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Commentary

The potential of endogenous neuronal replacement in developing cerebral cortex following hypoxic injury

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Neurogenesis normally occurs throughout life in two regions of the adult mammalian brain—the olfactory bulb and the dentate gyrus (Altman, 1969; Altman and Das, 1965; Eriksson et al., 1998; Hinds, 1968; Kaplan and Hinds, 1977; Kornack and Rakic, 1999; Lois and Alvarez-Buylla, 1994; Luskin, 1993). Existence of continual neurogenesis in the mature mammalian brain has raised the hope that neuronal replacement in non-neurogenic regions by endogenous progenitors might be conceivable. Moreover, recent work has supported the possibility for endogenous neuronal replacement by demonstrating evidence of neurogenesis in the adult brain after injury (Snyder et al., 1997; Arvidsson et al., 2002; Magavi et al., 2000; Nakatomi et al., 2002). Even so, the evidence for neurogenesis in many studies is not always unequivocable, making the neurogenic potential of regions such as the neocortex a controversial issue (Gould and Gross, 2002; Rakic, 2002a,b; Nowakowski and Hayes, 2000). Here, we will review some of the recent progress that has been made in studies of neurogenesis and regeneration in the postnatal and adult cerebral cortex and discuss some of the methodological considerations that should be appreciated in these studies. Furthermore, we will consider a report by Fagel et al. (2006) in this issue of *Experimental Neurology* which may give us additional insight into the regenerative and neurogenic potential of the postnatal neocortex.

Neurogenesis in the postnatal brain. It had been widely held by neuroscientists for most of the last century that the adult brain is in some respects static and has little or no ability for regeneration. This belief was supported by the limited recovery seen in patients with neurodegenerative disorders or traumatic brain injuries. In contrast, the developing brain can show considerable plasticity and compensatory recovery of function after damage (Hack et al., 2002; Ment et al., 2003; Kolb, 1987). The degree to which such recovery in neonates could be due to addition of new neurons has been a relatively unstudied area of neuroscience. Sensitive methods for detecting newly generated cells came into use in the 1960s with the advent of ^{3}H-thymidine autoradiography to label dividing cells during S-phase of the cell cycle. Studies using this method demonstrated that the vast majority of neurogenesis occurs prenatally, but that neurogenesis continues in the postnatal hippocampus (Altman and Das, 1965) and olfactory bulb (Hinds, 1968; Altman, 1969). These early reports of postnatal and adult neurogenesis were confirmed by later studies in postnatal rodent forebrain (Kaplan and Hinds, 1977; Bayer, 1983).

The newly generated neurons in the postnatal and adult olfactory bulb originate in the anterior subventricular zone (SVZ) of the lateral ventricle (Luskin, 1993; Lois and Alvarez-Buylla, 1994). Neuroblasts generated in the SVZ undergo tangential chain migration (Lois et al., 1996) within the rostral migratory stream (RMS). The neuroblasts in the RMS migrate millimeters away from their site of origin into the olfactory bulb where they differentiate into functional granule or periglomerular interneurons (Belluzzi et al., 2003; Carleton et al., 2003).

Hippocampal neurogenesis has been observed in all adult mammalian species examined including macaque monkey (Kornack and Rakic, 1999) and human (Eriksson et al., 1998). Adult generated neurons arise in the subgranular zone of the hippocampus where they migrate a short distance into the dentate gyrus and differentiate into granule neurons. Within 3–4 weeks, newly generated granule neurons assume morphological and physiological characteristics of mature dentate granule cells (Hastings and Gould, 1999; Markakis and Gage, 1999; van Praag et al., 2002). Moreover, dentate neurogenesis appears to be affected by changes in hippocampal activity (Cameron et al., 1995; Gould et al., 1999a; Kempermann et al., 1997; Shors et al., 2001). Given the importance of the hippocampus for learning and memory, the presence of continuous neuronal turnover in the

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dentate gyrus has generated considerable excitement with the notion of neurogenesis having a possible role in memory formation.

The evidence for postnatal neocortical neurogenesis. Though studies of proliferation in the adult mammalian brain have repeatedly demonstrated neurogenesis in the SVZ and in the dentate gyrus, reports of newly generated neurons in neocortex of adult rat (Altman, 1963; Kaplan, 1981; Dayer et al., 2005) and macaque monkey (Bernier et al., 2002; Gould et al., 1999b, 2001) have not been as readily accepted. One reason that these reports have been controversial is that original studies on the development of the mammalian cerebral cortex using ^{3}H-thymidine autoradiography report neocortical neurons as originating during restricted periods of embryonic development (Angevine and Sidman, 1961; Berry and Rogers, 1965; Hicks and D'Amato, 1968; Rakic, 1974, 1985). Furthermore, attempts to replicate the results of Gould et al. (1999b) using similar 5′-bromodeoxyuridine (BrdU) labeling methodologies have been unsuccessful (Koketsu et al., 2003; Kornack and Rakic, 2001) and recent work utilizing a retrospective birth dating strategy in adult human occipital cortex has demonstrated that neocortical neurons are as old as the individual (Spalding et al., 2005).

