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Patterns of cell death in the perinatal mouse forebrain

Morgan Mosley¹, Charisma Shah¹, Kiriana A. Morse², Stephen A. Miloro², Melissa M. Holmes³, Todd H. Ahern^{2,*}, and Nancy G. Forger^{1,*}

¹Neuroscience Institute, Georgia State University, Atlanta, GA 30302

²Department of Psychology, Center for Behavioral Neuroscience, Quinnipiac University, Hamden, CT 06518

³Department of Psychology, University of Toronto, Mississauga, Ontario L5L 1C6

Abstract

The importance of cell death in brain development has long been appreciated, but many basic questions remain, such as what initiates or terminates the cell death period. One obstacle has been the lack of quantitative data defining exactly when cell death occurs. We recently created a “cell death atlas,” using the detection of activated caspase-3 (AC3) to quantify apoptosis in the postnatal mouse ventral forebrain and hypothalamus, and found that the highest rates of cell death were seen at the earliest postnatal ages in most regions. Here we have extended these analyses to prenatal ages and additional brain regions. We quantified cell death in 16 forebrain regions across nine perinatal ages from embryonic day (E) 17 to postnatal day (P) 11 and find that cell death peaks just after birth in most regions. We find greater cell death in several regions in offspring delivered vaginally on the day of parturition compared to those of the same post-conception age but still *in utero* at the time of collection. We also find massive cell death in the oriens layer of the hippocampus on P1, and in regions surrounding the anterior crossing of the corpus callosum on E18, as well as the persistence of large numbers of cells in those regions in adult mice lacking the pro-death *Bax* gene. Together, these findings suggest that birth may be an important trigger of neuronal cell death, and identify transient cell groups that may undergo wholesale elimination perinatally.

Graphical Abstract

*Co-Corresponding authors: Nancy G. Forger, Neuroscience Institute, Georgia State University, Atlanta, GA 30302, Phone: (404) 413-5888, nforger@gsu.edu, Todd H. Ahern, Department of Psychology, Center for Behavioral Neuroscience, Quinnipiac University, Hamden, CT 06518, todd.ahern@quinnipiac.edu

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Keywords

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Introduction

Two waves of cell death shape normal mammalian brain development. The first is confined to the ventricular and subventricular proliferative zones, and eliminates many neural precursor cells within hours of their birth (Blaschke et al., 1996; 1998; Thomaïdou et al., 1997; Kuan et al., 2000). The second wave, known as “post-mitotic cell death,” occurs throughout the brain and eliminates roughly 50% of the neurons that have migrated, differentiated, and begun to make axonal connections (Oppenheim et al., 1985; Buss et al., 2006). Post-mitotic cell death sculpts developing circuits, contributes to sex differences in neuronal cell number (Forger, 2009), and may identify a window of vulnerability during which exposure to injuries, infections, or variations in environmental factors have greatest impact (McDonald et al., 1988; Ikonomidou et al., 1989, 1999; Yakovlev et al., 2001; Olney, 2002).

Although the importance of cell death in neural development has been appreciated for decades, many surprisingly basic questions remain (see Yamaguchi and Miura, 2015 for a recent review). For the most part, we still do not know what initiates the cell death period, what terminates it, or what accounts for the large regional differences in the magnitude of cell death in the developing brain. The classical view, that developmental neuronal cell death is a cell suicide program triggered by a lack of trophic factors (Purves, 1988; Barde, 1989; Burek and Oppenheim, 1996), is best supported for neurons with axonal connections in the periphery (i.e., motoneurons and ganglion cells), but less well established for the brain. The

death of interneurons in the cerebral cortex, for example, may be intrinsically determined (i.e., independent of trophic factors; Southwell et al., 2012), and non-neuronal cells such as microglia may kill otherwise viable neurons in some developing brain regions (e.g., Marin-Teva et al. 2004, Wakselman et al. 2008).

One obstacle to resolving basic questions about neuronal cell death is that, with the exception of a few well-studied regions (e.g., cerebellum, substantia nigra pars compacta, and some cortical regions; Jackson-Lewis et al., 2000; Verney et al., 2000; Stankovski et al., 2007; Cheng et al., 2011), systematic data on the timing and magnitude of postmitotic cell death in the mammalian brain has been lacking. We recently addressed this by creating a “cell death atlas of the postnatal mouse brain” (Ahern et al., 2013), comprising brains of male and female C57BL/6 mice collected during the first two postnatal weeks. Postmitotic neuronal cell death is crucially controlled by members of the Bcl-2 family of proteins (Yuan and Yankner, 2000; Roth and D’Sa, 2001), and culminates in the activation of caspases, including caspase-3 (Porter and Jänicke, 1999; Hengartner, 2000). Using immunohistochemical detection of activated caspase-3 (AC3) to detect dying cells, we found the highest density of cell death in the ventral forebrain and hypothalamus at the earliest age(s) examined (postnatal day (P)1 to P3; Ahern et al., 2013). This raised the possibility that “peak” cell death actually occurs earlier – i.e., at P0 or prenatally.

Here we examined this possibility by extending the cell death atlas to earlier ages. In addition, we present both pre- and postnatal cell death data from brain regions not included in the previous study. We find that AC3 cell number is very low in most forebrain regions at embryonic day (E)17 and increases over the next few days, with remarkably steep rises in some areas. In most regions examined, the highest rates of cell death are observed just after birth. We also identify several forebrain regions with intense accumulations of AC3-positive cells perinatally, and confirm an excess of neurons in these regions in adult mice lacking the pro-death gene *Bax*. Taken together, these findings clearly define the period of post-mitotic cell death in many regions of the mouse forebrain, suggest a possible role for parturition in triggering neuronal cell death, and point to the perinatal elimination of several transient cell groups.

