles of PCD as a mechanism for quantitative matching, sculpting, and deleting anatomical structures, regulating cell number, and eliminating error-prone or defective cells (Buss et al., 2006; Fuchs and Steller, 2011; Yeo and Gautier, 2004). One of the exciting findings, for instance, is that dying cells can actively affect the behaviors of neighboring cells both in invertebrate and vertebrate animals (Bergmann and Steller, 2010; Morata et al., 2011). However, many important and interesting questions are unanswered. Why do such a large number of cells die during development? What determines the decision between life and death in cells of developing embryos? Here, we review recent advances in the understanding of the regulation and functions of PCD in neural development.

PCD, Apoptosis, and Regulated Cell Death: Similar but Different

PCD is a widely observed and evolutionary conserved trait. As indicated by the operative phrase "programmed" cell death, the pattern and timing of cell death during development are strictly scheduled and tightly regulated (Lockshin and Williams, 1964, 1965). The concept that PCD depends on cell differentiation program was suggested by early work in insects and chicken (Lockshin and Williams, 1964; Saunders, 1966). Next came ground-breaking findings describing genetic control of PCD in *Caenorhabditis elegans*. It was determined that in *C. elegans*, 131 cells undergo programmed cell death, with 105 of them from the neuronal lineage (Ellis and Horvitz, 1986; Ellis et al., 1991; Hedgecock et al., 1983; Sulston and Horvitz, 1977). Surprisingly, worms lacking all PCD develop normally and appear healthy, suggesting that PCD itself is not essential for the survival of the nematode, at least in laboratory culture conditions (Ellis and Horvitz, 1986). In contrast, both flies and mice that lack pro-apoptotic genes exhibit lethality and many defects in various organs, including the CNS (Buss et al., 2006; Miura, 2012; Oppenheim, 1991; White et al., 1994). Thus, PCD must play a pivotal role in constructing a functional nervous system in development.

Apoptosis is the major form of PCD in the developing nervous system. Apoptosis was originally defined as a form of cell death characterized morphologically by chromatin condensation, DNA fragmentation, intact plasma membranes at its early phase, and cell shrinkage and/or fragmentation (Kerr et al., 1972). Apoptosis is executed by members of an evolutionarily conserved family of cysteine proteases called caspases, which cleave a wide range of cellular substrates (Degterev and Yuan, 2008). Executioner caspases, including Caspase-3/7, are physiologically activated by extrinsic death ligands or intrinsic signals such as DNA damage, survival factor deprivation, ER stress, abnormal ion flux, or reactive oxygen overproduction (Green et al., 2014) (Figure 1). In fact, the term PCD has been often used to indicate apoptosis

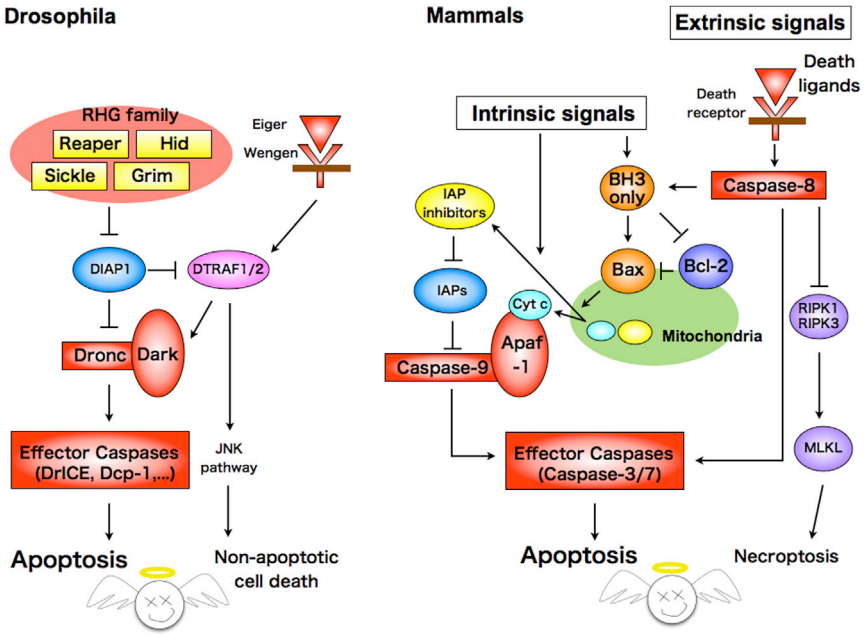

Figure 1. Pathways Leading to Apoptosis and Other Regulated Cell Death Processes

Schematic comparison of fly and mammals molecular pathways leading to apoptosis by effector caspase activation. RHG, Reaper, Hid, Grim, and Sickle; all of which act as DIAP1 inhibitors. IAPs, Inhibitors of Apoptosis; DIAP1, *Drosophila* IAP-1; and DRONC, *Drosophila* Nedd2-like Caspase, which is a counterpart of mammalian caspase-9. Dark, *Drosophila* apaf-1-related-killer, which is a counterpart of mammalian apaf-1. DrICE and Dcp-1, *Drosophila* ICE(Interleukin-1 Converting Enzyme) and Death caspase-1, which are considered to be fly counterparts of mammalian caspase-3. Dtraf1, *Drosophila* TNF-receptor-associated factor 4. Eiger and Wengen are fly counterparts of mammalian TNF and TNF receptor, respectively. JNK, c-Jun N-terminal kinases. BH3 only, Bcl-2 homology 3-only proteins, including Bid, Puma, and Noxa, etc. Apaf-1, Apoptotic protease activating factor 1. Cyt c, Cytochrome *c*, which is released from mitochondria and triggers formation of apoptosome that consists of Cyt c, Apaf-1, and Caspase-9 and activates effector caspases. IAP inhibitors, Omi/HtrA2 and Smac/DIABLO, which are translocated from mitochondria in response to intrinsic death stimulus. Suppression of caspase-8 causes necroptosis via RIPK (receptor-interacting serine/threonine-protein kinase)1, RIPK3, and MLKL (mixed lineage kinase domain-like) in certain conditions.

induced by pathological conditions or toxic insults, because death in these cases relies on caspases, which are "installed" (i.e., "programmed") cellular protein components. However, as the original definition of "programmed" cell death is to indicate developmentally programmed (or naturally occurring) cell death in embryonic and postembryonic development, applying the term PCD to cell death observed in such pathological conditions is inaccurate. It has therefore been recently suggested that pathological cell death, which activates "programmed" gene cascades, should be denoted as "regulated cell death" (Galluzzi et al., 2012, 2014). "Regulated cell death" can include apoptosis, necroptosis (in other words, regulated necrosis), pyroptosis, netosis, ferroptosis, cornification, and autophagic cell death, all of which have distinct molecular mechanisms for executing cell death. The in vivo significance and regulation of these cell death processes is less understood and is now being actively explored.

