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Induction of neuronal type-specific neurogenesis in the cerebral cortex of adult mice: manipulation of neural precursors in situ

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Abstract

Over the past 3 decades, research exploring potential neuronal replacement therapies have focused on replacing lost neurons by transplanting cells or grafting tissue into diseased regions of the brain [*Nat. Neurosci.* 3 (2000) 67–78]. Over most of the past century of modern neuroscience, it was thought that the adult brain was completely incapable of generating new neurons. However, in the last decade, the development of new techniques has resulted in an explosion of new research showing that neurogenesis, the birth of new neurons, normally occurs in two limited and specific regions of the adult mammalian brain, and that there are significant numbers of multipotent neural precursors in many parts of the adult mammalian brain [*Mol. Cell. Neurosci.* 19 (1999) 474–486]. Recent findings from our laboratory demonstrate that it is possible to induce neurogenesis de novo in the adult mammalian brain, particularly in the neocortex where it does not normally occur, and that it may become possible to manipulate endogenous multipotent precursors in situ to replace lost or damaged neurons [*Nature* 405 (2000) 951–955; *Neuron* 25 (2000) 481–492]. Recruitment of new neurons can be induced in a region-specific, layer-specific, and neuronal type-specific manner, and newly recruited neurons can form long-distance connections to appropriate targets. Elucidation of the relevant molecular controls may both allow control over transplanted precursor cells and potentially allow the development of neuronal replacement therapies for neurodegenerative disease and other central nervous system injuries that do not require transplantation of exogenous cells. © 2001 Published by Elsevier Science B.V.

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1. Introduction

Neural cell replacement therapies are based on the idea that neurological function lost to injury or neurodegenerative disease can be improved by introducing new cells that can replace the function of lost neurons. Theoretically, the new cells can do this in one of two general ways [11]. New neurons can anatomically integrate into the host brain, becoming localized to the diseased portion of the brain, receiving afferents, expressing neurotransmitters, and forming axonal projections to relevant portions of the brain. Such neurons would function by integrating into the microcircuitry of the nervous system and replacing lost

neuronal circuitry. Alternatively, newly introduced cells could more simply constitutively secrete neurotransmitters into local central nervous system (CNS) tissue or they could be engineered to produce growth factors to support the survival or regeneration of existing neurons. Growing knowledge about the normal role of endogenous neural precursors, their potential differentiation fates, and their responsiveness to a variety of cellular and molecular controls suggest that neuronal replacement therapies based on manipulation of endogenous precursors either in situ or ex vivo may be possible.

Neuronal replacement therapies based on the manipulation of endogenous precursors in situ may have advantages over transplantation-based approaches, but they may have several limitations as well. The most obvious advantage of manipulating endogenous precursors in situ is that there is no need for external sources of cells. Cells for transplanta-

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tion are generally derived from embryonic tissue, non-human species (xenotransplantation), or cells grown in culture. Use of embryonic-derived tissue aimed toward human diseases is complicated by limitations in availability and by both political and ethical issues; e.g., current transplantation therapies for Parkinson's disease require tissue from several embryos. Xenotransplantation of animal cells carries potential risks of introducing novel diseases into humans, and questions about how well xenogenic cells will integrate into the human brain. In many cases, cultured cells need to be immortalized by oncogenesis, increasing the risk that the cells may become tumorigenic. In addition, transplantation of cells from many of these sources risk immune rejection and may require immunosuppression, if they are not derived from the recipient.

However, there are also potential limitations to the potential of manipulating endogenous precursor cells as a neuronal replacement therapy. First, such an approach may be practically limited to particular regions of the brain, since multipotent neural precursors are densely distributed only in particular subregions of the adult brain (e.g., the subventricular zone, SVZ, and hippocampal subgranular zone). In some cases, it is possible that there simply may not be sufficient precursor cells to effect functional recovery. In addition, the potential differentiation fates of endogenous precursors may be too limited to allow their integration into varied portions of the brain. Another potential difficulty is that it may be difficult to provide the precise combination and sequence of molecular signals necessary to induce endogenous precursors to efficiently and precisely proliferate and differentiate into appropriate types of neurons deep in the brain.

The substantial amount of prior research regarding constitutively occurring neurogenesis provides insight into the potential and limitations of for endogenous precursor based neuronal replacement therapies. Recent work has partially elucidated the normal behavior of endogenous adult precursors, including their ability to migrate to select brain regions, differentiate into neurons, integrate into normal neural circuitry, and, finally, functionally integrate into the adult brain. Research is also beginning to elucidate biochemical and behavioral controls over constitutively occurring neurogenesis. The location, identity, and differentiation potential of endogenous adult precursors are beginning to be understood. In this review, we will first review research from a variety of labs regarding normally occurring neurogenesis, then review some of our experiments demonstrating that endogenous neural precursors can be induced to differentiate into neurons in regions of the adult brain that do not normally undergo neurogenesis.

2. Constitutively-occurring adult mammalian neurogenesis

Ramon y Cajal [136] has been widely quoted as writing:

“In the adult centers the nerve paths are something fixed, ended and immutable. Everything may die, nothing may be regenerated.” The relative lack of recovery from CNS injury and neurodegenerative disease and the relatively subtle and extremely limited distribution of neurogenesis in the adult mammalian brain resulted in the entire field reaching the conclusion that neurogenesis does not occur in the adult mammalian brain. Joseph Altman was the first to use techniques sensitive enough to detect the ongoing cell division that occurs in adult brain. Using tritiated thymidine as a mitotic label, he published evidence that neurogenesis constitutively occurs in the hippocampus [4] and olfactory bulb [3] of the adult mammalian brain. These results were later replicated using tritiated thymidine labeling followed by electron microscopy [61]. However, the absence of neuron-specific immunocytochemical markers at the time resulted in identification of putatively newborn neurons being made on purely morphological criteria. These limitations led to a widespread lack of acceptance of these results, and made research in the field difficult.

The field of adult neurogenesis was rekindled in 1992, when Reynolds and Weiss showed that precursor cells isolated from the forebrain [103] can differentiate into neurons *in vitro*. These results, and technical advances, including the development of immunocytochemical reagents that could more easily and accurately identify the phenotype of various neural cells, led to an explosion of research in the field. Normally occurring neurogenesis in the olfactory bulb, olfactory epithelium, and hippocampus have now been well-characterized in the adult mammalian brain.

3. Olfactory bulb neurogenesis

The cells contributing to olfactory bulb neurogenesis originate in the anterior peri-ventricular zone, and thus undergo a fascinating and intricate path of migration to reach their final position in the olfactory bulb. Adult olfactory bulb neurogenesis has been most extensively studied in the rodent, though there is *in vitro* [99,66] and *in vivo* [10] evidence suggesting that such neuronal precursors exist in humans. Several experiments show that the precursors that contribute to olfactory bulb neurogenesis reside in the anterior portion of the subventricular zone. When retroviruses [75], tritiated thymidine [75], vital dyes [26,75], or virally labeled SVZ cells [26,79] are microinjected into the anterior portion of the SVZ of postnatal animals, labeled cells are eventually found in the olfactory bulb. Upon reaching the olfactory bulb, these labeled neurons differentiate into interneurons specific to the olfactory bulb, olfactory granule cells and peri-glomerular cells. To reach the olfactory bulb, the neuroblasts undergo tangential chain migration through the rostral migratory stream (RMS) into the olfactory bulb [105]. Once in the olfactory bulb, the neurons migrate along radial glia away

from the RMS and differentiate into interneurons.

Of considerable interest have been the factors that contribute to the direction of migration of the neuroblasts, as well as factors involved in initiating and controlling migration itself. *In vitro* experiments show that caudal septum explants secrete a diffusible factor, possibly the molecule Slit [88] that repels olfactory bulb neural precursors [53]. Consistent with the idea that SVZ precursor migration is directed by repulsion is the finding that SVZ precursors migrate anteriorly along the RMS even in the absence of the olfactory bulb [64]. The tangential migration of the cells seems to be at least partially mediated by PSA-NCAM, expressed by the neuroblasts themselves [54]. This may be modified by tenascin and chondroitin sulfate proteoglycans that are located near the SVZ [33]. Neuroblasts undergoing chain migration along the RMS do not travel along radial glia, although glia may play a role in their migration. Garcia-Verdugo et al. presented anatomical evidence that SVZ neuroblasts migrate within sheaths of slowly dividing astrocytes [32]. However, the astrocyte sheaths may not be necessary for tangential migration, since a great deal of tangential migration occurs in the first postnatal week, before astrocytes can be found in the RMS [71]. Understanding the factors that contribute to normal SVZ precursor migration could be important in developing approaches to induce such precursors to migrate to injured or degenerating regions of the brain.

The effort to identify the neural precursors that contribute to olfactory bulb neurogenesis has generated a great deal of controversy. Two potential sources of olfactory bulb neuroblasts have been suggested: astrocytes in the subventricular zone, and ependymal cells lining the ventricles. It has been reported that single ependymal cells are capable of producing neurospheres [58], free-floating spheres of cultured multipotent neural precursors, neurons, and glia. In contrast, it has also been reported that ciliary ependymal cells form spheres that consist of only astrocytes [21]. Other investigators have been unable to generate neurospheres from single ciliary ependymal cells, and, instead, suggest that the multipotent neural precursors found in the adult brain are a type of astrocyte, expressing astrocytic morphology and glial fibrillary acidic protein (GFAP) [27]. Another, independent report provides support for the concept that multipotent neural precursors with similarities to astrocytes contribute to adult neurogenesis [72]. While the majority of currently available evidence suggests that GFAP-expressing cells in the SVZ are the source of olfactory bulb neurogenesis, it is important to distinguish between true astroglia and a distinct class of precursor cells that may also express GFAP. GFAP, while generally a reliable marker for activated astrocytes, has been used as a sole phenotypic marker in reports suggesting that astrocytes are multipotent neural precursors, or stem cells. It is quite possible that at least some multipotent neural precursors may also express GFAP, while remaining distinct from astroglia. Further clarifying the identity of potentially multiple classes of multipotent

neural precursors that contribute to adult neurogenesis will increase our ability to manipulate such cells.