Materials and Methods

Mouse Breeding and Collection of Brains, E17 – P1

Methods throughout followed as closely as possible those used in the generation of the postnatal cell death atlas (Ahern et al., 2013). Wildtype C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, Maine) were housed in a 12:12 light:dark cycle (lights on at 0600 h) at 22°C with food and water available *ad libitum*. All procedures were in accordance with National Institutes of Health animal welfare guidelines and approved by the Georgia State University Institutional Animal Care and Use Committee.

The forebrain regions studied here differentiate as discernable nuclei between E15 - E17 in the mouse (Niimi et al., 1962; Creps, 1974; Henderson et al., 1999). We therefore collected brains on E17, E18, E19/P0 and P1, including a one-day overlap with the postnatal atlas (Ahern et al., 2013) to allow us to calibrate our counts with those reported previously.

To generate timed pregnancies, males and females were paired overnight. The males were removed the following morning at ~0900 hours and females checked for sperm plugs; this was designated day E0. Both male and female offspring from a total of 17 litters were collected. All fetuses/pups were collected at the same time of day (between 1400–1500 h), and pups from at least three different litters were used for each time point with the exception of E17 (two litters).

Fetuses collected on E17 and E18 were removed by Cesarean (C) section after briefly exposing the dam to CO₂ (< 1 minute). Fetuses are refractory to CO₂ exposure and are relatively unaffected by such short exposure times (Leary, 2013; Pritchett et al., 2005). A large incision was made in the dam's ventral midline, fetuses were extruded from the uterine horns, and all brains collected within 10 minutes.

At the time of collection on the 19th day after conception, some offspring had been born vaginally earlier that day, whereas others were still *in utero* and were delivered by C-section. In the analyses below, we refer to the combined group as “E19/P0.” When examined separately, the C-sectioned group is referred to as “E19” and the vaginally-born group as “P0.” All pups collected on P1 had been vaginally delivered on day 19 (P0).

Fetuses and neonates of both sexes were rapidly decapitated, and brains removed and fixed overnight in 5% acrolein (Alfa Aesar, Ward Hill, MA) in 0.1M phosphate buffer (PB). The tissue was then transferred to 30% sucrose in 0.1M PB and stored at 4°C. Two series of 40µm coronal sections were collected, placed in cryoprotectant (30% sucrose, 1% polyvinylpyrrolidone, 30% ethylene glycol in 0.1M PB), and stored at –20°C until use.

Previously Generated Material

Postnatal Cell Death Atlas—We collected new data from the previously generated postnatal atlas material (Ahern et al., 2013), including data from five hippocampal regions and the core and shell of the nucleus accumbens. In addition, some data from the postnatal cell death atlas that were published previously (Ahern et al., 2013) are reprinted below along with the expanded age range. The previously published data are clearly indicated as such in the figures.

***Bax* ^{+/+} and *Bax* ^{–/–} brains**—*Bax* is a pro-death member of the Bcl-2 protein family that is required for the death of developing neurons; virtually all naturally-occurring neuronal cell death is eliminated in mice with a targeted disruption of the *Bax* gene (White et al., 1998; Ahern et al., 2013). The *Bax* ^{+/+} and *Bax* ^{–/–} brains examined in this study were drawn from previously collected brains of *Bax* knockout mice and their wildtype siblings (Forger et al., 2004; Holmes et al., 2009). The *Bax* deletion was on a C57BL/6 background and mice were adult at the time of sacrifice. For thionin-stained sections, animals were intracardially perfused with formalin, and brains were frozen sectioned at 30µm. For immunocytochemistry, animals were perfused with 4% paraformaldehyde and frozen sectioned at 30µm.

Immunohistochemistry

Alternate sections from all perinatal animals were immunohistochemically stained for AC3, closely following the protocol of Ahern et al. (2013). Free-floating sections were extensively rinsed in 1X Tris-buffered saline (TBS; pH 7.6), and immersed in 0.05M sodium citrate for 30 minutes. Sections were again rinsed, transferred to 0.1M glycine in 1X TBS for 30 minutes, rinsed, then incubated in a concentrated blocking solution (1X TBS, 20% normal goat serum, 1% hydrogen peroxide, 0.3% Triton-X). Sections were incubated overnight in primary antibody solution (Cleaved Caspase-3, RRID:AB_231409, Cell Signaling, Danvers, MA; 1:20,000 in 1XTBS, 2% normal goat serum, 0.3% Triton), then washed in a dilute blocking solution (1X TBS, 1% normal goat serum, 0.02% Triton-X), incubated for one hour in secondary antibody solution (goat anti-rabbit (Vector Laboratories, Burlingame, CA; 1:250, 1X TBS, 2% normal goat serum, 0.3% Triton-X), followed by rinses in 1X TBS with 0.2% Triton-X, before incubating for one hour in an ABC solution (Vectastain Elite ABC Kit, Vector Laboratories). Reagent A and B of the ABC kit were added to 1X TBS at a dilution of 0.8% based on pilot optimization runs. Sections were incubated for 2–5 minutes in diaminobenzidine-nickel (DAB) solution (Vector Laboratories), followed by rinses in 1X TBS. Sections were mounted onto microscope slides and counterstained with thionin.

We also used immunohistochemical detection of the microglial marker, ionized calcium-binding adaptor molecule 1 (Iba1), to examine the distribution of microglia in a subset of animals on E18. Activated microglia with an amoeboid morphology are responsible for the phagocytosis of cell corpses, and accumulate in regions of high cell death (reviewed in Pont-Lezica et al., 2011; Wake et al., 2012). The immunohistochemical procedure followed that described for AC3 above, except that the primary antibody used was anti-Iba1 (RRID:AB_2314667, Wako Chemicals, Osaka, Japan, 1:20,000 in 1X TBS, 2% normal goat serum, 0.3% Triton), and the sodium citrate incubation was increased to one hour.