Regulation of Developmental Apoptosis

An Intrinsic Death Timer

Scheduled PCD depending on cell lineage was clearly shown in *C. elegans* (Sulston and Horvitz, 1977). Is this also the case in other animals such as insects and vertebrates that have significantly larger numbers of cells? Classic studies proposed the existence of a "ticking death timer" coupled with cell lineage determination within cells or tissues. For example, cells undergo death in isolated chicken limb buds in culture according to specific time line, which corresponds to the time schedule of in vivo development (Maghsoudi et al., 2012; Saunders, 1966). A similar developmental scheme was recently observed in the mammalian brain. During postnatal mouse development, about half of γ-aminobutyric acid (GABA)-secreting interneurons, which are marked by GAD67 expression, undergo BCL2-associated X protein (Bax)-dependent apoptosis (Figures 1 and 2A). Intriguingly, interneuron precursors cultured in vitro or transplanted into the cortex was found to die at a cellular age similar to that at which endogenous interneurons die during normal development (Song et al., 2012; Southwell et al., 2012) (Figure 2A). A constant percentage of cells die intrinsically, irrespective of the number of transplanted cells, suggesting that the cells die in a cell-autonomous or a population-autonomous manner. Thus, developmental cell death in cortical interneuron precursors is apparently regulated by or coupled with a "ticking" intrinsic developmental timer either within the cells themselves or the population. However, the underlying mechanism has yet to be determined.

Timing by Transcription Factor Expression

Recent studies of neuroblasts (NBs) in the *Drosophila* CNS have revealed the molecular basis underlying one such "ticking" developmental timer, and its correlation with cell death in neural development (Homem and Knoblich, 2012; Kohwi and Doe, 2013). NBs resemble mammalian neural stem cells in that they sequentially generate progenitor cells with different fate potentials, because these progenitors initially produce a specialized subset of neurons and later produce glia. The embryonic *Drosophila* CNS can be subdivided into the cephalic region, brain, and the thoracic/abdominal region, ventral nerve cord (VNC). During embryonic neurogenesis, the NBs asymmetrically divide and generate one self-renewing NB and another daughter cell to produce all embryonic and larval neurons. At present, patterns of NBs division are classified into several types, including type 0, type I, and type II (Figure 2B) (Baumgardt et al., 2014; Homem and Knoblich, 2012; Kohwi and Doe, 2013). Most NBs in the abdominal region die by the end of embryonic stages. In contrast, NBs in the cephalic and thoracic regions arrest their cell-cycle and enter a G0-like quiescent state and reinitiate division during the late first instar stage to form the adult CNS (Cheng et al., 2011; Homem and Knoblich, 2012; Sousa-Nunes et al., 2011). One-fourth of the neuronal cells, which are generated from dorsomedial (DM) amplifying type II NBs, undergo apoptosis

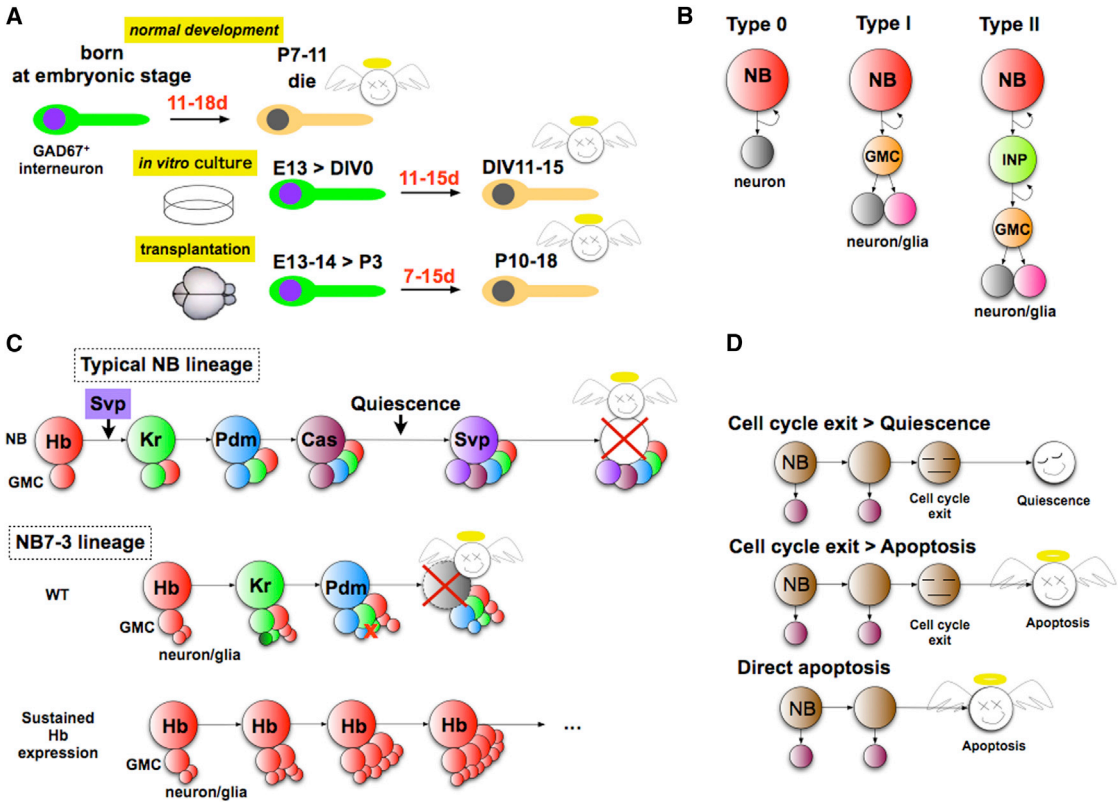

Figure 2. Apoptosis Governed by Intrinsic Developmental Clock

(A) Intrinsic timer for cell death of interneurons in mouse cerebral cortex. GAD67$^+$ interneurons that are cultured in vitro or transplanted into embryonic brain undergo cell death within a specific time window.

(B) Types of NBs in *Drosophila*. In type 0 NB division, the daughter cell directly differentiates into neurons. Type I NBs divide asymmetrically and generate one self-renewed NB and a daughter ganglion mother cell (GMC) that can divide only once to produces two post-mitotic cells, which can be either neurons or glia. Type II lineage NBs, which are observed in posterior regions of the brain, generate an NB and a transit-amplifying cell (intermediate neural progenitor, INP). INPs generate another INP to self-renew as well as a GMC, thereby generating a larger population of neural cells in the central brain (Bello et al., 2008).