As a possible explanation, it has been suggested that reports of new neurons in adult neocortex are instead due to methodological difficulties. One such problem is the accidental identification of newly generated satellite glia nuclei as belonging to adjacent neurons (Koketsu et al., 2003; Kornack and Rakic, 2001). Satellite glia are closely apposed to cortical neurons and are sometimes pressed up against the neuronal cell body. Therefore, without careful inspection, it may appear in ^{3}H-thymidine and BrdU studies that a newly generated glial nucleus belongs to a neuronal soma. Another methodological problem with the identification of newly generated neurons has been the difficulty in the use of definitive neuronal markers. The use of neuronal markers is necessary because the ^{3}H-thymidine and BrdU techniques mark the nucleus of a newly generated cell with little indication of cellular phenotype. For example, some of the neuronal markers that have been used in recent studies (NSE, MAP2, TOAD-64) can label reactive glia (Rakic, 2002a,b). Another neuronal marker, NeuN, is often used as a definitive neuronal label, even though it is not reactive with all neurons (such as Purkinje and mitral cells) and under some protocols can label non-neuronal cells (Koketsu et al., 2003; Rakic, 2002a,b). In summary, recent evidence seems to indicate that under normal conditions adult neocortical neurogenesis does not occur or that if it does occur, it is not readily detectable with current methods.

The report by Fagel et al. (2006) in this issue of *Experimental Neurology* both highlights the structural plasticity of the neonatal brain in response to injury and illustrates some of the remaining challenges pertaining to studies of neurogenesis. These new data suggest that unlike the adult neocortex, neocortical neurogenesis is readily apparent in the neonatal neocortex and moreover is greatly enhanced following hypoxic injury. If this finding is substantiated by further research, it may represent an important breakthrough in our understanding of the limits of neurogenesis in the neocortex and mechanisms of regeneration.

Fagel et al. (2006) use a mouse model of chronic sublethal hypoxia to study the regenerative capacity of the neocortex during the early postnatal period. First, neonatal mice aged P3 were subjected to a low oxygen environment for either 24 h or 8 days then perfused at P11. The mice exposed to sublethal hypoxia for 24 h had no apparent change in brain weight, while the mice raised in the low-oxygen environment for 8 days exhibited a statistically significant 24% decrease in brain weight with respect to control reared mice at P11 as well as increased ventricular volume and cortical thinning. Next, they used stereological techniques combined with immunocytochemistry for the neuronal marker NeuN to estimate cortical volume and neuronal number. Hypoxic-reared mice at P11 were found to have a 30% decrease in NeuN immunoreactive neurons and a decrease in cortical volume. Remarkably, when they analyzed brains at P18 from hypoxic-reared mice that had been allowed to recover normal oxygen environment for 7 days, the changes in brain weight, cortical volume, and NeuN-positive cells appeared to have been reversed, with no differences being detected between the hypoxic and normoxic conditions. This apparent recovery persisted in hypoxic-reared mice that were allowed to survive until P49. Next, they immunostained tissue from normoxic and hypoxic-reared mice for the neurofilament protein SMI-32 at P11, P18, and P49 as a way to assess for changes in dendritic arborization. Examination of cortex at P11 revealed decreased SMI-32 immunostaining in hypoxic-reared mice and less prominent staining of the pyramidal cell apical dendrites at P18 and P49. These results are in agreement with previous studies indicating that an enhanced regenerative potential may exist in the neonatal cerebral cortex (Kolb et al., 1996, 1998; Kolb, 1987). In addition, this model of sublethal hypoxia may be useful in identifying the mechanisms of recovery seen in young patients suffering from respiratory distress syndromes that result in cerebral hypoxia (Hack et al., 2002; Ment et al., 2003).

A second feature of the report by Fagel et al. (2006) is in their analysis of proliferation and differentiation of newly generated cells in the neocortex of normal and hypoxic-reared mice. First, they administered multiple pulses of 50 mg/kg BrdU between P16 to P18 in normal ($N = 3$) and hypoxic-reared mice ($N = 3$) and then sacrificed the animals at P49. They then combined BrdU immunocytochemistry with immunostaining for neuronal or glial cell antigens and then analyzed between 100–200 BrdU-positive cells in the cerebral cortex per brain. They found that approximately 43% of newly generated cells were positive for the oligodendrocyte marker Rip and 36% of BrdU-positive cells expressed the astrocyte marker GFAP in normal and hypoxic-reared mice. Unexpectedly, they also report that approximately 10% of BrdU-positive cells were colabeled with the neuronal nuclei marker, NeuN in both control mice and hypoxic-reared mice. Moreover, BrdU-positive cells were also found to express neuronal markers at shorter survivals as well, such as colabeling with the neuronal antigen Hu at P23 (5 days after last BrdU injection) and colabeling with NeuN at P29 (11 days after last BrdU injection). Last, they tested for BrdU incorporation during DNA repair in cortical neurons by giving successive BrdU injections at P18 and then sacrificing the animals just 20 h later. Indeed, they did find evidence for BrdU incorporation into