To determine the phenotype of cell groups persisting in *Bax*^{-/-} mice, we examined *Bax*^{+/+} and *Bax*^{-/-} brains that were processed for double-label immunohistochemistry for NeuN and GFAP, markers of mature neurons and astrocytes, respectively. These brains came from animals in our previous study (Holmes et al., 2009) and were labeled using sequential staining for NeuN [1:1000 mouse anti-NeuN monoclonal antibody (RRID:AB_2298772, Chemicon International, Temecula, CA)] using a DAB reaction (brown reaction product), and GFAP (1:1000 rabbit anti-GFAP polyclonal antibody; RRID:AB_2109645, Chemicon International), using Vector SG (blue reaction product; Vector Laboratories), as described (Holmes et al., 2009).

Antibody specificity

Details of primary antibodies are given in Table 1. The AC-3 antibody used here recognizes the active fragment of cleaved caspase-3. It detects a couplet on Western blots of the expected size and does so only when apoptosis-initiating proteases are active (Cheong et al., 2003). It does not cross-react with full-length (inactive) caspase-3 in human or mouse cells (Hu et al., 2000; Sammeta and McClintock, 2010). Moreover, virtually all labeling with this antibody is abolished in the forebrain of newborn mice lacking the pro-death gene *Bax* (Ahern et al., 2013), supporting its specific detection of dying cells in this material.

The specificity of the GFAP antibody was demonstrated in Western blots of rat retina lysate (Chang et al., 2007) where it labeled a band of the expected size (50 kDa); quantification of the labeled band in Western blots also correlated with quantification of immunocytochemical labeling of cells with astrocytic morphology in tissue sections.

The Iba1 antibody used here recognizes a single band of 17 kDa on immunoblots, corresponding to the size of Iba1 protein, and does not recognize neurons or astrocytes (Imai et al., 1996; Ito et al., 1998). It has been used extensively as a microglial marker in perinatal mouse brain (e.g., Nilsson et al., 2008; Shrivastava et al., 2012).

The mouse monoclonal against neuron-specific nuclear antigen (NeuN) was originally made against cell nuclei purified from mouse brain (Mullen et al., 1992). The antibody does not recognize oligodendrocytes or astrocytes in mouse brain but labels neurons *in vivo* and *in vitro*. Western blotting with this antibody shows three bands of 46–48-kDa (Mullen et al., 1992).

Digital analysis of AC3-labeled regions of interest

All slides were digitally scanned using a NanoZoomer Digital Pathology slide scanner (Hamamatsu Photonics, Bridgewater, NJ) and analyzed using the Aperio ImageScope program (version 12.1.0.5029, Leica Biosystems, Nussloch, Germany) as previously (Ahern et al.; 2013). Digitized slides were coded and analyzed by investigators blind to group membership.

We counted AC3+ cells bilaterally in a total of 16 brain regions. Regions were chosen that had clearly identifiable borders at all ages examined. For nine of the regions, AC3+ cells had previously been quantified from P1–P11, and we expanded the age range to E17 here. This included four hypothalamic regions [anteroventral periventricular nucleus (AVPV), medial preoptic nucleus (MPON), paraventricular nucleus (PVN), and suprachiasmatic nucleus (SCN)], the lateral and principal nuclei of the bed nuclei of the stria terminalis (BNSTl, BNSTp), two regions in the amygdala [central amygdala (CeA), and medial amygdala (MeA)], and the lateral septum (LS). In addition, we quantified cell death at nine ages in seven brain regions not previously examined in the postnatal atlas: the core and shell of the nucleus accumbens (NAcc core and NAcc shell) and five hippocampal regions [cornu ammonis (CA)1, CA2/3, CA1 oriens layer, CA2/3 oriens layer, and the dentate gyrus (DG)]. Newly generated data are plotted with solid lines in the figures below, whereas reprinted data are indicated by dotted lines.

Most regions were examined in their entirety (i.e., in each section in which they occurred throughout their rostrocaudal extent). For the much larger hippocampal regions, we sampled four sections from the dorsal hippocampus of each animal. In the younger animals (E17–P1) these were four consecutive (alternate) sections, starting with the anterior-most section in which the DG was clearly visible. For older animals with larger hippocampi (~P3–P11), we identified the first section in which the DG was clearly visible (anterior anchor), and the first section in which the lateral extent of the hippocampus begins to dip ventrally (posterior anchor, i.e., Figure 69 in Paxinos et al. 2007); these sections and two additional sections spaced evenly between them were traced and counted. This allowed us to sample

approximately the same rostro-caudal extent of the hippocampus in each subject. The NAcc shell could not be distinguished from the NAcc core prior to E18; counts on E17 therefore represent the combined area.

An observer blind to the sex or age of the subject traced the outline of each region of interest using Aperio ImageScope, and the cross-sectional area was recorded. Labeled AC3+ cells within the region were counted as in Ahern et al. (2013). The measure “total AC3 cells” is the sum of these counts times two (because alternate sections were processed for AC3 immunohistochemistry). The “density of AC3+ cells” was calculated by dividing the total number of AC3+ cells by the total measured volume of each region, and is expressed as AC3+ cells per mm³.

Cell number in the hippocampal oriens of *Bax*^{+/+} and *Bax*^{-/-} mice

We quantified the number of cells with a neuronal morphology (large and multipolar) in thionin-stained sections, as well as the number of NeuN+ and GFAP+ cells in immunohistochemically stained sections of the CA1 oriens of adult *Bax*^{+/+} and *Bax*^{-/-} mice (N = 3 of each). Two consecutive sections were counted, and the first section was the most anterior section in which the DG was clearly visible in each animal.