(C) A temporal series of transcriptional factor expression in NBs. Typical NBs lineage in the VNC exhibits the expression of a series of transcription factors at progressive embryonic stages: First Hunchback (Hb), then Kruppel (Kr), then the co-expressed POU domain proteins Nubbin and Pdm2, which are together designated as Pdm, and then the zinc-finger transcription factor Castor (Cas). Orphan-nuclear receptor protein Seven up (Svp) is necessary for the transition from Hb$^+$ to Kr$^+$. NB7-3 lineage undergoes apoptosis after Pdm expression, leading to lineage-stop. One of the daughter cells from Kr$^+$ GMC cells also dies by apoptosis in a programmed manner. Sustained Hb expression abolishes this cell death event.

(D) Three lineage-stop mechanisms observed in NBs. DIV, days in vitro; NB, neuroblast; GMC, ganglion mother cell; INP, intermediate neural progenitor.

during the pupal stage. Therefore, the arrest of a lineage (lineage-stop) by apoptosis at a specific developmental time point is crucial for the establishment of an appropriate number of cells in the nervous system. Recent studies have revealed that both generation of a variety of neurons and glia with distinct identities from a single NB lineage and apoptosis of those cells at a specific timing are regulated by an intrinsic developmental ticking program, namely transcription factor switching (Figure 2C).

Transcription factor switching is a phenomena that distinct sets of several transcription factors are expressed in a temporal series in NBs and their progeny inherit the transcription factors expressed by the mother NBs (Figure 2C) (Homem and Knoblich, 2012; Isshiki et al., 2001; Maurange et al., 2008). Studies indicate that the cell identity defined by the temporal transcription switching also determines the dying fate of NBs and its progeny, although its timing and pattern vary among NB lineage. For instance, in the well-studied NB7-3 lineage, Ikaros family zinc-finger transcription factor Hunchback (Hb$^+$) NBs generate a neural progenitor, GMC, which then produce two daughter cells. Both of the daughter cells are post-mitotic motor neurons that survive. On the other hand, the zinc-finger transcription factor Kruppel (Kr$^+$) NBs produce a GMC that generates two post-mitotic interneurons, one of which undergoes apoptosis (Karcavich and Doe, 2005). Interestingly, the continuous forced expression of Hb$^+$ NBs results in progeny that have the characteristics of the Hb$^+$ lineage and therefore do not undergo apoptosis (Isshiki et al., 2001; Karcavich and Doe, 2005) (Figure 2C). Thus, apoptosis of the progeny of NB7-3 Kr$^+$ NBs depends on cell identity, which is governed by the switch of expression from Hb$^+$ to Kr$^+$.

Overproduction of neural cells occurs if the NB lineage-stop fails. In addition to inhibition of apoptosis by alternation of NBs identity by mutation in genes involved in temporal transcriptional series as mentioned previously (Figure 2C), NB lineage-stop is also prevented by loss of cell-cycle inhibitor Dacapo, a fly ortholog of mammalian p21CIP1/p27KIP1/p57KIP2 (Baumgardt et al., 2014). Thus, it is proposed that there are three mechanisms for "NB lineage-stop": (1) cell-cycle exit leading to quiescence, (2)

cell-cycle exit leading to apoptosis, or (3) direct induction of apoptosis (Baumgardt et al., 2014) (Figure 2D).

Several lines of evidence imply that an intrinsic developmental timer-dependent lineage-stop by cell death or quiescence is also important in vertebrates. Temporal series of gene expression cascades are also observed in the ordered production of neurons in the cerebral cortex, retina, hindbrain, and spinal cord in the vertebrate CNS development (Dehay and Kennedy, 2007; Kohwi and Doe, 2013; Okano and Temple, 2009). In the cerebral cortex, radial glia, the neural progenitor cells of the telencephalon, are initially neurogenic and then switch to a gliogenic phase during perinatal stages (Hirabayashi and Gotoh, 2010). Robust cell death has been suggested to shortly follow this neuron-glial switch (Takahashi et al., 1999). It will be interesting to examine if PCD in neural stem cells and their progeny in the postnatal cortex is intrinsically determined by the expression of transcription factors.

Triggers for PCD

Triggers that directly induce PCD during development are associated with a variety of developmental events. However, there are many examples of PCD for which direct triggers have not been specified. Triggers of PCD in nervous system development can be classified into several groups that are not always mutually exclusive: induction of death ligands and/or pro-apoptotic proteins, loss of survival signals, growth factor signaling, cell-cell interaction, and intrinsic transcription factor expression.

Upregulation of pro-apoptotic genes expression by transcription factors is commonly observed in invertebrates and vertebrates (Miguel-Aliaga and Thor, 2004). In *C. elegans*, the pro-apoptotic gene *egl-1* is upregulated in specific cells fated to die by PCD, and this upregulation is mediated by the transcription factors Eya1 and ceh-34, a six-family homeodomain gene (Hirose et al., 2010). In *C. elegans*, most of the genes known to control cell death specification encode transcription factors, some of which are known to directly regulate the transcription of cell death genes (Hirose et al., 2010). For instance, the induction of pro-apoptotic BH3-only proteins (e.g., Noxa, Puma) plays a role in activation of downstream apoptotic pathways in vertebrates (Chipuk and Green, 2008) (Figure 1). In the fly, the RHG pro-apoptotic family genes are transcriptionally upregulated and responsible for PCD, but their distinct roles in PCD vary, depending on cell type and context (Tan et al., 2011) (Figure 1). Considering that PCD is an evolutionary conserved trait, such cell-type-specific transcriptional regulation is hypothesized to exist in vertebrates. Indeed, in *Xenopus*, transcriptional activity of the homeobox transcription factor Barhl2 gene is necessary for induction of apoptosis in the prospective notochord and floor plate (Offner et al., 2005). Apoptosis is prevented by overexpression of Bcl2, but the mechanisms by which Barhl2 induces apoptosis are unclear.

Competence for PCD

Competence is defined as the ability of a cell or tissue to respond to a specific inductive signal and thereby opt for a specific cell fate. Competence can change as development proceeds. Several factors that are developmentally regulated are found to be essential for cellular competence for PCD. In the fly, regulation of NB apoptosis under the control of a temporal series of transcription factor-expression patterns is observed at the end of the larval stages, as well as during embryogenesis. In larval stages, repression of Dichaete (D, a SOXB family member), expression of Grainyhead (Grh), and repression of Castor (Cas) occurs sequentially in type II NBs (Maurange et al., 2008). Transient expression of Abdominal-A (AbdA) at the late larval stage induces the RHG pro-apoptotic family genes, causing apoptosis of NBs (Figure 3A). The competence to pulsed expression of AbdA for apoptosis induction depends on Grh, an evolutionarily conserved transcription factor. In the abdomen, Grh is necessary for apoptosis induction in two ways: first Grh maintains AbdA expression and second Grh is necessary for competence to AbdA expression-induced apoptosis (Almeida and Bray, 2005; Bello et al., 2003; Cenci and Gould, 2005; Maurange et al., 2008).