Although the identity of the adult multipotent neural precursors in the SVZ is still controversial, a number of experiments have been performed to manipulate their fate and examine their potential, both *in vitro* and *in vivo*. These results will guide attempts to manipulate endogenous precursors for brain repair. *In vitro*, subventricular zone precursors have been exposed to a number of factors to determine their responses. Generally, precursor cells have been removed from the brain, dissociated, and cultured in EGF (epidermal growth factor) and/or bFGF (basic fibroblast growth factor). The EGF and/or bFGF is then removed, and the cells are exposed to growth factors of interest. The details of this process, including the particular regions the cells are derived from, the media they are plated in, and the substrate they are plated on, can have significant effects on the fate of the precursors [129]. EGF and bFGF [44–46] both induce the proliferation of subventricular zone precursors, and can influence their differentiation. EGF tends to direct cells to a glial fate, and bFGF more toward neuronal fate [129]. BMPs (bone morphogenetic proteins) promote differentiation of SVZ precursors into astroglial fates [47], while PDGF (platelet derived growth factor) [129,131] and IGF-I [6] promote SVZ precursors to differentiate into neurons. There are conflicting results regarding whether BDNF (brain derived neurotrophic factor) promotes the survival [35,65] or differentiation [2,6] of SVZ precursors *in vitro* [2]. *In vitro* results show that it may be possible to influence the proliferation and differentiation of adult SVZ precursors.

The effects of several growth factors have also been tested *in vivo*, to investigate their effects under physiological conditions. Intracerebroventricularly (i.c.v.) infused EGF or TGF- α induce a dramatic increase in SVZ precursor proliferation, and bFGF induces a smaller increase in proliferation [23,69]. Even subcutaneously delivered bFGF can induce the proliferation of SVZ precursors in adult animals [126]. But despite the fact that newborn, mitogen-induced cells disperse into regions of the brain surrounding the ventricles, it is generally accepted that none of the newborn cells differentiate into neurons [69]. Intraventricularly infused BDNF increased the number of newly born neurons found in the olfactory bulbs of adult animals [135]. These results extend the *in vitro* results, and suggest that it may be possible to use growth factors to manipulate adult endogenous precursors *in vivo* for brain repair.

Several reports have attempted to establish the differentiation potential of SVZ multipotent precursors, but these have yielded conflicting results. Postnatal mouse SVZ precursors can differentiate into neurons in a number of regions in the developing neuraxis [73], while their fate is more limited to astroglia when they are transplanted into adult brain [51]. Adult mouse SVZ precursors injected intravenously into sublethally irradiated mice have been reported to differentiate into hematopoietic cells, inter-

puted as demonstrating the broad potential of neural precursors for differentiation and interlineage ‘trans-differentiation’. However, it is possible that a chance transformation of cultured SVZ cells led to a single transformant precursor accounting for this finding. In this event, it could be concluded that this result is not generally the case. However, labeled multipotent neural precursors derived from adult mouse and transplanted into stage 4 chick embryos or developing mouse morulae or blastocysts, can integrate into the heart, liver, and intestine, and express proteins specific for each of these sites [22]. Adult multipotent neural precursors may not be totipotent, but they appear to be capable of differentiating into a wide variety of cell types under appropriate conditions. These results indicate that the local cellular and molecular environment in which SVZ neural precursors are located can play a significant role in their differentiation. Providing the cellular and molecular signals for appropriate differentiation and integration of new neurons will be critical for neuronal replacement therapies in which endogenous neural precursors are either transplanted or manipulated *in situ*.

4. Olfactory epithelium neurogenesis

Sensory neurons in the olfactory epithelium are continually generated in adult rodents. The globose basal cells of the olfactory epithelium divide, differentiate into neurons, and send their axons through the olfactory nerve to the olfactory bulb [15,56]. Of all the neurons in the mammalian body, olfactory epithelium sensory neurons are most directly exposed to potentially damaging influences, interpreted as necessitating their continual replacement. The constant flow of air over the epithelium brings a continuously varying combination of new odorants and potential insults to the olfactory sensory neurons. Despite the precarious position of olfactory receptor neurons, the population of olfactory receptor neurons is maintained, and mammals maintain a fairly consistent sense of smell throughout life.

Neurogenesis in the olfactory epithelium is strongly modulated by neuronal death in the epithelium. Olfactory nerve lesions or olfactory bulb lesions, which lead to the degeneration of axotomized neurons [19,43], result in an increase in proliferation of precursors [15,16,19,43,107]. The new neurons that form in the adult olfactory epithelium send axons through the olfactory nerve and into the mature olfactory bulb [8,24]. The ability of the newborn neurons to re-form long-distance projections is probably due to both their immature state and the environment through which they extend their axons.

In vitro experiments suggest that mature olfactory sensory neurons produce a signal that inhibits neurogenesis. Olfactory epithelial precursor cells undergo dramatically reduced neurogenesis in the presence of

mature olfactory receptor neurons *in vitro* [17], suggesting that when olfactory sensory neurons are lost *in vivo*, the factors inhibiting neurogenesis are reduced, allowing the formation of new sensory neurons. Understanding the signals that inhibit neurogenesis and neuronal integration may be as important as understanding the signals that foster neurogenesis.

However, the factors that inhibit neurogenesis in the adult olfactory epithelium have not yet been discovered, and research in the field has focused on many of the growth factors that influence neurogenesis in the SVZ and olfactory bulb. Many of the same factors influence both olfactory bulb and olfactory epithelial neurogenesis. IGF-I increases the rate of neurogenesis of olfactory epithelium precursors, both *in vivo* and *in vitro* [100]. *In vitro*, FGF-2 stimulates the proliferation of globose basal cells, TGF- α induces their differentiation, and PDGF promotes their survival [91].

Research in the olfactory epithelium highlights the role of inhibitory factors in controlling neurogenesis; understanding these signals in degenerating portions of the brain may be instrumental in developing neuronal replacement therapies.

5. Hippocampal neurogenesis

Neurogenesis in the adult hippocampus has been extensively studied, due at least partially to the tantalizing connection between the hippocampus and the formation of memory. Does hippocampal neurogenesis play a part in memory formation? This question has only begun to be answered, but our understanding of hippocampal neurogenesis is already quite significant. Of particular interest is the fact that hippocampal neurogenesis can be modulated by physiological and behavioral events such as aging, stress, seizures, learning, and exercise. These properties of hippocampal neurogenesis may provide novel methods of studying neurogenesis and may elucidate broader influences that may be relevant to neuronal replacement therapies.

Hippocampal neurogenesis has been described *in vivo* in adult rodents [4], monkeys [39,42,67] and adult humans [28]. Newborn cells destined to become neurons are generated along the innermost aspect of the granule cell layer, the subgranular zone, of the dentate gyrus of the adult hippocampus. The cells migrate a short distance into the granule cell layer, send dendrites into the molecular layer of the hippocampus, and send their axons into the CA3 region of the hippocampus [49,85,117]. Adult-born hippocampal granule neurons are morphologically indistinguishable from surrounding granule neurons [31]. The precursor cells appear to mature rapidly, and they extend their processes into the CA3 region as early as 4 days after division [49]. The properties of both the precursor cells

and the hippocampal environment likely contribute to the rapid maturation observed.

Much research on hippocampal precursors has been performed *in vivo*, but many *in vitro* results are also useful for understanding the effects of growth factors on the differentiation of hippocampal precursors. Hippocampal precursor cells are studied *in vitro* much like SVZ precursors: they are removed from the brain, dissociated, and typically cultured in either EGF or bFGF; the mitogen is then removed; and the cells are exposed to the growth factors of interest. Hippocampal precursors proliferate in response to FGF-2 and can differentiate into astroglia, oligodendroglia, and neurons *in vitro* [31]. BDNF increases both neuronal survival and differentiation, while NT-3, NT-4/5, and CNTF have more limited effects [77]. Further demonstrating the existence of precursors in the adult human, multipotent precursors derived from the adult human brain can be cultured *in vitro* [70,106].

Hippocampal neurogenesis occurs throughout adulthood, but declines with age [68]. This age-related decline could be due to a depletion of multipotent precursors with time, a change in precursor cell properties, or a change in the levels of molecular factors that influence neurogenesis. Understanding what causes this age-related decrease in neurogenesis may be important in assessing the potential utility of potential future neuronal replacement therapies based in manipulation of endogenous precursors. Although aged rats have dramatically lower levels of neurogenesis than young rats, adrenalectomized aged rats have levels of neurogenesis very similar to those of young adrenalectomized rats [18,89]. These results suggest that it is at least partially increased corticosteroids, which are produced by the adrenal glands, and not a decrease in the number of multipotent precursors, that leads to age-related decreases in neurogenesis. At least in the hippocampus, multipotent precursors survive with advancing age.

Seizures can also increase hippocampal neurogenesis. However, it appears that seizure-induced neurogenesis may contribute to inappropriate plasticity, highlighting the fact that newly introduced neurons need to be appropriately integrated into the brain in order to have beneficial effects. Chemically or electrically induced seizures induce the proliferation of subgranular zone precursors, the majority of which differentiate into neurons in the granule cell layer [9,98]. However, some newborn cells differentiate into granule cell neurons in ectopic locations in the hilus or molecular layers of the hippocampus and form aberrant connections to the inner molecular layer of the dentate gyrus, in addition to the CA3 pyramidal cell region [55,98]. It is hypothesized that these ectopic cells and aberrant connections may contribute to hippocampal kindling [97,102].

Hippocampal cell death or activity-related signals resulting from seizures may modify signals that lead to increased neurogenesis. Induced seizures lead to degeneration of hippocampal neurons [83,101,130], which is fol-

lowed by neurogenesis [9,98]. Excitotoxic or physical lesions of the hippocampal granule cell layer induce precursor cell proliferation within the dentate gyrus and the formation of neurons that have the morphological and immunocytochemical properties of granule cell neurons [41]. These results suggest that hippocampal granule cells either inhibit neurogenesis, as do neurons in the olfactory epithelium, or that they or surrounding cells produce signals that induce neurogenesis as they die. However, since neurogenesis is also increased by less pathological levels of electrophysiological activity [25], it is also possible that signals induced by electrophysiological activity play a role in seizure-induced hippocampal neurogenesis.