Cingulate cortex/indusium griseum and subcallosal sling

Two striking clusters of AC3+ cells were visible with the naked eye in AC3-stained sections of late prenatal mice. One was located just dorsal and lateral to the midline crossing of the corpus callosum, in an area approximately corresponding to cingulate cortex area 2/indusium griseum (Cg2/IG; Adamek et al., 1984); the other was just ventral to the corpus callosum and may correspond to the subcallosal sling (Silver et al., 1982). Due to the sheer density of AC3 staining in these two areas, we were unable to individually count labeled cells. Instead, we captured images of the areas and used the thresholding function of ImageJ (Version 1.47; National Institutes of Health) to compare AC3 immunostaining across perinatal ages. The sections analyzed spanned from just anterior to the most rostral crossing of the corpus callosum to the appearance of the forceps major. Each region was outlined in ImageJ and the number of pixels above threshold determined. Threshold was based on background staining of an adjacent brain area with no AC3+ cells and was determined separately for each animal. Total number of pixels above threshold was then divided by the volume sampled, and converted to pixels above threshold per mm³.

Photomicrographs

For figures including photomicrographs, Adobe Photoshop was used to crop images, adjust brightness, and balance color.

Statistical Analysis

We included an overlapping day (P1) between analyses performed on the previously generated material (postnatal atlas; Ahern et al., 2013) and the new material (E17-P1), so that we could “calibrate” between the two data sets. Across the 16 brain regions examined, AC3+ cell densities on P1 in the two sets of material were highly correlated, with counts from the previously generated material accounting for 95% of the variance between brain

regions in the new material ($R^2 = 0.95$; $df = 14$, $p < 0.0001$). The datasets were therefore combined and the values plotted for P1 below represent the P1 animals from both collections.

We first performed two-way ANOVAs (sex-by-age) for each brain region. No significant sex differences were found for any of the newly generated data presented here, so counts from males and females were combined and 1-way ANOVAs performed for each brain region. (We note that in the previously published data, there was an effect of sex on AC3+ cell number in the BNSTp only, with more AC3+ cells in females from about P5–P7; Ahern et al., 2013). Planned comparisons using Fisher's least significant difference were performed only following significant main effects.

The number of animals per age group varied from 12 (E17) to 35 (P1; the combined P1 animals from the postnatal atlas and new material). However, a given brain region was not included in the analysis if tissue damage or staining artifacts prevented an accurate count of AC3+ cells. The final median number of animals per age (followed by the range in parentheses) across all brain regions was as follows: E17, 6 (4–12); E18, 9 (6–12); E19/P0, 20 (12–31); P1, 29 (21–35); P3, P5, and P7, 23 (20–24); P9 and P11, 21 (19–22).

Two-tailed, independent t-tests were used to compare the number of cells (thioninstained, NeuN+ or GFAP+) in the hippocampal oriens layer of *Bax*^{+/+} and *Bax*^{-/-} mice.

Results

AC3+ cell density

All 16 regions exhibited a significant effect of age on AC3+ cell density between E17-P11 (Table 2). For 13 of the 16 regions, AC3+ cells were very sparse on E17 and increased over the next several days (Figures 1 and 2). Highest AC3+ cell densities were seen between birth and P5, followed by a decline to very low levels in all regions on P9 and P11 (Figure 2). Exceptions to this pattern included the DG, CA1, and PVN: for each of these regions AC3+ cell density was high at E17 and remained high over the next several days (Figure 2E, G); density then declined between E19/P0 and P1 for the PVN ($P < 0.0001$), and between P1 and P3 for the DG and CA1 ($P < 0.0001$). The difference in AC3+ cell density between the CA1 and CA2/3 during late prenatal life was striking; this can be seen not only in the quantitative analysis (Figure 2G), but also in photomicrographs (Figure 4).

As seen previously (Ahern et al., 2013), there were large differences in the density of AC3+ cells across brain regions. In the CeA, for example, densities were below 600 AC3+ cells per mm^3 at all ages examined (Figure 2D), whereas peak densities of $>5,000$ AC3+ cells per mm^3 were seen in the CA1 and CA2/3 oriens (Figure 2H).

Total number of AC3+ Cells

AC3+ density measures allow for comparisons of the rate of cell death across brain regions of very different overall size, but can obscure changes in the *total* number of dying cells when brain regions change significantly in size, as they do during perinatal development. Figure 3 presents total AC3+ cells for the 16 brain regions analyzed above; all regions

showed a significant main effect of age on total number of AC3+ cell number (Table 1). As was seen for AC3+ cell density, the highest total numbers of AC3+ cells are seen soon after birth. In fact, the pattern is more consistent for total counts than it was for density: even in the regions with high *densities* of AC3+ cells prenatally (DG, CA1, and PVN), the total number of AC3+ cells is low at E17, with increases over the next several days (Figure 3E, G), followed by a decline to very low levels by P11. Peak total numbers of AC3+ cells were seen “early” (P0, P1) in the PVN, BNSTl, DG, CA1, CA2/3, CA1 oriens and CA2/3 oriens, and “late” (P5) in the BNSTp, AVPV, SCN, LS, MPON, CeA, NAcc core, and NAcc shell.

Persistence of cells in the hippocampal oriens layer of *Bax* knockout mice

As noted above, the CA1 and CA2/3 oriens layers of the hippocampus were remarkable for the exceptionally high density of AC3+ cells observed on P1 (~6,000 AC3+ cells per mm³), which was twice that of the next highest brain region (Figures 2H and 3H). Both oriens regions also exhibited abrupt, three- to four-fold increases in AC3+ cell density in the 24h between P0 and P1 ($P < 0.0001$ for both CA1 and CA2/3 oriens; Figures 2H, 3H and Figure 4).

The hippocampal oriens layer is cell sparse in adulthood, and populated mainly by the basal dendrites of CA pyramidal neurons and their afferents (Reznikov, 1991). The very high rate of cell death in the newborn oriens therefore suggested the elimination of a transient cell population. To investigate this further, we examined the oriens layers in adult *Bax*^{-/-} mice, in which developmental neuronal cell death is nearly completely prevented (White et al., 1998; Ahern et al., 2013). We find a large population of multipolar, neuronal-like cells in the CA1 and CA2/3 oriens layers of thionin-stained sections of *Bax*^{-/-} mice; quantification indicates 8 times more cells in the CA1 oriens of *Bax*^{-/-} than of wildtype mice (Figure 5; $P < 0.0005$). This far exceeds the ~1.5- to 2-fold increases in cell number seen in other brain and spinal cord regions of *Bax* knockouts (Deckwerth et al., 1996; Sun et al., 2003; Forger et al., 2004; Jacob et al., 2005).