Competence to a specific signal that triggers apoptosis can vary depending on developmental context. For example, depending on the cell type, Notch can either induce neurons to die or allow them to survive. Activation of Notch triggers apoptosis of the vmd1a sensory organ lineage and supernumerary interomatidial precursors in *Drosophila* (Miller and Cagan, 1998; Orgogozo et al., 2002). In the developing antennal lobes of *Drosophila*, Notch triggers apoptosis in the antero-dorsal projecting neuron lineage (adPN), whereas it specifies ventral projecting neurons lineage (vPN) cells (Figure 3B) (Lin et al., 2010). Likewise, life-or-death decisions by Notch signaling are also observed in the developing *Drosophila* optic lobe (Figure 3B).

How is it possible for the same input (i.e., Notch levels) to regulate the binary decision of cell death or cell survival in opposing ways depending on cell lineage? Interestingly, determination of whether $Notch^{ON}$ or $Notch^{OFF}$ cells survive or die is strictly dependent in cell lineage, suggesting that pro-survival versus pro-death responses in Notch-activated cells are intrinsically determined by cellular identity. A recent study has elucidated the molecular mechanism governing competence to apoptotic triggers in the developing *Drosophila* optic lobe, wherein another transcriptional factor cascade determines cell survival versus cell death decision mediated by Notch signaling (Figure 3B). This differential response is achieved by the concerted action of Dll, Ey, and Slp, wherein NBs are preinstalled with two independent apoptotic programs, Notch-induced *reaper*-dependent apoptosis and Ey-induced *hid*-dependent apoptosis, which is inhibited in the presence of Dll and unknown factor(s) in the Ey time window. In the Slp/D time window, Ey induced *hid*-dependent apoptosis and Slp inhibited Notch-induced *reaper*-induced apoptosis (Figure 3C). Interestingly, it seems that these transcription factors independently contribute to distinct developmental events, such as the determination of neuronal identity, cell survival decisions, and the mode of cell division. This suggests that temporal dynamics in transcription factor expression determines the competence of Notch-mediated cell survival versus cell death decisions (Bertet et al., 2014).

Developmentally Regulated Susceptibility to Apoptotic Triggers

Susceptibility to apoptosis indicates the extent to which cells that receive either endogenous or exogenous pro-apoptotic stimuli undergo apoptosis. Susceptibility to apoptosis can differ among regions and developmental stages. For instance, in imaginal discs of the developing fly eye, susceptibility to the proapoptotic gene *hid* differs in a region-specific manner (Figure 3D). Cell fate in the eye imaginal disc is sequentially determined. Mitotic

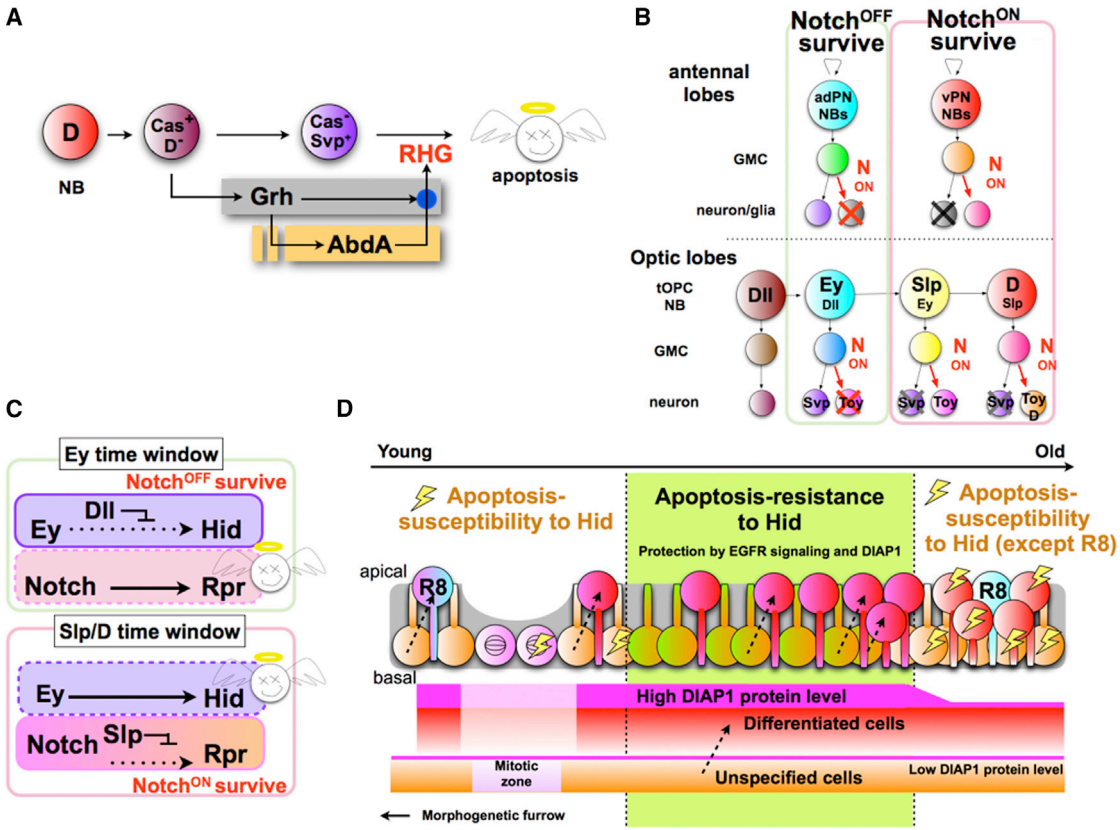

Figure 3. Mechanisms of Competence and Susceptibility to Apoptosis

(A) Competence to AbdA-induced apoptosis in NBs. Grh expression induced by Cas is necessary for maintaining AbdA expression and installing the competence to induce RHG expression by AbdA expression in NBs.

(B and C) Transcriptional factor cascades responsible for binary life-or-death decision by Notch signaling. (B) Schematic views of a temporal series of transcriptional factor expressions coupled with cell death in optic lobes and antennal lobes. In optic lobes, another temporal series of differential transcription factor expression governs the sequential production of distinct neuronal subtypes: first Distalless (Dll), then Eyeless (Ey), then Sloppy-paired (Slp), then Dichaete (D) (Bertet et al., 2014). During periods when Ey is expressed, NBs divide in type I mode to produce two daughter progeny. One of the daughters is positive for Notch signaling activity ($Notch^{ON}$), which induces *reaper* expression and apoptosis, and another is negative for the Notch signaling ($Notch^{OFF}$) and survives. In contrast, at the later periods, when transcription of Ey is shut off and Slp and/or D are expressed, the $Notch^{ON}$ daughter cell survives and the $Notch^{OFF}$ cell undergoes apoptosis. (C) Mechanisms for the binary fate decision in optic lobes. Two apoptotic pathways underlie the binary life-or-death decision.