Events occurring in the hippocampus dramatically demonstrate that behavior and environment can have a quite direct influence on the brain's microcircuitry. Animals living in an enriched environment containing toys, running wheels, and social stimulation contain more newborn cells in their hippocampus than control mice living in standard cages [62]. Experiments to further assess which aspects of the enriched environment contribute to increased neurogenesis reveal that a large portion of the increase can be attributed to simply exercise via running [123,124]. Associative learning tasks that involve the hippocampus also appear to increase neurogenesis [36]. Stress, on the other hand, can reduce neurogenesis in both rodents [38,120] and primates [42]. An intriguing, but completely speculative, idea that has been advanced by some in this field is that the processes mediating these effects on neurogenesis may underlie some of the benefits that physical and social therapies provide for patients with stroke and brain injury.

Some of the molecular mechanisms that mediate behavioral influences on hippocampal neurogenesis have begun to be elucidated. For instance, IGF-I, which increases adult hippocampal neurogenesis [1], is preferentially transported into the brain in animals that are allowed to exercise. Blocking IGF-I activity in exercising animals reduces hippocampal neurogenesis, suggesting that IGF-I at least partially mediates the effects of exercise on neurogenesis [121]. Stress increases systemic adrenal steroid levels and reduces hippocampal neurogenesis [120]. Adrenalectomy, which reduces adrenal steroids, including corticosteroids, increases hippocampal neurogenesis [18,37], suggesting that adrenal hormones at least partially mediate the effects of stress on hippocampal neurogenesis. Intriguingly, some antidepressant medications also appear to increase neurogenesis [20,84]. Together, these results demonstrate that adult neurogenesis can be modified by systemic signals, suggesting that modifying such systemic signals, and not only local ones, may be useful in developing potential future neuronal replacement therapies involving manipulation of endogenous precursors [63].

Adult hippocampal multipotent precursors can adopt a variety of fates *in vivo*, suggesting that they may be able to

appropriately integrate into neuronal microcircuitry outside of the dentate gyrus of the hippocampus. Hippocampal precursors transplanted into neurogenic regions of the brain can differentiate into neurons, while precursors transplanted into non-neurogenic regions do not differentiate into neurons at all. Adult rat hippocampal precursors transplanted into the rostral migratory stream migrate to the olfactory bulb and differentiate into a neuronal subtype not found in the hippocampus, tyrosine-hydroxylase-positive neurons [118]. However, although adult hippocampal precursors transplanted into the retina can adopt neuronal fates and extend neurites, they do not differentiate into photoreceptors, demonstrating at least conditional limitation of their differentiation fate potential [92,134]. These findings demonstrate the importance of the local cellular and molecular microenvironment in determining the fate of multipotent precursors. These results also highlight that, although adult hippocampal precursors can adopt a variety of neuronal fates, they may not be able to adopt every neuronal fate.

Some recent correlative evidence suggests that newly generated neurons in the adult hippocampus may participate in some way in hippocampal-dependent memory. Non-specifically inhibiting hippocampal neurogenesis using a systemic mitotic toxin impairs trace conditioning in a manner not seen in relevant controls, suggesting a role for newly born neurons in the formation of memories [114]. These results, though correlative so far, suggest that adult-born hippocampal neurons integrate functionally into the adult mammalian brain. Ongoing research in multiple laboratories is exploring the precise role they play in the adult hippocampal circuitry.

An interesting, but as yet unproven, hypothesis concerning the role of hippocampal neurogenesis in human depression has been proposed. Jacobs et al. propose that insufficient hippocampal neurogenesis causally underlies depression [57]. Consistent with this hypothesis, stress-related glucocorticoids are associated with a decrease in neurogenesis, and increased serotonin levels [13,57], or anti-depressants [84] are associated with an increase in neurogenesis. However, the hippocampus is generally thought to be involved in memory consolidation, and less involved in the generation of mood, suggesting that altered hippocampal neurogenesis may be secondary to depression, rather than causative. This subject is reviewed in more depth in a review by Duman et al. in this issue.

6. Cortical neurogenesis

The vast majority of studies investigating potential neurogenesis in the neocortex of the well-studied rodent brain do not report normally occurring adult cortical neurogenesis. Our own results demonstrate a complete absence of constitutively occurring neurogenesis in murine neocortex [82]. However, two studies reported low level,

constitutively-occurring neurogenesis in specific regions of the neocortex of adult primates [40] and in the visual cortex of adult rat [61]. In Gould et al. [40] neurogenesis of 2–3 new neurons/mm³ was reported in prefrontal, inferior temporal, and posterior parietal cortex of the adult macaque, but not in striate cortex, a presumably simpler primary sensory area. It appears that other groups have not yet been able to reproduce these findings in rodents or primates. There exists a single report of neurogenesis in the visual cortex of the adult rat [61], but this study used tritiated thymidine and purely morphological cell type identification, and has not been confirmed by any other group, including our own. It is unclear whether neurogenesis occurs normally in the neocortex of any mammals, but further examination of potential constitutively-occurring neurogenesis in classically non-neurogenic regions will be required to definitively assess the potential existence of perhaps extremely low level neurogenesis.

7. Functional adult neurogenesis occurs in non-mammalian vertebrates

Functional adult neurogenesis also occurs in many non-mammalian vertebrates. The medial cerebral cortex of lizards, which resembles the dentate gyrus of mammals, undergoes postnatal neurogenesis and can regenerate in response to injury [76]. Newts can regenerate their tails, limbs, jaws, and ocular tissues, and the neurons that occupy these regions [14,60]. Goldfish undergo retinal neurogenesis throughout life [59] and, impressively, can regenerate surgically excised portions of their retina in adulthood [52]. Although lower animals can undergo quite dramatic regeneration of neural tissue, it is unclear how relevant this is to mammals. It is thought that selective evolutionary pressures have led mammals to lose such abilities during normal life.

Birds, complex vertebrates whose brains are much closer to mammals in complexity, also undergo postnatal neurogenesis. Lesioned postnatal avian retina undergoes some neurogenesis, with the new neurons most likely arising from Muller glia [29]. In songbirds, new neurons are constantly added to the high vocal center [5,93], a portion of the brain necessary for the production of learned song [94,115,132], as well as to specific regions elsewhere in the brain, (but not all neuronal populations). It is possible to experimentally manipulate the extent to which new neurons are produced in at least one songbird, the zebra finch, which does not normally seasonally replace HVC-RA projection neurons in the song production network, e.g., as canaries do [108]. Inducing cell death of HVC-RA neurons in zebra finches leads to deterioration in song. Neurogenesis increases following induced cell death and birds variably recover their ability to produce song coincident with the formation of new projections from area

HVC to area RA, suggesting that induced neuronal replacement can restore a learned behavior.

8. The location of adult mammalian multipotent precursors

If adult multipotent precursors were limited to the two neurogenic regions of the brain, the olfactory bulb and hippocampal dentate gyrus, it would severely limit the potential of neuronal replacement therapies based on *in situ* manipulation of endogenous precursors. However, adult multipotent precursors are not limited to the olfactory epithelium, anterior SVZ, and hippocampus of the adult brain; they have been cultured *in vitro* from caudal portions of the SVZ, septum [96], striatum [96], cortex [95], optic nerve [95], spinal cord [112,128], and retina [122]. The precursors derived from all these regions can self-renew and differentiate into neurons, astroglia, and oligodendroglia *in vitro*. It is thought that they normally differentiate only into glia or die *in vivo*. Cells from each region have different requirements for their proliferation and differentiation. Precursors derived from septum, striatum, cortex, and optic nerve are reported to require bFGF to proliferate and differentiate into neurons *in vitro*. There are conflicting reports on whether bFGF is sufficient to culture spinal cord precursors [112,128]. Retinal precursors do not require any mitogens in order to divide *in vitro*, though they do respond to both EGF and bFGF. As with all primary cultures, the particular details of the protocols used can strongly influence the proliferation, differentiation, and viability of the cultured cells, so it is difficult to compare results from different labs. It is estimated that adult multipotent precursors can be found in small-but-significant number in various regions of the brain; e.g., separating cortical neuronal precursors by Percoll gradient yields about 140 multipotent precursors per mg, vs. 200 per mg in the hippocampus [95]. Understanding the similarities and differences between the properties of multipotent precursors derived from different regions of the brain will be instrumental in potential developing neuronal replacement therapies based on manipulation of endogenous precursors.

Though it is not generally accepted, there are reports that, in addition to the undifferentiated multipotent precursors that are found in various portions of the brain, mature neurons themselves can be induced to divide [12,48]. While it seems unlikely that a neuron could maintain the elaborate neurochemical and morphologic differentiation state of a mature neuron while replicating its DNA and remodeling its nucleus and soma, it is still theoretically possible. Though it is generally accepted that other neural cells, such as astroglia, can divide, most reports suggest that any attempt by differentiated neurons to re-enter the cell cycle results in aborted cycling and ultimately, death [133]. Significant evidence will need to

be presented to convincingly demonstrate that mature neurons in the adult brain are capable of mitosis.

9. Manipulating the cortical environment

Endogenous multipotent precursors in the adult brain can divide, migrate, differentiate into neurons, receive afferents, and extend axons to their targets. Multipotent precursors are concentrated in the olfactory epithelium, anterior SVZ, and the dentate gyrus of the hippocampus, but they can be found in lower densities in a number of other regions of the adult brain. In addition, these precursors also have a broad potential; they can differentiate into at least three different cell types, astroglia, oligodendroglia, and neurons, given an appropriate *in vitro* or *in vivo* environment. These properties of endogenous multipotent precursors led us to explore the fate of these precursors in an adult cortical environment that has been manipulated to support neurogenesis.