Double-labeling for NeuN and GFAP established that the large majority of rescued cells in the *Bax* knockouts are neuronal: the number of NeuN+ cells in the CA1 oriens of *Bax*^{-/-} animals was 5.6-fold greater than the number in wild-type controls (Figure 5; $P < 0.05$), whereas the number of GFAP+ cells did not differ significantly between genotypes (not shown). This supports the existence of a transient layer of neurons in the hippocampal oriens that is eliminated by an explosive period of cell death coinciding with birth.

Cingulate cortex area 2/ Indusium griseum and subcallosal sling

Next we analyzed two forebrain areas that were not *a priori* identified as regions of interest, but stood out as having the highest levels of AC3 labeling in any brain region of perinatal animals, visible with the naked eye in stained sections. These cell groups were located just dorsal and ventral to the midline crossing of the corpus callosum and are identified here as Cg2/IG and the subcallosal sling (Figure 6A,B). In both regions we found significant effects of age on AC3+ cell labeling ($P < 0.0001$ and $P = 0.0002$ for the Cg2/IG and subcallosal sling, respectively; Figure 7), with the highest density of AC3+ cells seen on E18. The cluster in the Cg2/IG was remarkable for the abrupt increase between E17–E18 and the

equally abrupt decrease between E18-E19/P0 ($P < 0.0001$ in both cases; Figure 7). The subcallosal sling displayed a similar pattern, but with more modest differences in cell death density between E17-E18 and E18-E19/P0 ($P = 0.006$ and $P = 0.04$, respectively). Few AC3+ cells were seen in these regions at P3 or later ages (not quantified).

The morphology of AC3+ cells in the Cg2/IG could be seen more clearly in high-powered views of sections that were not counterstained (Figure 5C), and processes could be seen projecting into, or perpendicularly through, the corpus callosum in this material (Figure 5C). In support of the interpretation that the intense AC3 staining in the Cg2/IG and subcallosal sling is indicative of cell death, we observed many pyknotic cells in this regions in thionin-stained sections of E18 animals not processed for immunohistochemistry (not shown), as well as a dense accumulations of microglia with an activated morphology (Figure 6C).

Examination of adult *Bax*^{-/-} mice suggests an accumulation of thionin-stained and NeuN+ cells in the Cg2/IG region that far exceeds differences in cell density between *Bax*^{-/-} and wildtype animals in adjacent regions (Figure 6E-H). As discussed below, the AC3 cells in the perinatal Cg2/IG may represent the elimination of a transient neuronal cell group that pioneers the midline crossing of the corpus callosum or hippocampal commissure.

AC3+ density at 19 days post-conception in offspring vaginally born or still in utero

Most C57BL/6 dams give birth at 19 days post-conception (e.g., Murray et al., 2010). At our usual collection time between 1400 and 1500 h on E19, some litters had been born vaginally earlier that day (P0 group), whereas others were still *in utero* (and removed by C-section; E19 group). The pups are combined as the E19/P0 group in the analyses above. When analyzed separately, there was no significant difference for most cell groups. However, we found greater AC3+ cell density in vaginally delivered pups in the suprachiasmatic nucleus ($P = 0.033$), AVPV ($P = 0.013$) and the subcallosal sling ($P = 0.003$) (Figure 7). No brain regions showed the opposite pattern. Body weights between the groups did not differ (1.32 ± 0.02 g and 1.33 ± 0.02 g for E19 and P0 offspring, respectively; two-tailed t-test, $P = 0.756$).

Discussion

Despite the fact that the role of cell death in brain development is now textbook material, systematic analyses of cell death in the developing mammalian brain have been lacking. With few exceptions, most previous studies have quantified dying cells in one, or at most a few, brain regions at one or a few ages. Here we present cell death dynamics in 16 forebrain regions across 9 perinatal ages, providing the most comprehensive view to date of the patterning of post-mitotic neuronal cell death in the mouse brain. These data may help guide the search for factors that account for regional differences in the magnitude of cell death or that trigger the onset and termination of the cell death period.

Limitations

We opportunistically chose to quantify brain regions with clearly defined borders at all ages in the current study and, although we examined a relatively large number of areas, there are obviously many more that we did not. Missing from this analysis, for example, are any

thalamic or cortical regions (with the exception of Cg2). Limited data on the timing of cell death in the mouse cortex have previously been published and analyses of multiple cortical areas using the current material are in progress (T. Ahern, unpublished). We believe the regions quantified here to be reasonably representative because the lack of AC3+ cells at the earliest ages examined (E17–E18), and peak rates of cell death during first few days of postnatal life, were clearly widespread phenomena throughout the forebrain based on qualitative assessments of the material.

Another limitation of this work is that the identification of cell death relied almost exclusively on the detection of AC3. This could be a concern if some dying cells do not express AC3, or if AC3 immunoreactivity labels cells that are not undergoing apoptosis. Although caspase-independent cell death has been described for neuronal precursor cells or after neuronal injury (Zhan et al., 2001; Rideout and Stefanis, 2001; Cregan et al., 2002), it does not, as far as we know, occur during naturally-occurring death of post-mitotic neurons. The other possibility (non-apoptotic roles for AC3 in our labeled cells) is not likely to be a concern at the ages studied here: although AC3 is involved in dendritic pruning in *Drosophila* and synaptic plasticity in weanling rats (Li et al., 2010; Hyman and Yuan, 2012), the large majority of cells expressing caspase-3 in the neonatal rodent nervous system also show signs of pyknosis (Srinivasan et al., 1998; Zacharaki et al., 2010; Zuloaga et al., 2011). More important, almost all (>95%) activated caspase-3 (AC3) cells are eliminated throughout the perinatal forebrain in mice lacking the pro-death gene, *Bax* (Ahern et al., 2013). Double labeling studies are limited in number, but indicate that the majority of AC3+ cells in the perinatal rodent brain are neuronal (Zuloaga et al., 2011), in accord with our observation that many of the AC3+ cells in perinatal animals in the current study had a neuronal morphology. In addition, the “extra” cells in *Bax*^{−/−} mice were NeuN+ in two brain regions that had high AC3+ cell densities perinatally. Taken together, AC3 immunoreactivity in the late prenatal and neonatal brain is a reliable marker for naturally occurring cell death and primarily labels dying neurons.