NeuN positive neurons after this short survival, but reasoned through stereological estimates that the number of neurons undergoing DNA repair was not sufficient to account for the number of BrdU/NeuN-positive cells seen at longer survivals. These results contrast with earlier studies that report neurogenesis in the rodent as occurring during a restricted period during development (Angevine and Sidman, 1961; Berry and Rogers, 1965; Caviness and Sidman, 1973; Hicks and D'Amato, 1968; Smart and Smart, 1982). Nevertheless, these results would support recent work reporting that neurogenesis may continue well past embyronic development in the rodent neocortex (Dayer et al., 2005; Gould et al., 2001).

Does neurogenesis continue in the neocortex? One of the reasons that the notion of postnatal and adult cortical neurogenesis is still controversial is due methodological concerns with the use of thymidine analogs such as BrdU to label newly generated cells. Since BrdU incorporation is a measure of DNA synthesis, one concern has been that some cells are labeled by BrdU as a result of DNA repair rather than cell division. This concern has been confirmed in some studies of DNA repair (Moore et al., 2002; Pang et al., 2003), while in others, it has been argued that DNA repair is not a significant source of BrdU incorporation (Cooper-Kuhn and Kuhn, 2002; Zitnik and Martin, 2002). Furthermore, recent studies have demonstrated that dying neurons can undergo cell cycle events and incorporate BrdU during progression of neurodegenerative disease or hypoxic–ischemic injury (Herrup and Busser, 1995; Katchanov et al., 2001; Kuan et al., 2004; Yang et al., 2001). Therefore, it is important that BrdU-positive cells be assessed for expression of cell cycle markers and markers for apoptosis in studies of injury and regeneration.

Another concern is that care must be taken in studies of injury models and regeneration where measurements of neuronal loss and dendritic change must be obtained. For example, it is possible that cell type specific markers can be developmentally regulated or exhibit reduced antigenicity without cell loss after injury, such as immunoreactivity of the neuronal marker NeuN after hypoxia–ischemia (Unal-Cevik et al., 2004). Therefore, use of a general nuclear markers such as DAPI or alternative histochemical detection of cell populations is necessary for quantification of cell loss. Furthermore, one must be cautious when using the neuronal subtype specific neurofilament SMI-32 as a marker of change in cortical projection neurons and their arborizations, since expression of this neurofilament is largely absent in the callosal pyramidal neurons of layers II and V and is not expressed in all neocortical areas (Boire et al., 2005; van der Gucht et al., 2001; Voelker et al., 2004). In these studies where detection of morphological changes must be detected, it may be more useful to utilize methods that display the full morphology of a range of different neuronal cell types, such as with lipophilic dyes, retrograde tracers, or by use of the Golgi staining method as per Park et al. (2002).

Another important implication for studies of postnatal and potentially rare neurogenesis originates from studies of bone marrow stem cells which indicate that bone marrow derived cells can express neural antigens (Priller et al., 2001; Corti et al., 2002; Kopen et al., 1999; Mezey et al., 2003; Muñoz-Elias et al., 2004). Currently, the mechanism by which bone marrow derived cells come to express neural markers is a matter of debate (Lu and Tuszynski, 2005; Wurmser and Gage, 2002; Cogle et al., 2004). Reports that cells of the bone marrow derived lineage are able to adopt the phenotype of target tissues by cell fusion (Terada et al., 2002; Ying et al., 2002) and can cross the blood brain barrier and fuse with neurons (Alvarez-Dolado et al., 2003; Priller et al., 2001) complicates the ability to tell whether a BrdU-positive cell is truly newly generated without further testing for cell lineage and DNA ploidy. Furthermore, the resident tissue macrophages of the brain, the microglia, are derived from the bone marrow lineage, and can undergo replacement throughout life (Bechmann et al., 2001; Corti et al., 2002; Eglitis and Mezey, 1997; Hess et al., 2004; Hickey and Kimura, 1988; Simard and Rivest, 2004; Vallières and Sawchenko, 2003). Given the close interaction between phagocytotic microglia and dying neurons (Marín-Teva et al., 2004), it will be important in studies of cell proliferation and neurogenesis that researchers use a combination of neuronal and glial cell-type-specific markers along with markers of the microglial and mesenchymal lineages.

In spite of the possibility that some of the recovery described by Fagel et al. (2006) could be the result of altered cell cycle events or antigen expression, their results suggest the exciting possibility that endogenous neuronal progenitors in the developing brain may be capable of regeneration after damage by extended hypoxia. A companion paper by Park et al. (2006, this issue) employing entirely different techniques—i.e., introduction of the "reporter cell" concept—supports this possibility. If this latent regenerative potential can be understood in more mechanistic detail, it may open up a new era of therapies for the developing brain that promote enhancement of endogenous neurogenesis.

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