Our laboratory has previously shown that cortex undergoing synchronous degeneration of apoptotic projection neurons forms an instructive environment that can guide the differentiation of transplanted immature neurons or neural precursors. Immature neurons or multipotent neural precursors transplanted into targeted cortex migrate selectively to layers of cortex undergoing degeneration of projection neurons [50,80,111], differentiate into projection neurons [50,80,111,116], receive afferent synapses [80,116], express appropriate neurotransmitters and receptors, and re-form appropriate long-distance connections to the original contralateral targets of the degenerating neurons [50] in adult murine neocortex. Immature neurons or neural precursors transplanted into control intact or kainic acid-lesioned cortex do not migrate, differentiate into projection neurons, or integrate into cortex. Together, these results suggested to us that cortex undergoing targeted apoptotic degeneration could direct endogenous multipotent precursors to integrate into adult cortical microcircuitry. But before discussing the fate of endogenous precursors in such an environment, we will review in more detail the results indicating that neocortex undergoing targeted apoptosis forms an instructive environment for neurogenesis.

Biophysically targeted apoptotic neurodegeneration produces highly specific cell death of selected projection neurons within defined regions of neocortex (Fig. 1). Importantly, surrounding glia, interneurons, and projection neurons that have not been targeted are not injured by this approach [80,81]. Degeneration results from the photoactivation of retrogradely transported nanospheres carrying the chromophore chlorin e_6 [80,110,111]. First, nanospheres carrying the chromophore chlorin e_6 are injected into the axonal terminal fields of the targeted projection neurons. The nanospheres are retrogradely transported to the soma of the projection neurons and stored non-toxicly within

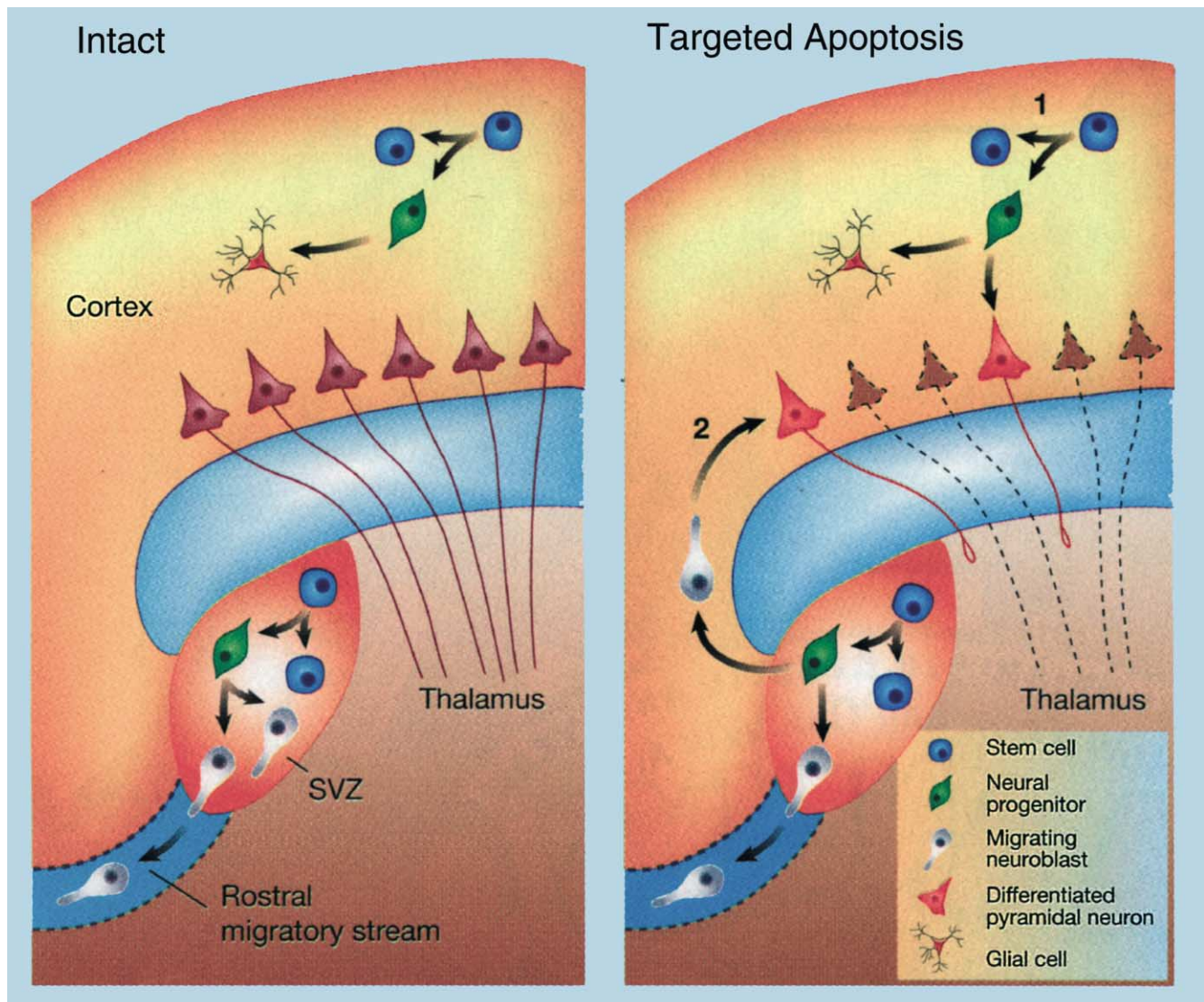


Fig. 1. Induction of neurogenesis in the neocortex of adult mice. Cartoon showing targeted apoptosis of corticothalamic projection neurons and subsequent recruitment of new neurons from endogenous neural precursors, without transplantation, in adult mouse neocortex. (a) In intact adult mouse neocortex, endogenous precursors exist in the cortex itself, and in the underlying subventricular zone (SVZ). Normally, these precursors produce only glia in cortex. Neurons produced in the anterior SVZ migrate along the rostral migratory stream to the olfactory bulb (not shown). (b) When corticothalamic projection neurons are induced to undergo synchronous targeted apoptosis, new migratory neuroblasts are born from endogenous precursors. These migrate into cortex, differentiate progressively into fully mature neurons, and a subset send long-distance projections to the thalamus, the appropriate original target of the neurons being replaced. The new neurons appeared to be recruited from SVZ precursors and potentially also from precursors resident in cortex itself. Adapted from Bjorklund and Lindvall, *Nature* (news and views), 405 (2000) 892–844. Reprinted by permission from *Nature* copyright 2000 Macmillan Magazines Ltd.

neuronal lysosomes. The projection neurons are then noninvasively exposed to long wavelength light through intact dura and/or skull, which specifically induces the chlorin e_6 to produce singlet oxygen, and induces apoptosis of chlorin e_6 containing cells. Chlorin e_6 only produces the reactive oxygen species when excited by near-infrared light. The long wavelength light used is not absorbed by neural tissue and does not cause damage by either heating or radiation-based effects. Since neither the chlorin e_6 nor the long wavelength light cause degeneration by themselves, only neurons both selectively labeled

with the chromophore and located in the controlled light path undergo apoptotic degeneration.

We have applied this approach in several mammalian and avian CNS systems, effecting 50 to 99% degeneration (typically ~70%) of the targeted populations [80,82,108,111]. In neocortex, we have produced selective degeneration of pyramidal neurons with callosal projections (laminae 2/3 and/or 5 neurons), cortico-thalamic projections (lamina 6 neurons), or cortico-spinal projections (lamina 5 neurons) [80–82,111]. Targeted neurons die by apoptosis, via a cascade of characteristic apoptotic

cellular events. Targeted neurons undergo nuclear and internucleosomal DNA fragmentation, undergo heterochromatin condensation, and form apoptotic bodies [109,110]. Targeted neurons are TUNEL positive and are phagocytically removed from the brain without inflammation or gliosis. Neuronal death depends on both new protein synthesis and intrinsic endonuclease activity. This relatively synchronous initiation of neuronal apoptosis leads to prolonged alterations in gene expression [127], that may not be induced by the relatively sporadic cell death that occurs in neurodegenerative disease.

The instructive environment that exists in apoptotically degenerating cortex can alter the differentiation fate of transplanted immature neurons or multipotent precursors. To examine the fate of heterotopically transplanted immature neurons in cortex undergoing apoptotic degeneration, immature neurons derived from E14 mice, which normally contribute to layers 5 and 6 of cortex, and E17 mice were transplanted into cortex undergoing the targeted apoptosis of layer 2/3 callosal projection neurons. The transplanted immature neurons differentiated into neurons in layer 2/3 and formed contralateral callosal projections, which are typical of layer 2/3 neurons but absent in layer 6 neurons. We also tested the potential of two multipotent precursor cell lines: cerebellar-derived C17.2 precursor cells and hippocampus-derived HiB5 precursor cells, derived from an even earlier stage. Both of these multipotent precursor cell lines can differentiate into neurons and glia when transplanted into the embryonic developing brain, and differentiate only into glia (or remain undifferentiated) when transplanted into adult intact or kainic acid lesioned cortex. C17.2 cells differentiated into neurons at low efficiencies when transplanted into regions of cortex undergoing targeted apoptosis. In contrast, similarly transplanted HiB5 cells did not differentiate into neurons, perhaps due to the absence of functional TrKB receptors. These results highlight the fact that, although cortex undergoing apoptotic degeneration is instructive for neuronal differentiation and integration, the newly introduced cells must be competent to respond to such environmental signals.

Immature neurons or precursors transplanted near targeted regions of cortex migrated up to 800 μm to occupy degenerating regions of cortex, while neurons or precursors transplanted into control mice remain within approximately 40 μm of their injection site. Approximately 45% of transplanted E17 neurons underwent directed migration and pyramidal neuron differentiation; the rest remained in the injection site, surviving as small, presumptive, interneurons. In living slice cultures, transplanted immature neurons adopted classic migratory morphologies, with leading and trailing processes, elongated cell bodies, and underwent saltatory migration. They migrated across intact layers 5 and 6, toward targeted regions of cortex. The immature neurons migrated along axons and host-derived, RC-2 positive radial glial-like cells. Host as-

trocytes, identified by the expression of a host-specific transgene, de-differentiated and formed transitional radial glia-like cells only in targeted cortex that contained donor cells. Most neurons completed their migration by 2 weeks after transplantation and had already begun extending axons towards the corpus callosum.