Does birth orchestrate cell death?

The observation that cell death peaks within a few days of birth in most forebrain regions raises the possibility that parturition orchestrates patterns of neuronal cell death. The final stage of mammalian gestation is characterized by major hormonal shifts and a state of sterile inflammation (Soares and Talamantes, 1984; Bernal, 2001; Thomson et al., 1999; Golightly et al., 2011); these are followed by the mechanical stimuli of a vaginal delivery, a transition to breathing air, and marked changes in energy metabolism, feeding, temperature regulation, and new challenges to sensory-motor systems (Turgeon and Meloche, 2009; Hillman et al., 2012). Peri-parturitional events trigger major adaptive changes in peripheral organs that are required for adaptation to *ex utero* life (e.g., Thilaganathan et al., 1994; Fowden et al., 1998; Jain and Eaton, 2006) and could also influence brain development, including the timing or magnitude of neuronal cell death. Alternatively, cell death may be pre-programmed and occur at a set time after conception regardless of the timing of birth. As far as we know, data are not currently available to discriminate between these two possibilities. Whether birth plays a role in controlling neuronal cell death could be addressed by examining mice in which birth is experimentally advanced or delayed by hormonal or genetic manipulations

(Sugimoto et al., 1997; Langenbach et al., 1997; Gross et al., 1998; Jeff et al., 2000; Hashimoto et al., 2010). Similarly, by carefully equating time *ex utero* after a vaginal or cesarean delivery, one could determine whether mode of birth makes a difference. Both types of studies have the potential to identify novel factors controlling the timing and magnitude of cell death in the mammalian brain.

Exceptions to the “rule”

Exceptions to the pattern of peak rates of cell death soon after birth were seen in the DG, PVN, and CA1, which all had high densities of AC3+ cells prenatally.; even in these regions, however, *total* AC3+ cell number conformed to the pattern.

Our counts of total AC3+ cells in the DG are in good agreement with those reported previously for perinatal mice (Kim et al., 2009). The DG forms quite late, with the earliest neurons born at E16 (Bayer, 1980; Altman and Bayer, 1990; Stanfield and Cowan, 1979). As would be expected, DG volume increases steeply during the next few days (a nearly 5-fold increase (487%) in volume in the three days between E17 and P1, based on our material; data not shown). Similarly, although many neurons in the CA1 of rodents are born by E16, neurogenesis continues in this region through the last days of gestation and first few postnatal days (Stanfield and Cowan, 1979; Soriano et al., 1989; Bayer, 1980; Bowers et al., 2010). Because regions of neurogenesis are often associated with high cell turnover (Blaschke et al., 1998; Morshead and van der Kooy, 1992; Jabès et al., 2010; Kim et al., 2011), the relatively late histogenesis of the DG and CA1 could explain the high density of AC3+ cells during late prenatal life.

The PVN, on the other hand, forms quite early in the mouse (Karim and Sloper, 1980), so late histogenesis cannot explain high rates of cell death prenatally. The PVN is one of the most important autonomic control centers in the brain, and plays a central role in the stress response. Many studies have demonstrated “programming” effects of late prenatal stressors on the hypothalamic-pituitary-adrenal axis and, in particular, long-term changes in PVN function (e.g., McCormick et al., 1995; Hossain et al., 2008; Zohar and Weinstock, 2011). Late prenatal stress also increases apoptosis in the fetal PVN of rats (Fujioka et al., 1999; 2003; Tobe et al., 2005). It is possible that the cell turnover occurring just prior to birth in the PVN of mice contributes to the susceptibility of this brain region to prenatal programming.

Unusually high rates of cell death represent the elimination of transient cell groups in the hippocampus, subcallosal sling, and cingulate cortex

One unexpected finding of the current study was the large number of AC3+ cells in the oriens layers of the hippocampus (normally a cell-poor region in adulthood). The CA1 and CA2/3 oriens layers of the hippocampus had by far the highest densities of AC3+ cells in any of the brain regions quantified in this or the previous study (Ahern et al., 2013). Also of note were the abrupt changes in the number and density of AC3+ cells in the oriens regions: a 300–400% increase in AC3+ cells between P0 and P1, followed by a return to baseline several days later. Because a population of large neurons persisted in the oriens layers in *Bax*^{−/−} mice, this suggests the relatively sudden elimination of a transient cell group in the

developing hippocampus. The elimination of cell groups that may play a temporary role during development of the vertebrate nervous system has been described previously (e.g., Whitlock and Westerfield, 1998; Reyes et al., 2004; Jung et al., 2008). As far as we can tell, the rapid die-off of a cell group in the hippocampal oriens has not previously been described, although Stanfield and Cowan (1979) noted a decrease in cell density in the stratum oriens and stratum radiatum between E18 and P1 in mice, based on appearance in a Nissl stain. The identity or function of the eliminated cells is not known and is an interesting question for future study.