(D) Differences in and mechanisms for the susceptibility to *hid*-induced apoptosis in developing eye disc. Dotted arrows indicate cell differentiation from unspecified cells to neurons. RHG, *reaper*, *hid*, and *grim*; tOPC, the tips of the outer proliferation center; N, Notch; adPN, antero-dorsal projecting neuron lineage; vPN, ventral projecting neurons lineage.

progenitor cells are extremely sensitive to the exogenously induced apoptotic signal, *hid* expression. On the other hand, differentiating but yet unspecified cells exhibit resistance to *hid* expression through epidermal growth factor receptor (EGFR) signaling, and differentiated photoreceptor neurons do so by accumulating the apoptosis inhibitory protein, DIAP1. Interestingly, developmentally older, matured photoreceptor cells degrade DIAP1 and become susceptible to apoptosis again. Remarkably, R8 photoreceptor cells develop a resistance mechanism to apoptosis that is distinct from DIAP1 accumulation and EGFR signaling (Fan and Bergmann, 2014). Developmental susceptibility to apoptosis is also regulated in *Drosophila* by microRNAs or epigenetic modification of chromatin (Hilgers et al., 2010; Zhang et al., 2008). In mammals, it is proposed that the susceptibility to neuronal apoptosis is controlled at the various levels: protection by growth factor signalings, the amount of IAP protein, redox regulation of cytochrome *c*, and downregulation of the apoptosome machinery and executioner caspases during differentiation and aging (Braunger et al., 2013; Donovan and Cotter, 2002; Donovan et al., 2006; Ohsawa et al., 2009; Stoka et al., 2006; Vaughn and Deshmukh, 2008; Wright et al., 2004, 2007). Thus, distinct mechanisms that account for differential susceptibility to apoptotic triggers exist in a differentiation state and cell-type-specific manner.

Function of Developmental Apoptosis in the Nervous System

Morphogenetic Movement Dynamics

Based on histological observations, it has long been suggested that PCD is involved in morphogenesis (Teng and Toyama, 2011). In amphibian and amniote development, many cells die by apoptosis in the boundary regions between the neural plate and non-neural ectoderm during and after the neural tube closure (NTC), a dynamic process through which the neural plate bends and fuses to form the neural tube and brain (Hensey and Gautier, 1998; Nonomura et al., 2013; Yamaguchi and Miura,

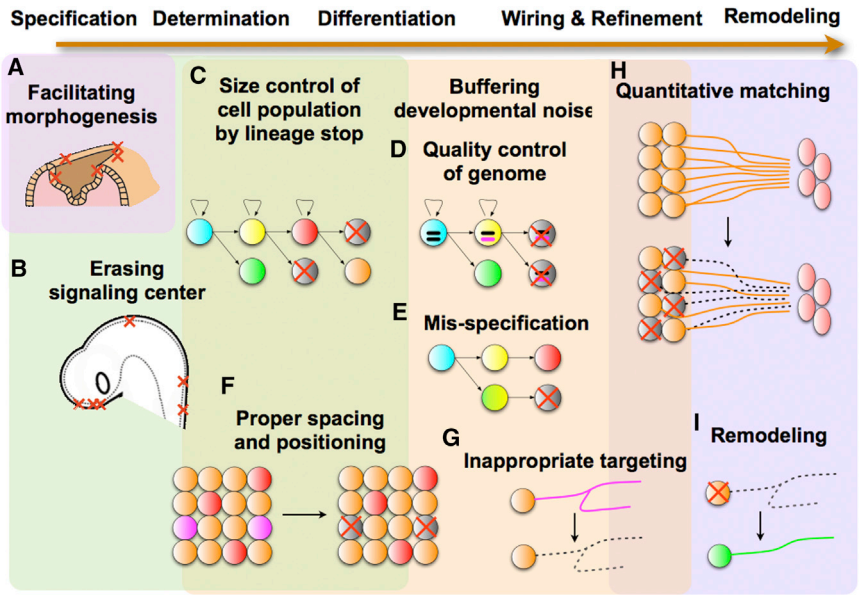

Figure 4. Roles of PCD during Nervous System Development
Schematic representation of functions of cell death during neural development. X indicates cells that undergo PCD.

2013; Yamaguchi et al., 2011). It is reported that inhibition of apoptosis during NTC does not affect epithelial fusion and separation of the neural tube from the non-neural ectoderm (Massa et al., 2009). This suggests that apoptosis is not required for epithelial fusion but is rather a parallel phenomenon or a consequence of NTC. Recent progress in live-imaging techniques reveals that inhibition of apoptosis prevents the normal progression of events in cranial NTC (Yamaguchi et al., 2011), consistent with the observations that apoptotic cells facilitate epithelial remodeling by affecting the movement of neighboring cells (Lubkov and Bar-Sagi, 2014; Teng and Toyama, 2011). Thus, apoptosis can affect the dynamics of neuroepithelial morphogenesis (Figure 4A). Determining what exactly triggers and regulates apoptosis during morphogenesis is an important challenge to fully understanding the significance of this phenomenon.

Sculpting and Eliminating Signaling Centers

In the developing mouse embryo, the anterior neural ridge (ANR) is the most rostral boundary region between the neural plate and non-neural ectoderm. The ANR also exhibits massive cell death before and after neural tube closure (Figure 4B). Genetic-tracing studies in apoptosis-deficient mutants (*apaf-1* or *caspase-9 knockouts*) have demonstrated that apoptosis effectively eliminates cells expressing Fgf8, which diffuses from the rostral half of the ANR to the surrounding regions and acts as a morphogen crucial for forebrain patterning (Nonomura et al., 2013). Apoptosis deficiency results in abnormally prolonged persistence of Fgf8-expressing cells in the rostral ANR, which leads to abnormal distribution of Fgf8 protein within the surrounding regions and perturbed ventral forebrain development. Sculpting and elimination of signaling centers by apoptosis is also observed in *Xenopus* neural development (Offner et al., 2005). It is thus considered to be an efficient and well-conserved mechanism for diminishing cells or tissues that transiently function in normal development, but are unnecessary or even harmful at other stages of development (Nonomura et al., 2013; Offner et al., 2005). Consistent with this idea, many signaling center exhibit substantial apoptosis (Gibson et al., 2011; Homma et al., 1994; Miller and Briglin, 1996; Sanz-Ezquerro and Tickle, 2000) and cell death is proposed to play a role in their disappearance after they carry out their developmental functions. However, confirmation of this hypothesis requires further empirical evidence.

Cell Number Regulation

Proper organ and tissue size determination requires the coordination of cell proliferation, cell size regulation, and cell death. The most notable example of cell number regulation by cell death is described by the neurotrophic theory, as mentioned in introduction (Figure 4H) (Cowan, 2001; Levi-Montalcini, 1987; Oppenheim, 1991; Purves et al., 1988; Raff, 1992). This mechanism serves as a means of quantitative matching of not only neurons and glia with targets and afferents in vertebrates, but also in the *Drosophila* (Barres et al., 1992; Bergmann et al., 2002; Buss et al., 2006; Hidalgo et al., 2001; Zhu et al., 2008). The determination of cell viability versus death depends on the amount of neurotrophins and the timing of their reception in target tissue, as mediated by the dependence receptor system, as extensively discussed in many excellent reviews on neurotrophic theory and dependence receptors (Buss et al., 2006; Dekkers et al., 2013; Jiang and Reichert, 2012).