After migrating, transplanted immature neurons integrated into the microcircuitry of the targeted cortex. Synaptophysin staining and electron microscopy demonstrated that more than 65% of donor neurons accepted afferent input. In addition, transplanted neurons expressed neurotransmitters and neurotransmitter receptors appropriate to the region of cortex into which they were transplanted. Donor neurons expressed the same complement of neurotransmitters (glutamate, aspartate, and GABA) and receptors (kainate-R, AMPA-R, NMDA-R, and GABA-R) as endogenous cortical projection neurons [113]. Taken together, these results demonstrate that targeted cortex induces immature neurons to differentiate into neurons that closely resemble the neurons that were induced to degenerate.

Donor neurons or neural precursors transplanted into cortex undergoing apoptotic degeneration re-form specific axonal projections to the original targets of the degenerating projection neurons. Cells transplanted into control intact or kainic acid lesioned cortex do not form any long distance projections. To examine the specificity of the donor neuron's long distance projections, we transplanted immature neurons into cortex undergoing degeneration of transcallosal, cortico-cortical layer 2/3 projection neurons. Using a retrograde label, we found that donor cells re-formed specific callosal connections 6 to 10 mm long to contralateral cortex, the original targets of the degenerating projection neurons. The donor cells did not re-form connections to closer, alternate targets of other nearby populations of S1 neurons, including ipsilateral thalamus, motor cortex, and secondary somatosensory cortex. The efficiency of this anatomic reconnectivity appears to be highly dependent on the developmental stage of the donor neuroblasts or precursors; >40% of donor E19 neuroblasts, 21% of donor E17 neuroblasts, 10–15% of donor E14 neuroblasts, and only 1–3% of transplanted multipotent precursors make long distance projections. The properties of the donor cells are important in determining whether they can respond to the instructive signals produced by targeted apoptosis in neocortex.

To begin to identify the molecular signals that are responsible for the instructive environment produced by cortex undergoing targeted apoptosis of projection neurons, we analyzed the gene expression of candidate neurotrophins, growth factors, and receptors in regions of targeted neuronal death. We compared gene expression to that of intact adult cortex using Northern blot analysis, *in situ* hybridization, and immunocytochemical analysis. The genes for BDNF, NT-4/5, and NT-3 are upregulated only in degenerating regions of cortex, specifically during the

period of projection neuron apoptosis [127]. The expression of a variety of other growth factors that are not as developmentally regulated is not altered. These results are in contrast to the less specific, more immediate, and short-lived changes in gene expression observed in response to non-specific necrotic injuries or seizure-induced injury. Surrounding glia and neurons may change their gene expression in response to activity dependent changes at their synapses or factors released by degenerating projection neurons. For example, BDNF is upregulated specifically by local interneurons adjacent to degenerating projection neurons. We are currently using polymerase chain reaction (PCR)-based suppression subtraction hybridization and DNA microarray approaches to examine the expression of other known and novel factors that contribute to the instructive environment formed by cortex undergoing targeted apoptosis of projection neurons.

10. Induction of neurogenesis in the neocortex of adult mice

Based on the results outlined above, we investigated the fate of endogenous multipotent precursors in cortex undergoing targeted apoptotic degeneration. Although endogenous multipotent precursors exist in the adult brain, including cortex, neurogenesis does not normally occur in postnatal mouse cortex. We examined the fates of newborn cells in targeted neocortex, an environment that is instructive for neurogenesis by exogenous precursors. In these experiments, we addressed the question of whether the normal absence of constitutive cortical neurogenesis reflects an intrinsic limitation of the endogenous neural precursors' potential, or a lack of appropriate microenvironmental signals necessary for neuronal differentiation or survival. In addition, these experiments provide information regarding whether endogenous neural precursors could potentially be manipulated in situ, toward future neuronal replacement therapies.

We found that endogenous multipotent precursors, normally located in the adult brain, could be induced to differentiate into neurons in the adult mammalian neocortex (Fig. 1). We induced synchronous apoptotic degeneration [80,111] of corticothalamic neurons in layer 6 of anterior cortex and examined the fates of dividing cells within cortex, using BrdU and markers of progressive neuronal differentiation. BrdU+ newborn cells expressed NeuN, a mature neuronal marker, and survived at least 28 weeks. First, subsets of BrdU+ precursors expressed Doublecortin, a protein expressed exclusively in immature, typically migrating neurons [30,34], and Hu, an early neuronal marker [7,87]. We observed no new neurons in control, intact cortex. Retrograde labeling from thalamus demonstrated that newborn, BrdU+ neurons can form long-distance corticothalamic connections. Together, these results demonstrate that endogenous neural precursors can be induced in situ to differentiate into cortical neurons,

survive for many months, and form appropriate long-distance connections in the adult mammalian brain.

We induced synchronous apoptosis of layer 6 corticothalamic projection neurons via chromophore-targeted neuronal degeneration (Fig. 2). We targeted layer 6 corticothalamic projection neurons in the anterior neocortex because they are located close to the subventricular zone, a region that contains a relatively large number of multipotent neural precursors. Chlorin e_6 conjugated nanospheres were injected into the thalamus in adult female mice, from which they were retrogradely transported to the somata of

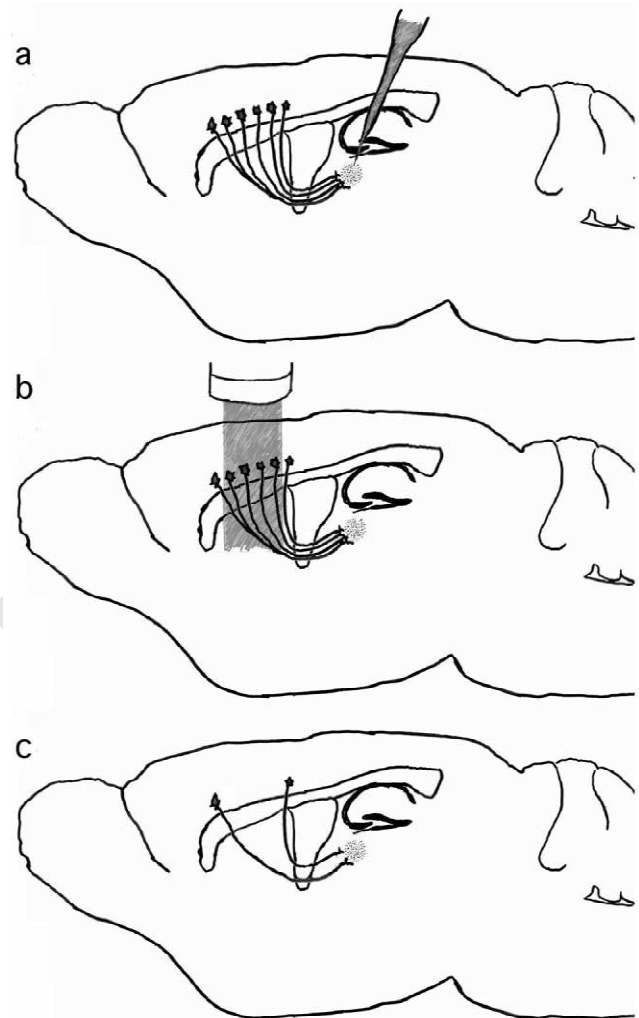


Fig. 2. Biophysically targeted apoptotic neurodegeneration produces highly specific cell death of selected projection neurons within defined regions of neocortex. Degeneration results from the photoactivation of retrogradely transported nanospheres carrying the chromophore chlorin e_6 [80,110,111]. (a) Nanospheres carrying chlorin e_6 are injected into the axonal terminal fields of the targeted projection neurons. The nanospheres are retrogradely transported to the somata of the projection neurons and stored non-toxicly within neuronal lysosomes. (b) The projection neurons are then exposed to long wavelength light, which specifically induces the chlorin e_6 to produce singlet oxygen, and induces apoptosis of neurons containing chlorin e_6 . (c) Since neither the chlorin e_6 nor the long-wavelength light cause apoptosis by themselves, only neurons both selectively labeled with the chromophore and located in the controlled light path undergo apoptotic degeneration.

layer 6 corticothalamic projection neurons. Layer 6 anterior cortex was noninvasively exposed to long wavelength light via intact dura, inducing apoptosis of the corticothalamic projection neurons in anterior cortex. We treated mice with BrdU for the following 2 weeks. After 2, 5, 9, and 28 weeks of survival, we examined the phenotype of BrdU-positive (BrdU+) newborn cells using markers of developing and mature neurons and glia, including Doublecortin, Hu, NeuN, glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP). We examined their axonal projections using the retrograde label FluoroGold.

We found that newborn cells differentiated into mature, NeuN-expressing, neurons and survived for at least 28 weeks. Recently born, BrdU+/NeuN+ neurons were found only in regions of cortex undergoing apoptotic degeneration of layer 6 corticothalamic projection neurons 2–28 weeks after inducing degeneration (Fig. 3). To confirm that newborn cells were double-labeled and not merely closely apposed to pre-existing neurons, we imaged them using laser scanning confocal microscopy and produced 3-dimensional digital reconstructions. The reconstructions confirmed that these newborn cells were neurons. Some newborn neurons had pyramidal neuron morphology (large, 10–15 μm diameter somata with apical processes; Fig. 3a–d) characteristic of neurons that give rise to long-distance projections. In order to examine the theoretical possibility that these newborn cells were inappropriately expressing multiple phenotypic markers, we triple-labeled sections with antibodies to GFAP, an astroglial marker, and MBP, an oligodendroglial marker. BrdU+/NeuN+ neurons were negative for GFAP (Fig. 3j) and MBP, confirming that they had specifically differentiated into mature neurons. We found approximately 50–100 newborn neurons/ mm^3 following induction of apoptosis (see Table 1), while we found no newborn neurons in the neocortex of control mice at any time ($n=13$), confirming previous reports that neurogenesis does not occur in the adult rodent brain. These results demonstrate that endogenous neural precursors can be induced in situ to differentiate into mature neurons and survive for many months in the adult murine neocortex.