A massive accumulation of AC3+ cells was also seen in the subcallosal sling and (especially) Cg2/IG at E18; in fact, the density of AC3+ cells in these regions was so great that we could not count them individually. Both the subcallosal sling and Cg2/IG have been implicated in the early formation of the corpus callosum (CC) or hippocampal commissure (HC). The subcallosal sling (previously referred to as the “glial sling,” but renamed when it was shown to contain neurons) is present before the first CC axons cross the midline (E15–E16 in the mouse) and may function to guide pioneer axons that grow along its surface (Silver et al., 1982; Shu and Richards, 2001; Shu et al., 2003). The appearance of pyknotic cells just before birth in the subcallosal sling of the mouse has been reported previously (Hankin et al., 1988); our analyses confirm the interpretation that the disappearance of this structure is due to cell death. Niquille et al. (2009) more recently proposed the elimination of a transient population of neurons thought to guide axons crossing the CC. Although the cells they describe were located primarily within the CC itself, we note that some of the AC3+ labeling they observed on P0 (Niquille et al., 2009; Supplemental figure 3) appears ventrolateral to the CC and may also correspond, in part, to what we have identified here as the dying off of the subcallosal sling.

Based on work in the rat, cells in the Cg2 region were proposed to be the first cortical neurons to project their axons to the contralateral hemisphere (i.e., to pioneer the CC; Koester and O’Leary, 1994). Prenatally, however, the CC is closely apposed to the slightly more ventral HC, and work in mice suggests that the early projecting cingulate neurons instead enter the HC (Ozaki and Wahlsten, 1998). Regardless of which interpretation is correct, it is interesting that both Koester and O’Leary (1994) and Ozaki and Wahlsten (1998) raised the possibility that the Cg2 neurons that pioneer the midline crossing are later eliminated by apoptosis, although neither group favored that explanation. Many AC3+ labeled cell processes in the current study coursed perpendicular to the CC, in accord with the suggestion of Ozaki and Wahlsten (1998) that they projected through the CC to the more ventrally located HC; other AC3+ fibers ran parallel to the CC, however. The fact that so many cells in the Cg2/IG were AC3 labeled, and that this area was also filled with pyknotic cells and amoeboid microglia, suggests that many are eliminated at ~E18, after the CC and HC have been established.

Conclusions

Much of the recent work on cell death in the developing brain pertains to injury-induced cell death. Many years after its discovery, however, there is clearly much that remains to be learned about the control of naturally-occurring cell death during brain development. The

findings presented here, and previously (Ahern et al., 2013), demonstrate that the bulk of developmental cell death occurs within several days of birth. Whether birth (parturition) plays a causative role in timing cell death, although a seemingly basic question, is not known. The signals that trigger the elimination of cells in the Cg2/IG and SCS on E18 or hippocampal oriens cells on P1 also are not known, and are areas for future investigation.

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The authors quantify cell death in 16 regions of the mouse forebrain during late prenatal and early postnatal life. They find peak cell death just after birth in most regions. They also describe massive cell death perinatally in regions surrounding the corpus callosum, and the persistence of neurons in those regions in adult *Bax* knockout mice.

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Figure 1.

Photomicrographs of coronal sections immunohistochemically stained for activated caspase-3 (AC3) show many more dying cells on postnatal day (P) 1 than on embryonic day (E) 17. **A**, **C**, and **E** are from mice on E17; **B**, **D**, and **F** are from P1 mice. **A** and **B** are taken at the level of the nucleus accumbens; **C** and **D** are taken at the level of the lateral nucleus of the bed nucleus of the stria terminalis; **E** and **F** are higher magnification views of the regions indicated by the boxes in **C**, **D**. Scale bars = 100 μm in **A–D** and 25 μm in **E,F**.

Abbreviations: ac, anterior commissure; CC, corpus callosum; LV, lateral ventricle; 3V, third ventricle.

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Figure 2.

Mean ($\pm$ SEM) density of AC3+ cells in 16 regions of the mouse forebrain from embryonic day 17 (E17) to postnatal day 11 (P11). Filled symbols (E17-P1) represent data from new material generated for this study and open symbols (P1-P11) are data from previously generated material (Ahern et al., 2013). Previously published data from Ahern et al. (2013) are shown by dotted lines (A–D and PVN in E) and reprinted here with the permission of John Wiley & Sons; new data collected from the material generated in Ahern et al., (2013) are plotted in solid lines (DG in E, and F–H). Abbreviations: AVPV, anteroventral

periventricular nucleus; BNSTl and BNSTp, lateral and principal nuclei of the bed nuclei of the stria terminalis; CA1, cornus ammonis 1; CA2/3, cornus ammonis 2 and 3; CeA, central amygdala; DG, dentate gyrus; LS, lateral septum; MeA, medial amygdala; MPON, medial preoptic nucleus; NAcc, nucleus accumbens; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus.

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Figure 3.

Mean ($\pm$ SEM) total number of AC3+ cells in 16 regions of the mouse forebrain from embryonic day 17 (E17) to postnatal day 11 (P11). Filled symbols (E17-P1) represent data from new material generated for this study and open symbols (P1-P11) are data from previously generated material (Ahern et al., 2013). Previously published data from Ahern et al. (2013) are shown by dotted lines (A-D and PVN in E) and reprinted here with the permission of John Wiley & Sons; new data collected from the material generated in Ahern et al., (2013) are plotted in solid lines (DG in E, and F-H). Abbreviations: AVPV,

anteroventral periventricular nucleus; BNSTl and BNSTp, lateral and principal nuclei of the bed nuclei of the stria terminalis; CA1, cornus ammonis 1; CA2/3, cornus ammonis 2 and 3; CeA, central amygdala; DG, dentate gyrus; LS, lateral septum; MeA, medial amygdala; MPON, medial preoptic nucleus; NAcc, nucleus accumbens; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus.

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Figure 4.

Photomicrographs of coronal sections through the hippocampus from embryonic day (E) 17 through postnatal day (P) 5. Sections were immunohistochemically stained for activated caspase-3 (AC3) and counterstained with thionin. **A:** E17, **B:** E18, **C:** E19/P0, **D:** P1, **E:** P3, **F:** P5. A peak of cell death in the hippocampus is evident from P1–P3. Although not shown here, few to no AC3+ cells were present in the hippocampus of the older animals (P7, P9, and P11) examined in this study. Scale bars = 100 μ m.