Preceding PNS development, abundant apoptosis is observed in the early phases of CNS development (Figures 4A–4C). Loss of intrinsic apoptotic pathway genes (*caspase-3*, *caspase-9*, or *apaf-1*) in mouse results in brain malformations that include brain ventricle compression, indented neuroepithelial sheets, and exencephaly, a condition in which neuroepithelium protrudes outside of the skull at later embryonic stages. These phenotypes are observed in the 129 murine genetic background, a mouse strain used fortuitously for the first derivation of embryonic stem cells and frequently for the generation of targeted-knockout mouse strains, and have been interpreted as "brain overgrowth" in which neural cells are overproduced in the absence of apoptosis during embryogenesis in mouse (Kuan et al., 2000). However, a recent study indicated that the total numbers of brain cells do not increase much at early embryonic stages, indicating a need to revise this explanation of brain overgrowth (Nonomura et al., 2013). Initial causes of the embryonic brain malformations observed in apoptosis-deficient embryos can be explained simply by defects in neural tube closure (Figure 4A) because inhibition of apoptosis does not significantly affect total embryonic brain cell numbers at stages when this morphological abnormality has begun to appear (Nonomura et al., 2013).

The minimal impact of knocking out apoptotic genes on total embryonic brain cell numbers might be attributed to other lineage-stop mechanisms, including cellular senescence (see below) or cell-cycle exit, as local accumulation of non-proliferative Fgf8-expressing cells are observed in the ANR of apoptosis-deficient embryos (Nonomura et al., 2013). Alternatively, inhibition of PCD alone might be insufficient for expanding cell numbers drastically in mammals. For example, simultaneous manipulation of cell proliferation and cell survival by enhancement of Wnt/β-Catenin signaling, loss of α-N-catenin, or acceleration of the cell cycle through overexpression of Cdk4 and CyclinD1, results in drastic increases in total brain cell numbers (Chenn and Walsh, 2002; Lien et al., 2006; Nonaka-Kinoshita et al., 2013). This is in sharp contrast to *Drosophila*, wherein inhibition of PCD is sufficient to induce significant hyperplasia in the embryonic and larval CNS (Figure 2C) (Kanuka et al., 1999; Rogulja-Ortmann et al., 2007; Tan et al., 2011). In keeping with these findings, the regulation of PCD and organ size regulation in vertebrates appears to involve more regulatory processes than in invertebrates, as detailed in the following section (Fuchs and Steller, 2011).

Considering the relationship between cell death and cell number regulation, it is also worth taking into consideration that apoptotic cells can promote cell proliferation of surrounding cells by releasing growth factors including BMP, Wnt, and lipid mediators when they are dying in some contexts. This phenomenon, called compensatory proliferation, has been observed in injured tissues of various animals, including imaginal discs in the developing fly, regeneration of decapitated hydra, and damaged liver and irradiated tumor cells in mammals, and might be an adaptive mechanism to maintain appropriate numbers of cells for reconstructing the tissues (Chera et al., 2009; Fan and Bergmann, 2008; Li et al., 2010; Nishina et al., 2012). In the nervous system, it was reported that dying neurons can stimulate proliferation of neural stem cells both in vitro and in vivo (Agasse et al., 2004; Magavi et al., 2000). However, it remains unclear whether the compensatory proliferation indeed occurs and participates in normal neural development, and therefore further studies will be necessary.

Redundant Mechanisms that Limit Cell Numbers

Apoptosis-deficiency in vivo seems to be compensated in some cases by cell-cycle exit or by non-apoptotic cell death mediated by unknown mechanisms. This phenomenon is well demonstrated in *apaf-1* mutants, which exhibit an accumulation of non-proliferative cells that undergo apoptosis in normal development. However, most, if not all, of the excess cells ultimately undergo alternative forms of non-apoptotic cell death at later stages (Nonomura et al., 2013; Oppenheim et al., 2008; Yaginuma et al., 2001). In contrast, mice deficient for *caspase-3* or *bax* in C57BL6-dominant background, which do not show the morphological abnormalities observed in the 129 background and can survive to adulthood, exhibit higher cell density or cell numbers in the specific regions of cerebral cortex or hypothalamus than wild-type mice at adult stages (Forger et al., 2004; Gotsiridze et al., 2007; Le et al., 2002). These lines of evidence suggest that the redundant mechanisms for cell elimination may not likely operate in those cases, and that its operation is performed in a context-dependent manner.

Cell cycle exit acts upstream of or in parallel to cell death not only in apoptosis-deficient conditions, but also in the context of normal mammalian development. During mouse embryogenesis, senescence-associated β-galactosidase (SAβgal)-positive cells appear in developing organs including the roof plate of the neural tube in the CNS, as well as non-neural tissues (e.g., the ectodermal ridge in the limb buds) (Muñoz-Espín et al., 2013; Storer et al., 2013). The SAβgal$^+$ cells show some features of senescent cells, such as the expression of p21 (cyclin-dependent kinase inhibitor 1) and p53, universal markers for cellular senescence induced by non-physiological insults such as DNA damage or pathological states. However, SAβgal$^+$ cells do not show any signs of expression of p16 and DNA damage, two central mediators of replicative and oncogene-induced senescence, suggesting that the SAβgal$^+$ cells may not be prone to such errors or insults. Lack of p21 but not p53 results in lack of senescent (SAβgal$^+$) cells and causes subtle developmental abnormalities (Muñoz-Espín et al., 2013; Storer et al., 2013). Apoptosis might compensate for the lack of senescence in *cip1/p21* mutants, based on the facts that SAβgal$^+$ regions exhibit substantial apoptosis and it has been found that the number of cells double-positive for p21 and TUNEL increased in the AER of *cip1/p21* mutants (Storer et al., 2013). It should be taken into careful consideration, however, that SAβgal$^+$ staining does not always indicate cellular senescence, but may possibly mark acidic β-galactosidase activity that might be present in tissues involved in nutrient absorption, such as the visceral endoderm and intestines (Going et al., 2002; Huang and Rivera-Pérez, 2014). Further studies are needed to verify the significance of developmental senescence in mammals.

Taken together, several mechanisms, including apoptosis, alternative non-apoptotic cell death, and developmental senescence can compensate for one another in mammals in a context-dependent manner. To evaluate the precise contribution of PCD to brain cell number regulation during development, lineage-tracing experiments that can specifically label progeny of each cell lineage might be effective in tracing and comparing their respective fate with and without the possibility of cell death, as has been performed in previous studies in mouse (Chen et al., 2013; Nonomura et al., 2013). One would also expect that such approaches will reveal the identity of dying cells and thereby help address how early PCD is induced in and contributes to mammalian brain development.