To further understand the source of these newborn neurons, we examined where newly born cells were located in experimental and control animals. After 2 weeks

of BrdU treatment, newly born, BrdU+ non-neuronal cells were found throughout both experimental and control cortex. At 2 weeks, experimental mice ($n=6$) had 5400 ± 1500 BrdU+ cells per mm^3 in the experimental regions, not significantly more than control mice ($n=3$), which had 4100 ± 1700 newborn cells per mm^3 . Similarly, aspiration lesions of cortex have been reported to yield inconsistent increases in BrdU labeled cells in the subventricular zone [119]. Previous studies have established that proliferation normally occurs in the adult cortex; however, under normal circumstances, mitotic cells differentiate into glia, remain undifferentiated, or die.

Some newborn cells located within the cortex of experimental animals appear to originate from precursors located within cortex itself, whereas a second population, which was not present in control animals, appeared to originate in or near the subventricular zone. At 2 weeks, pairs of BrdU+ cells, apparently daughters of the same precursor, were found throughout both control and experimental cortex (Fig. 3i). It is possible that some of these newborn cells are the daughters of cortically located adult multipotent neural precursors that have been described in vitro [86,95]. The adult subventricular and ependymal zones, located subjacent to the deep layers of cortex containing degenerating projection neurons, also contain a population of constitutively dividing, multipotent neural precursors [3,74,78,90,103,104,125], and appear to be the source of the second population of newborn cells. Cells from the SVZ population, and perhaps both of these populations, may contribute to induced cortical neurogenesis.

We investigated the early differentiation and migration of the newborn neurons using a marker of early postmitotic neurons, Doublecortin (Dcx). In experimental mice only, newborn BrdU+/Dcx+ neurons with migratory morphologies appeared to migrate from the SVZ through the corpus callosum (Fig. 4a–d) and into experimental regions of cortex (Fig. 4e, f). Doublecortin is a microtubule-associated protein expressed in immature migrating and differentiating neurons [30,34]. Confocal analysis confirmed that these BrdU+ cells express Doublecortin (Fig. 4c, d). No BrdU+/Dcx+ cells were found in the corpus callosum or cortex of control animals. However, they exist in the rostral migratory stream of both controls and experimentals, as previously described [30,34]. Newly born BrdU+/Dcx+ neurons within the corpus callosum displayed morphologies characteristic of migrating neurons, while newborn Dcx+ neurons located in deep layers of cortex displayed more complex morphologies with apical processes that suggest their further differentiation. These results demonstrate the progressive differentiation of endogenous precursors: first into Dcx+ migratory neuroblasts, then into immature Dcx+ neurons with process extension, then into more mature neurons.

The location of Dcx+ neurons suggests that at least some of the newborn neurons that form in targeted cortex are derived directly from SVZ precursors. However, these

Table 1
Number of surviving newborn neurons from 2 to 28 weeks

Survival	Number of BrdU+/NeuN+ cells/ mm^3	No. of mice
2 weeks	97 ± 69	6
5 weeks	43 ± 20	3
28 weeks	78 ± 15	2

The difference between control (0 new neurons/ mm^3) and experimental mice is highly statistically significant ($P < 0.0001$, two-tailed t -test). These results demonstrate that endogenous neural precursors can be induced in situ to differentiate into mature neurons and survive for at least many months in the adult murine neocortex.

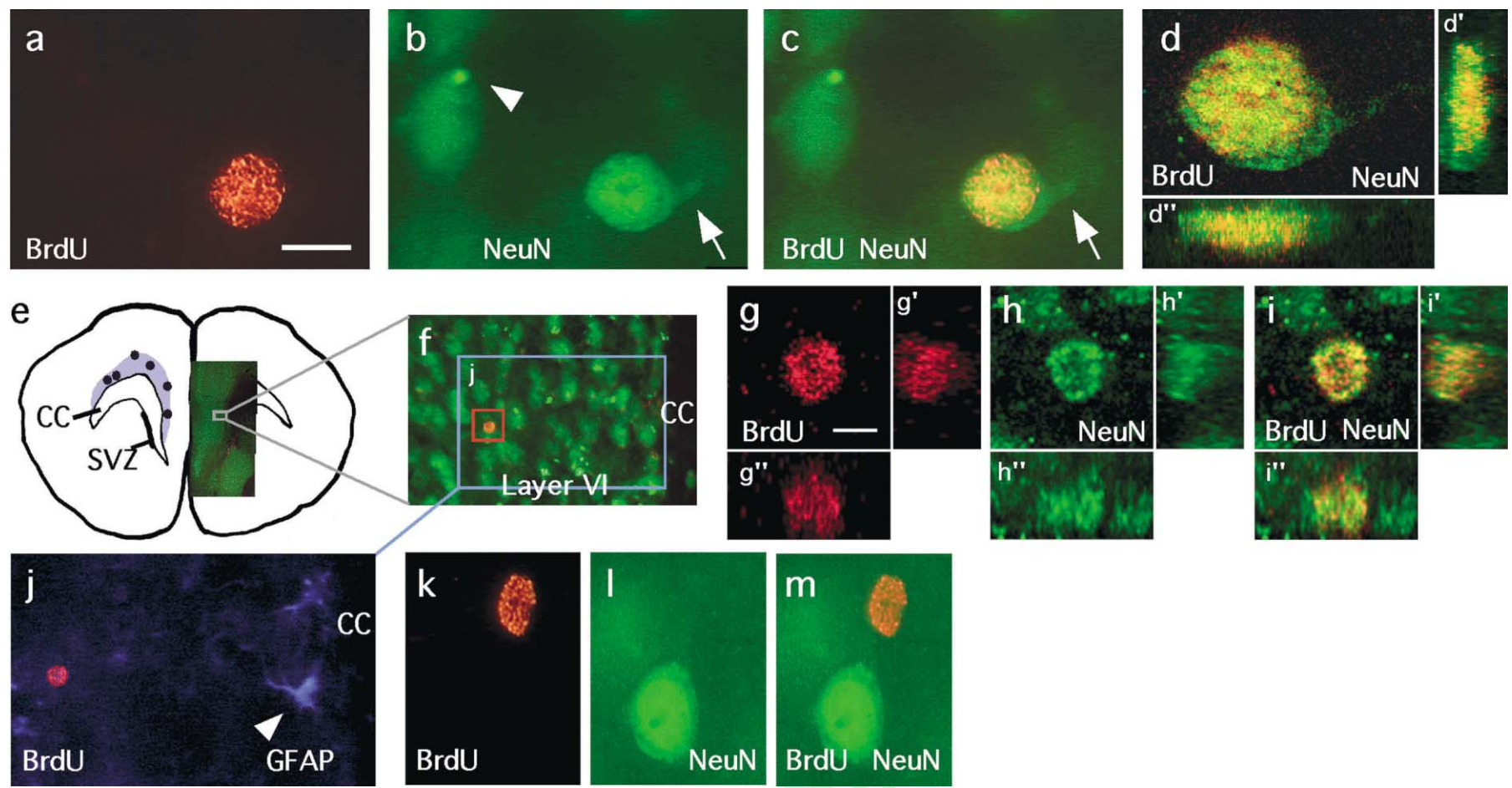


Fig. 3. Newborn, BrdU+ cells can be induced to differentiate into neurons, expressing NeuN, a mature neuronal marker, in regions of cortex undergoing targeted apoptotic degeneration of corticothalamic neurons. (a) A large, densely stained BrdU+ nucleus (red) 28 weeks after induction of apoptosis. Scale bar: 10 μ m. (b) The BrdU+ neuron, lower right, shows typical NeuN staining, with strong nuclear and weaker cytoplasmic labeling. Apical dendrite (arrow). The neuron at left in a different focal plane remains from the original nanosphere targeting for apoptosis; a lysosome containing nanospheres is indicated (arrowhead). No BrdU+/NeuN+ newborn neurons contained nanospheres, demonstrating that they are not original, targeted neurons. (c) Overlay. (d) Confocal images combined to produce a 3-dimensional reconstruction of this newborn neuron. (d', d'') cell viewed along the x-axis and y-axis, respectively, demonstrating colocalization of BrdU and NeuN. (e) Left, camera lucida showing location of BrdU+/NeuN+ cells (dots) within the targeted region (gray). Right, a sample newborn neuron in cortical layer VI. Corpus callosum (CC). Subventricular zone (SVZ). (f) Higher magnification view of layer VI shows a BrdU+ newborn neuron (red box). (g–i) Confocal three-dimensional reconstruction of red boxed region. (g', h', i') x-Axis. (g'', h'', i'') y-Axis. (g) BrdU (red), indicating the cell underwent mitosis during the 2 weeks following induction of apoptosis. Scale bar: 5 μ m. (h) NeuN (green). (i) Merged image. BrdU (red) and NeuN (green) labeling are coincident in all three dimensions. (j) The BrdU+/NeuN+ neuron (red) is GFAP-negative (blue). A GFAP+ astrocyte (arrowhead). (k, l, m) The presence of BrdU+/NeuN- and BrdU-/NeuN+ cells demonstrate that the double labeling protocol is specific. Image from same section as a–d. (k) BrdU (red) and (l) NeuN (green) do not show cross-reactivity. (m) Overlay. (Adapted from Magavi et al. [82]). Reprinted by permission from *Nature* copyright 2000 Macmillan Magazines Ltd.