Figure 5.

The elimination of developmental cell death reveals a prominent cell layer in the hippocampal oriens layers. **A, C.** Photomicrographs of thionin-stained sections of the hippocampus of adult wild-type (*Bax*^{+/+}) and *Bax*^{-/-} mice. **B, D.** Higher magnification views of the boxed areas in A and C. Many large, neuron-like cells are found in the CA1 and CA2/3 oriens of the *Bax* knockouts. **E, F.** Photomicrographs of the hippocampal oriens of adult *Bax*^{+/+} (E) and *Bax*^{-/-} (F) mice double-labeled for NeuN and GFAP. An accumulation of NeuN cells is seen in the *Bax*^{-/-} mice. Scale bars = 200 μ m in **A, B, E,** and

F; 25 μm in **C** and **D**. Quantification of thionin stained (**G**) and NeuN+ (**H**) sections of the CA1 oriens layer indicates many more cells with a neuronal morphology in *Bax*^{-/-} than *Bax*^{+/+} mice.

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Figure 6.

Striking accumulations of AC3+ cells are found in the cingulate cortex 2 / indusium griseum (Cg2/IG) region on E18. **A, B.** Photomicrographs of sections immunohistochemically stained for activated caspase 3 (AC3) and counterstained with thionin on E17 (A) and E18 (B). Photos in both cases are taken just posterior to the anterior-most crossing of the corpus callosum. Note the sudden increase in AC3+ cells in the Cg2/IG on E18 (B). **C.** Higher magnification view of the Cg2/Ig on E18 in a section without counterstain. AC3+ labeled cell bodies are seen in the Cg2/Ig (upper left) and several processes of labeled cells can be

see running perpendicular to the corpus callosum (arrows). **D.** Immunohistochemistry for Iba1 on E18, in a section rostral to **B**, shows an accumulation of amoeboid microglia just dorsal and ventral to the CC in the Cg2/IG and subcallosal sling, respectively (arrows). **E, F, G, H.** Thionin-stained (**E, F**) and NeuN/GFAP double-stained (**G, H**) sections of the Cg2/IG region in adult wild-type (*Bax*^{+/+}; **E, G**) and *Bax*^{-/-} (**F, H**) mice. Arrows point to an accumulation of cells in the *Bax*^{-/-} animals that is greatly reduced in the wild-type mice. Arrowhead in (**H**) points to an accumulation of cells around the lateral ventricle as previously described in *Bax*^{-/-} animals (Kim et al., 2007). Abbreviations: LV, lateral ventricle; CC, corpus callosum. Scale bars = 100 μ m in **A** and **B**, 10 μ m in **C**, 200 μ m in **D**; scale bar in **E** is 50 μ m for **E** and **F** and scale bar in **G** is 100 μ m for **G** and **H**.

Figure 7.

Quantification of AC3+ labeling in the cingulate cortex area 2/indusium griseum (Cg2/IG) and subcallosal sling (SCS) from embryonic day (E) 17 to postnatal day (P) 1. Highest rates of cell death were seen in both regions on E18. *, significantly different from E17 ($p < 0.0001$ and $p < 0.001$ for Cg2/Ig and SCS, respectively).

Figure 8.

Vaginally delivered E19/P0 pups exhibited significantly higher cell death density than cesarean delivered pups on E19/P0 in the (A) suprachiasmatic nucleus (SCN), (B) anteroventral periventricular nucleus of the hypothalamus (AVPV), and (C) subcallosal sling (SCS).

Table 1

Primary Antibodies

Antibody	Immunogen	Manufacturer data	Concentration
Activated caspase-3 (AC3)	Peptide sequence CRGTELDGCIETD, which is adjacent to Asp175 in human caspase-3	Cell Signaling Technology, Cat# 9661, Lot 36, RRID:AB_231409, Polyclonal antibody raised in rabbit	1:20,000
Glial fibrillary acidic protein (GFAP)	Purified bovine GFAP	Chemicon, Cat# AB5804, Lot 0512018283, RRID:AB_2109645, Polyclonal antibody raised in rabbit	1:1,000
Ionized calcium binding adaptor molecule 1 (Iba1)	Synthetic peptide corresponding to C-terminal sequence PTGPPAKKAISELP of Iba1	Wako, Cat# 27030, Lot PDJ0493, RRID:AB_2314667, Polyclonal antibody raised in rabbit	1:20,000
Neuronal nuclei (NeuN)	Purified cell nuclei from mouse brain	Chemicon, Cat# MAB377, Lot 0601019159, RRID:AB_2298772, Monoclonal antibody raised in mouse	1:1,000

Table 2

Results of ANOVAs examining the effects of age on cell death density and total cell death for 16 regions of interest. All p-values were significant ($p < 0.0001$).

	AC3+ CELL DENSITY	TOTAL AC3+ CELLS
REGION	F (df)	F (df)
AVPV	12.43 (8,165)	15.63 (8,165)
BNSTL	53.86 (8,179)	33.12 (8,179)
BNSTP	31.18 (8,175)	35.23 (8,175)
CA1	48.04 (8,164)	25.21 (8,164)
CA1 OR	29.01 (8,165)	27.44 (8,165)
CA2/3	30.29 (8,164)	30.03 (8,164)
CA2/3 OR	50.79 (8,165)	36.84 (8,165)
CEA	17.56 (8,161)	17.72 (8,161)
DG	26.15 (8,166)	23.85 (8,166)
LS	55.33 (8,143)	24.52 (8,143)
MEA	66.99 (8,168)	26.18 (8,168)
MPON	34.99 (8,144)	24.62 (8,144)
NACC CORE	35.81 (8,150)	32.85 (8,150)
NACC SHELL	25.55 (8,150)	41.48 (8,150)
PVN	21.93 (8,184)	10.39 (8,184)
SCN	19.80 (8,187)	27.38 (8,187)