PCD: A Reflection of Inevitable Developmental Error and Noise?

One important question is whether PCD is caused also by developmental errors and noise that are inevitable byproducts of developmental molecular program, or only induced by intrinsic cellular differentiation programs as discussed previously. The answer is both causes are true. It has been proposed that PCD plays an important role in buffering error or noise in several aspects of neural development. Indeed, it has been reported that many aneuploid cells are generated in mouse embryonic cerebral cortex development and that this phenomenon is greatly increased by inhibition of apoptosis during development (Bushman and Chun, 2013; Peterson et al., 2012; Rehen et al., 2001). Although how and why neural aneuploidy originates spontaneously in mammalian brain development remains unclear, these results suggest that endogenous PCD in the cerebral cortex, if not all of the nervous system, may arise from somatic

genomic alterations. PCD may thus serve as a quality control mechanism to eliminate cells that have undesirable genomic alterations (Figure 4D) (Bushman and Chun, 2013; Peterson et al., 2012). Hereafter, we will further discuss the quality control function, in other words, buffering action, of PCD by considering the potential triggers of PCD, namely developmental error, noise, or intrinsic developmental programs.

Misspecified, Mispositioned, or Aberrantly Migrated Cells

The developing *Drosophila* sensory organ is one of the essential models that enable studies of the process of neural cell-fate specification and proper cellular positioning. The molecular mechanisms by which the sensory organ precursors (SOPs) develop from proneural cell clusters have been well studied (Heitzler and Simpson, 1991; Simpson, 1990). Notch/Delta mediated lateral inhibition functions to generate a pattern of uniformly spaced SOPs, which can be identified by their expression of the neurogenic gene *neuralized* (Fichelson and Gho, 2003; Gho et al., 1999). Live-imaging analyses reveal that excess numbers of *neuralized*-positive cells are generated and eliminated during the cell-determination step. Approximately 20% of the *neuralized*-positive cell death is accompanied by high caspase activation and nuclear fragmentation. These *neuralized*-positive "SOP-like" cells are characterized by high Notch activation that is not observed in SOPs, suggesting that high Notch activation induces apoptosis (Koto et al., 2011). Interestingly, these SOP-like cells cannot be rescued from apoptosis even when neighboring normal SOPs are lost, suggesting that SOP-like cells are irreversibly fated for cell death. In contrast, some of the surrounding epithelial cells switch to an SOP fate, possibly due to the downregulation of lateral-inhibition. Thus, PCD helps to ensure proper spacing by erasing developmental noise, which is generated by the inevitable induction of SOP-like cells during Notch/Delta-mediated lateral-inhibition (Figures 4E and 4F).

The importance of PCD in proper spacing between cells is exemplified in visual system development both in invertebrates and vertebrates (Chen et al., 2013). The *Drosophila* compound eye is composed of ~750 ommatidia. Each ommatidium, which consists of eight photoreceptor neurons, four cone cells, and two primary pigment cells, is separated by interommatidial lattice cells. A 3-fold excess number of lattice cells are produced in development and those excess cells are eliminated by apoptosis to form a precise hexagonal lattice. Interommatidial lattice cell death is spatially regulated and executed in defined areas because 85% of lattice cell death was observed in two specific regions termed "death zones" that are located either between ommatidial units or adjacent to bristle groups (Monserrate and Brachmann, 2007). The death of lattice cells also depends on Notch signaling, which works to antagonize dEGFR survival signals in lattice cells (Cagan and Ready, 1989; Kooh et al., 1993; Wolff and Ready, 1991; Yu et al., 2002).

Likewise, during the development of the retina in mouse, many of intrinsically photosensitive retinal ganglion cells (ipRGCs), which contain melanopsin and function for circadian rhythms and papillary light responses, undergo Bax-dependent apoptosis (Chen et al., 2013). Bax mutant exhibits a 3.7-fold increase in density of ipRGCs and thereby shows disrupted ipRGC spacing, dendritic stratification, and ectopic synapses. The disruption of ipRGC spacing in Bax mutants does not affect melanopsin-driven circadian photoentrainment, but impairs rod/cone-driven photoentrainment, suggesting that PCD is essential for the establishment of specific aspects of neural circuit functions (Chen et al., 2013).

In addition to proper spacing, PCD also play a role in eliminating cells that migrate to inappropriate places in various organ systems. In the mouse brain, inhibition of cell death by deletion of *bax* causes misplacement of both Purkinje cells in the cerebellum, and neurons migrating to the olfactory bulbs and within the dentate gyrus during adult neurogenesis (Jung et al., 2008; Kim et al., 2007, 2009; Sun et al., 2004).

Neurons Targeting to "Inappropriate" Regions

Classic studies suggested that PCD eliminates neurons projecting to "incorrect" or "inappropriate" sites (i.e., areas that are not normally innervated by projections in the mature adult) in developing avian visual systems (Clarke, 1992). Recent studies using genetic manipulations in mouse and *Drosophila* have indeed shown that inhibition of apoptosis prevents regression of aberrant axonal projections in several neural systems, indicating a role for PCD in eliminating neurons that project to aberrant sites (Figure 4G) (Baek et al., 2013; Buss et al., 2006; Jiang and Reichert, 2012; Rogulja-Ortmann et al., 2007).

These findings raise the question: why do some neurons target to aberrant sites and degenerate during development? In addition to regulation based on the neurotrophic theory, one possible explanation is that such aberrant neurons possess errors that affect axonal targeting, and that PCD plays a role in error correction to eliminate such error-prone neurons (Clarke et al., 1998). This is the case for a number of gene mutations that lead to the mis-targeting of neural projections. For instance, flies harboring mutations in homeobox genes that determine neuronal identity, such as *Antp*, *labial*, *Dfd*, and *Scr*, exhibit aberrant axonal targeting and such aberrant neurons are eliminated by PCD in those mutants (Baek et al., 2013; Kuert et al., 2012, 2014). Thus, mechanisms that couple the control of neuronal identity with projection patterning might underlie regulatory processes that lead to the elimination of aberrant neurons by PCD.