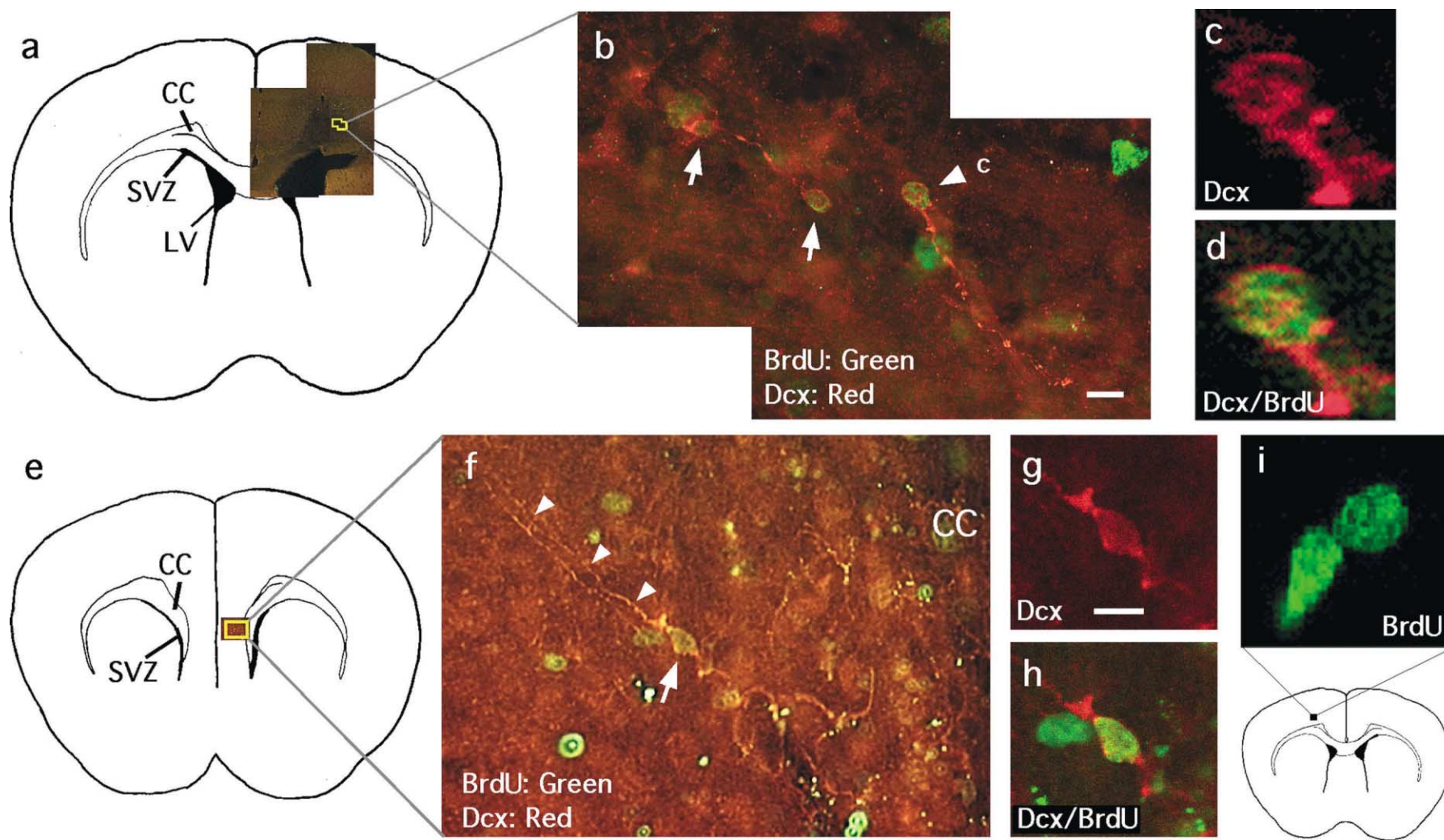


Fig. 4. Newborn cells express the immature, typically migratory neuronal marker Doublecortin (Dcx) 2 weeks after induction of apoptosis. BrdU+/Dcx+ neurons appear to migrate from the SVZ, through the corpus callosum, and into experimental regions of cortex. (a) Camera lucida showing BrdU+ (green)/Dcx+ (red) neurons within the superficial corpus callosum (CC). Lateral ventricle (LV). (b) Fluorescence micrograph of three BrdU+/Dcx+ neurons. Scale bar: 25 μ m. (c, d) Confocal micrographs confirm that the neuron in b (arrowhead) is BrdU+/Dcx+. (e) Camera lucida: a BrdU+/Dcx+ newborn neuron within cortex. (f) Higher magnification: the differentiating BrdU+/Dcx+ newborn neuron (arrow) extending processes (arrowheads) in cortex. No BrdU+/Dcx+ cells were found in the CC or cortex of control mice. Dcx staining is specific for migrating neurons [30,34]; it does not overlap with A2B5, O4, RIP, MBP, or GFAP staining. (g, h) Confocal micrographs of this newborn neuron confirm that it is BrdU+/Dcx+. Scale bar: 10 μ m. (i) Pairs of BrdU+ non-neuronal cells were found throughout cortex in experimental and control mice. (Adapted from Magavi et al. [82]). Reprinted by permission from *Nature* copyright 2000 Macmillan Magazines Ltd.

data do not rule out the possibility that other precursors contribute to neurogenesis. Newly born cortical neurons could theoretically be derived in three different ways. Newly born neurons could arise from endogenous cortical precursors, directly from SVZ precursors, or from endogenous precursors, such as SVZ precursors, that enter cortex and then divide within cortex. Neuronal replacement therapies that depend on precursors derived from normally neurogenic portions of the brain could be limited to regions of the brain that are adjacent to these neurogenic regions.

Thus, further understanding the source of the cells that can contribute to induced neurogenesis could be critical for deciding whether endogenous precursors could potentially form the basis of effective neuronal replacement therapies.

The continued differentiation of these newborn neurons was examined using antibodies to Hu, an RNA-binding protein that begins to be expressed in neuronal nuclei and somata soon after differentiation [7,87]. BrdU+/Hu+ neurons with large, ovoid nuclei were found in the cortex of experimental mice 2 weeks after induction of apoptosis (Fig. 5), whereas no BrdU+/Hu+ cells were found in control mice. We used Hoechst 33258, which stains DNA, as a nuclear counterstain that allowed us to confirm that a specific nucleus belonged to a particular cell. The dispersed heterochromatin pattern demonstrated by Hoechst staining coincident with the BrdU+ nuclei is characteristic of neuronal nuclei and further confirms BrdU+/Hu+ double labeling. The expression of Hu further confirms

induced cortical neuron differentiation by endogenous neural precursors.

These newborn neurons derived from endogenous precursors can re-form long distance projections. To examine the identities of the newborn, BrdU+ neurons and examine their potential ability to establish long distance connections, we injected the retrograde label FluoroGold into the same thalamic sites as the original nanosphere injections. Previous experiments from our laboratory show that large numbers of embryonic neurons transplanted into regions of adult cortex undergoing apoptotic degeneration of projection neurons can re-form long distance connections to the original targets of the degenerating neurons [50]. At 9 weeks, we observed newborn BrdU+ neurons retrogradely labeled with FluoroGold that had large nuclei and large cell bodies denoting pyramidal projection neuron morphology (Fig. 6). These results show that endogenous precursors that differentiate into mature neurons can establish appropriate, long-distance corticothalamic connections in the adult brain.

Several lines of evidence reinforce that BrdU+ neurons in these experiments are newborn neurons, and not pre-existing neurons artifactually labeled by BrdU due to DNA synthesis during apoptosis, as has been seen in some cell culture experiments. First, we directly controlled for this theoretical possibility, since the photoactive targeting nanospheres we employed also carry a fluorescent label, allowing us to identify all neurons targeted to undergo apoptosis. No BrdU+ cell was a targeted neuron, and no

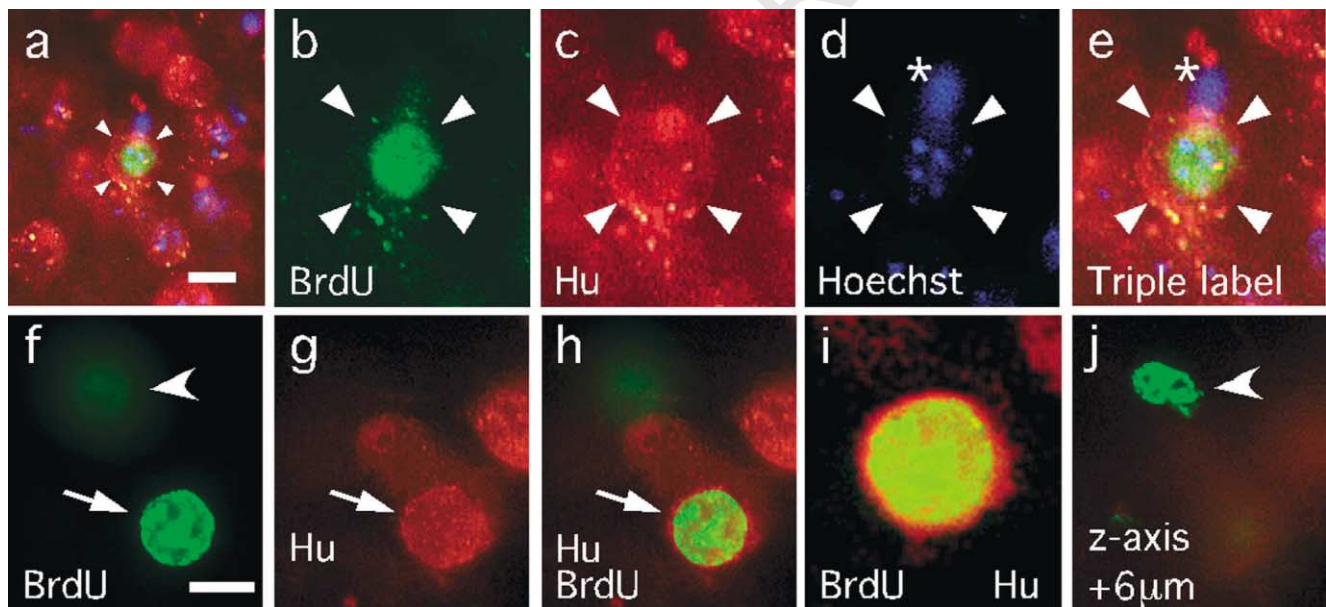


Fig. 5. Newborn, BrdU+ cells express the early neuronal marker, Hu in experimental cortex. (a) A BrdU+/Hu+ newborn neuron (arrowheads) at 2 weeks. Surrounding neurons are BrdU-negative. Scale bar: 10 μ m. (b–e) Higher magnification. (b) BrdU (green) labels the nucleus of a newborn neuron. (c) Hu (red) labels the BrdU+ newborn cell. (d) Hoechst (blue) shows the large, dispersed nucleus of the newborn neuron and the compact nucleus of an adjacent glial cell (asterisk). (e) Overlay. (f) Arrow indicates BrdU+ (green) nucleus of a BrdU+/Hu+ neuron at 28 weeks. Arrowhead indicates a BrdU+/Hu- cell in another plane of focus. (g) Hu+ soma (red) (h) Overlay. (i) Confocal confirmation of double labeling. (j) A BrdU+/Hu- cell located in a focal plane 6 μ m higher demonstrates specificity of staining. (Adapted from Magavi et al. [82]). Reprinted by permission from *Nature* copyright 2000 Macmillan Magazines Ltd.