Another possible explanation for the cause of aberrant projections leading to PCD in neurons that are not "error-prone" in normal development is that some neurons are programmed to innervate different sites from their final targets, but that this innervation then leads to intrinsically or extrinsically induced cell death. The elimination of projections by the death of cells programmed for aberrant targeting is considered to play a role in specific neuronal systems whose function is highly dependent on spatially precise topographic mapping (e.g., the visual system) (Buss et al., 2006; Clarke et al., 1998). For instance, in the developing mouse visual system, early-born Cadherin 3 (Cdh3)-expressing retinal ganglion cells (RGCs) first project to multiple targets before eventually restricting their target regions, which coincides with a significant decline in the number of Cdh3-expressing RGCs. In contrast, the axons of late-born Hoxd10- or dopamine receptor D4 (DRD4)-positive neurons project specifically to the correct target from the beginning of their development (Osterhout et al., 2014). These results suggest that birthdate-dependent intrinsic axon-targeting mechanisms coupled with PCD play a role in the establishment of proper neural circuit formation, although it has not been shown directly whether inhibition of PCD would cause persistence

of Cdh3-expressing neurons that had ectopically targeted multiple regions. The functional basis for these aberrant projections remains unclear, but it is compelling to speculate that they might play an adaptive role as pioneer neurons in guiding other neurons (Raper and Mason, 2010). Alternatively, they might be reminiscent of evolutionary traits that their ancestor possessed, developmental neural plasticity and the capacity to adapt to unexpected perturbation, or merely noise brought about as inevitable byproducts of birthdate-dependent developmental programs (Buss et al., 2006).

Elimination of aberrantly targeted neurons by PCD is not a universal aspect of neuronal targeting. Many developing neurons can retract or degenerate their projecting neurites without causing cell death via various signaling pathways (Buss et al., 2006). Several studies demonstrate that local activation of caspases is involved in axonal degeneration, arborization, and dendrite pruning (Campbell and Okamoto, 2013; Dekkers et al., 2013; Kuo et al., 2006; Lee et al., 2009; Nikolaev et al., 2009; Schoenmann et al., 2010; Williams et al., 2006), suggesting that apoptotic machinery itself is important for local refinement in neural circuit formation without causing cell death. Thus, it is assumed that cells exhibiting such local activation of caspases might have mechanisms that inhibit the propagation of local caspase activation to whole cell, which would presumably lead to cell death (Cusack et al., 2013; Dekkers et al., 2013; Kuo et al., 2006; Nikolaev et al., 2009; Williams et al., 2006). The relationship between local caspase activation in synaptic/axonal refinement versus apoptosis of aberrant neurons remains to be determined. Taken together, these studies indicate that although PCD is a common consequence of aberrant targeting in developing neurons, it is not a universal to all neuron types and the cause of PCD may vary depending on the context. Furthermore, the mechanisms underlying the spectrum of PCD phenomena need to be further elucidated.

PCD Remodeling of the Post-Embryonic CNS: Metamorphosis and Puberty

Finally, to extend unexplored roles of PCD both on structural and functional development of nervous system, we draw attention to two phenomena: metamorphosis in insects in which PCD is an essential player, and puberty and adolescence in mammals, which might potentially require PCD (Figure 4I). Metamorphosis is a fascinating phenomenon through which a juvenile animal remodels its body, including the nervous system, into an adult form to exhibit sexual behaviors and social adaptation. Metamorphosis is especially evident in animals such as holometabolous (which means "complete metamorphosis") insects and amphibians that both undergo large-scale remodeling of tissue architectures and conversion of cell types. Metamorphosis requires substantial PCD to eliminate juvenile structures such as the larval body in insects and tadpole tail in frogs. PCD is triggered by systemic hormones, such as ecdysone in insects and thyroid hormones in amphibians. During metamorphosis of the fly, adult-specific imaginal neurons do not die, but instead prune their dendrites and axons to remodel neural circuits. However, other larval neurons, including larval motor neurons, mushroom body neurons, neuropeptide-producing neurons, and optic neurons are eliminated during the pupal stage (Choi et al., 2006; Hara et al., 2013; Tasdemir-Yilmaz and Freeman, 2014; Togane et al., 2012; Winbush and Weeks, 2011). Most cell death during metamorphosis occurs in an Ecdysone receptor-dependent manner. As for sexual maturation important for reproductive behaviors, brain sexual dimorphism exists in *Drosophila* and is established by cell death during metamorphosis, as demonstrated by inhibition of cell death in females, resulting in male-like neural circuit formation. The survival of male-dominant mAL neurons is mediated by male-specific expression of the *fruitless* (*fru*) gene, which encodes the RNA binding protein FruM and regulates male-specific behaviors (Kimura et al., 2005). Similarly, the male-specific P1 neuron is eliminated in females by cell death (Kimura et al., 2008).

To what extent are these insights from studies on metamorphosis in insects and amphibian relevant to other animals that do not seem to undergo metamorphosis? There is a compelling idea that birds and mammals do in fact undergo a form of metamorphosis during puberty and adolescence, given that the definition of metamorphosis is to achieve maturation of reproductive ability and social adult behaviors under systemic regulation (Gilbert, 2013). For example, massive cell death, including apoptosis, occurs during steroid hormone-dependent global remodeling of the brain during sexual maturation in seasonally breeding songbirds (Thompson, 2011). In mammals, including humans, sexual and behavioral maturation also depends on the systemic action of gonadal steroid hormones during puberty and adolescence. In rodent brains, there are sexually dimorphic nuclei wherein different numbers of cells are observed between males and females. Sexual dimorphisms depend on sex steroids from gonads during postnatal brain development, and can be generated via differences in rates of proliferation, migration, or survival between males and females (Ahmed et al., 2008; Juraska et al., 2013). The dimorphisms in the principal nucleus of the bed nucleus of the stria terminalis and the anteroventral periventricular nucleus are mostly abolished by deletion of Bax. Thus, Bax-dependent PCD during postnatal brain development contributes to generation of sexual dimorphisms in these neurons (Forger, 2009). Interestingly, as mentioned previously, dying mammalian neurons can induce proliferation in cells that receive signals from the dying cells in some situations (Agasse et al., 2004; Magavi et al., 2000), which raises the possibility that PCD might be involved in remodeling or regenerative processes of nervous system in mammals. Precise lineage-tracing analysis with PCD mutants might be able to reveal the neural cell populations and the neural circuits whose size, formation, and architectures are regulated or remodeled through PCD during puberty and adolescence, the crucial periods for developing adult social behaviors in animals, including humans.

Conclusions

Recent studies highlight the old but essential concept of cell-lineage-dependent PCD during development. Such deterministic regulation of PCD contrasts with indeterministic life-or-death regulation, including competition for trophic factors, spontaneous genomic alternation, or "random" selection based on cell position and status. A combination of both regulatory processes is essential for proper development of a functional nervous system. In addition, recent studies have proposed that dying cells can actively affect the behavior of neighboring cells. However, it remains ambiguous whether PCD has such a function in the nervous system. Deciphering the full regulatory

roles and functions of PCD, including newly described types of regulated cell death other than apoptosis, is required to derive a deeper understandings of PCD not only in development and metamorphosis, but also in the developmental stages of puberty and adolescence, during normal aging processes, and in various pathological conditions (Chihara et al., 2014; Fuchs and Steller, 2011). All of these biological processes are accompanied by substantial cell death, a robust and inevitable event in multicellular organisms.

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