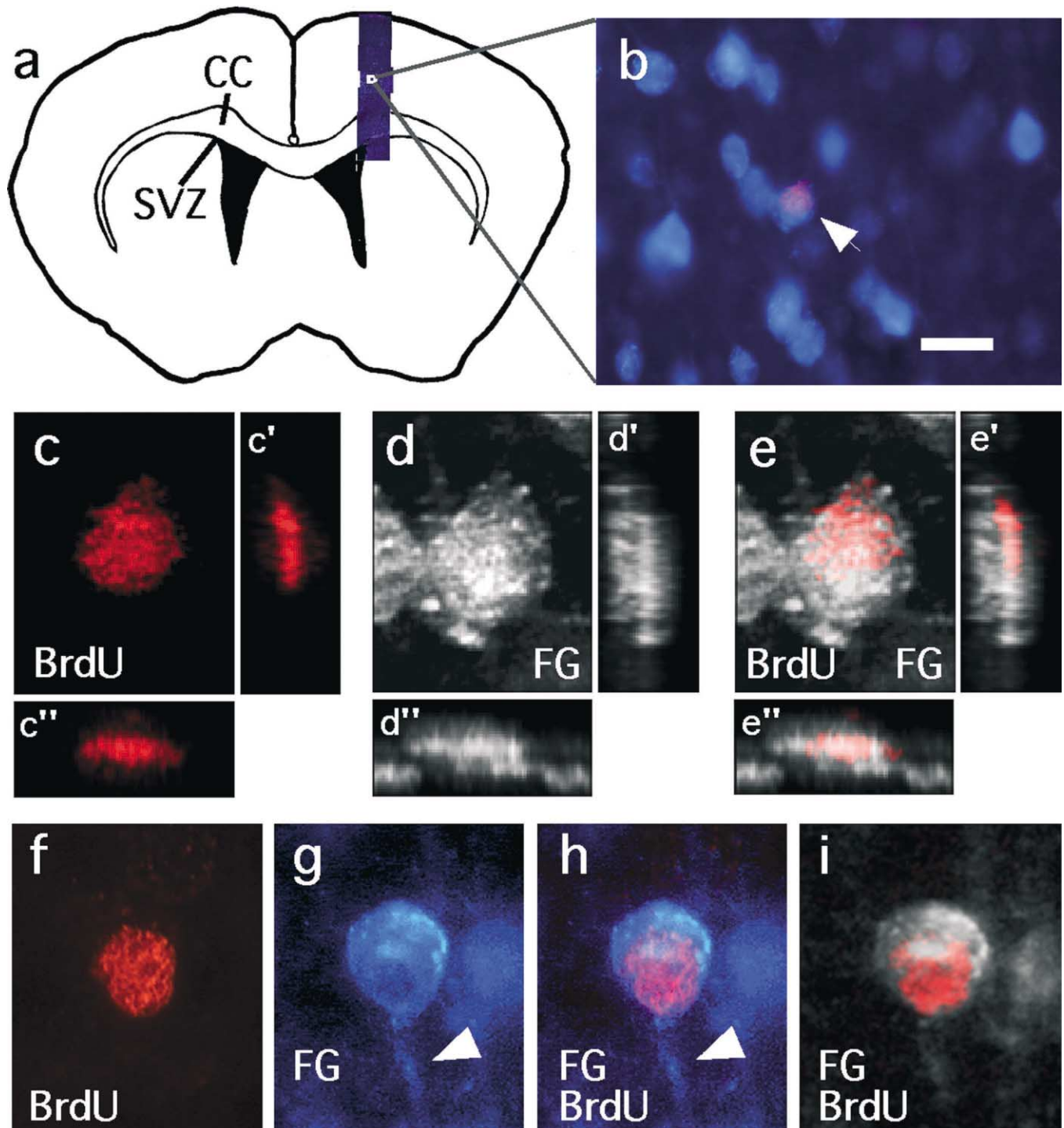


Fig. 6. Newborn, BrdU+ neurons in experimental cortex extend long-distance projections to thalamus. (a) Camera lucida of a BrdU+ (red)/FG+ (white) retrogradely labeled neuron in layer VI of cortex at 9 weeks. Corpus callosum (CC). (b) Higher magnification shows the newborn corticothalamic neuron (arrow) and FG+/BrdU- original neurons. Scale bar: 20 μ m. (c–e) Confocal three-dimensional reconstruction of the neuron. (c', d', e') x-Axis. (c'', d'', e'') y-Axis. (c) BrdU+ nucleus, indicating the cell underwent mitosis in the 2 weeks following induction of apoptosis. (d) FluoroGold+ soma, indicating that this neuron projects to thalamus. (e) Merged image, confirming double-labeling in three dimensions. (f) BrdU+ (red) nucleus of an adult-born FG+ corticothalamic neuron. (g) FG+ (blue) cell body with labeled axon (arrow). (h) Overlay. (i) Confocal microscopy confirms double labeling. (Adapted from Magavi et al. [82]). Reprinted by permission from *Nature* copyright 2000 Macmillan Magazines Ltd.

targeted neuron was BrdU+. Second, the appearance of BrdU incorporation during DNA repair is that of minor nuclear speckling, and not uniform, whole-nucleus labeling

as we observed in these experiments. Third, BrdU+ neurons were present in neocortex over 6 months after the end of the BrdU treatment, demonstrating their viability

and long-term survival. Fourth, and perhaps arguing most definitively for the identification of BrdU+ neurons as newborn neurons is the fact that we were able to trace the early developmental progression of these neurons using Doublecortin, an immature migratory neuronal marker. We found BrdU+/Dcx+ newborn neurons with migratory morphologies in the corpus callosum and appropriate cortical layer 6 of experimental mice, and we identified BrdU+/Dcx+ immature neurons extending early processes in these regions. Further, we have also conducted experiments investigating whether BrdU integrates into dying cells *in vivo*, or whether this is simply a theoretical concern from *in vitro* studies. We examined apoptotic neurons in early postnatal brain ($n > 1000$) and neurons undergoing excitotoxic degeneration induced by ibotenic acid in adult animals ($n > 5000$). Absolutely no apoptotic neurons, cleaved caspase-3 positive, or dying neurons with pyknotic, condensed nuclei integrated BrdU, even when BrdU doses three- to eight-times higher than normal were used. From this combination of evidence, we conclude that healthy, long-lived neurons with dense BrdU labeling throughout their nuclei can be reliably identified as newborn neurons.

Taken together, our results demonstrate that endogenous neural precursors can be induced to differentiate into neocortical neurons in a layer- and region-specific manner and re-form appropriate corticothalamic connections in regions of adult mammalian neocortex that do not normally undergo neurogenesis. It appears that these results are generalizable to other classes of projection neurons. The same microenvironment that supports the migration, neuronal differentiation, and appropriate axonal extension of transplanted neuroblasts and precursors also supports and instructs the neuronal differentiation and axon extension of endogenous precursors.

11. Conclusions

Recent research suggests that it may be possible to manipulate endogenous neural precursors *in situ* to undergo neurogenesis in the adult brain, toward future neuronal replacement therapy for neurodegenerative disease and other CNS injury. Multipotent precursors, capable of differentiating into astroglia, oligodendroglia, and neurons exist in many portions of the adult brain. These precursors have considerable plasticity, and, although they may have limitations in their integration into some host sites [134], they are capable of differentiation into neurons appropriate to a wide variety of recipient regions, when heterotopically transplanted [118]. Many adult precursors are capable of migrating long distances, using both tangential and radial forms of migration [26,75,79]. Endogenous adult neural precursors are also capable of extending axons significant distances through the adult brain [8,24,82]. In addition, *in vitro* and *in vivo* experiments have begun to elucidate the

responses of endogenous precursors to both growth factors and behavioral manipulations, and are beginning to provide key information toward manipulation of their proliferation and differentiation. Recent experiments from our laboratory have shown that, under appropriate conditions, endogenous precursors can differentiate into neurons, extend long distance axonal projections, and survive for long periods of time in regions of the adult brain that do not normally undergo neurogenesis. These results indicate that there exists a sequence and combination of molecular signals by which neurogenesis can be induced in the adult mammalian cerebral cortex, where it does not normally occur.

Together, these data suggest that neuronal replacement therapies based on manipulation of endogenous precursors may be possible in the future. However, several questions must be answered before neuronal replacement therapies using endogenous precursor become a reality. The multiple signals that are responsible for endogenous precursor division, migration, differentiation, and axon extension will need to be elucidated in order for such therapies to be developed efficiently. These challenges also exist for neuronal replacement therapies based on transplantation of precursors, since donor cells, whatever their source, must interact with the mature CNS environment in order to integrate into the brain. In addition, it remains an open question whether potential therapies manipulating endogenous precursors *in situ* would be necessarily limited to portions of the brain near adult neurogenic regions. However, even if multipotent precursors are not located in very high numbers outside of neurogenic regions of the brain, it may be possible to induce them to proliferate from the smaller numbers that are more widely distributed throughout the neuraxis. Potentially, it may be possible to induce the repopulation of diseased mammalian brain via the specific activation and instructive control over endogenous neural precursors along desired neuronal lineages. However, the field is just at the beginning of understanding the complex interplay between particular neural precursors' potential, their heterogeneity and how to take advantage of what may be partial cell-type restriction, permissive and instructive developmental signals, and modulation of specific aspects of neuronal differentiation and survival. Progress over the past decade has been great, and the coming decades promise to offer significant insight into these and other critical issues for the field.

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