Chapter 7

Curcuma oil: neural plasticity

"I have been impressed with the urgency of doing. Knowing is not enough; we must apply. Being willing is not enough; we must do."

- Leonardo da Vinci (1452-1519) -
7.1 Introduction

The adult brain harbors a population of neural stem cells (NSCs) throughout life in the subgranular zone (SGZ); (Palmer et al., 1997) of the hippocampus and adjacent to the lateral ventricles in the subventricular zone (Reynolds and Weiss, 1992). These NSCs are self-renewing, with the potential to generate all three basic cell types of the central nervous system: neurons, oligodendrocytes and astrocytes. In vivo they provide a constant supply of new cells to particular regions of the adult brain, including the hippocampus and the olfactory bulb.

Adult neurogenesis is often studied using bromodeoxyuridine (BrdU) or other thymidine analogues to label DNA synthesis in nascent neurons during the S-phase of the cell cycle.

Previous studies showed that endogenous precursors can generate new neurons in the ischemic striatum but not in the cortex, despite a large lesion burden at this site (Arvidsson et al., 2001; Parent et al., 2002). However, newer studies show that repair mechanisms may allow cortical recovery as well, (Magavi et al., 2000; Gotts and Chesselet, 2005b; Thored et al., 2006), and thus cortical regeneration after ischemia remains controversial.

Functional recovery from ischemic damage is the ultimate goal of any therapeutic effort, and inspiring evidence suggests that this is possible. Behavioral evidence also suggests a recovery of brain function after growth factor treatment in ischemic animals. Ischemia has a deleterious effect on learning in a Morris water maze, a hippocampus-dependent learning task. We observed in our previous chapter that C.oil treated rats performed significantly better compared with I/R after the insult. Our previous study thus prompted us to explore the C.oil dependent cortical recovery from transient ischemia.

7.2 Results

7.2.1 Stroke Alters Proliferation in the SVZ

Rats received injections of BrdU were made ischemic by occlusion of MCAo for 2hrs and were euthanized 24 hours after reperfusion. After24hrs, the number of BrdU-labeled cells in sham control animals was scanty. In sham-operated rats killed 24hrs after the surgery, 97%
of BrdU+ cells were seen in the SVZ. Low numbers of BrdU+ cells were observed in the cortex. Most of these cells were NeuN (78%) at the SVZ.

**7.2.2 C. oil enhances accumulation of newly born cells: expression of Doublecortin in Brain Neurons**

Many BrdU+ cells were colabeled with doublecortin (DCX), a marker for migrating neuroblasts. In animals subjected to stroke, few DCX processes were oriented into the cortex (Fig.1). In MCAo animals BrdU+/DCX+ staining was confined to the SVZ, suggesting that in 24 hours after stroke onset the distant injury triggers process extension or migration toward the injury. Most cells that expressed BrdU in the cortex (85%) after 24hrs of treatment with C. oil were also positive for DCX marker for new neuron (Fig.1).

**Fig.1:** Photomicrographs (representative of 5 photomicrographs) showing double cortin (DCX) immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24 hrs of reflow in I/R, and I/R-C. oil treated groups. Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, & K) and neuroblast marker DCX shown as red fluorescence (A, D, G, & J). The merger is shown in C, F, I, &L. (A, B & C) represent sham; (D, E & F) I/R day 24 hrs; (G, H & I), (J, K & L)
represent I/R-C. oil group at 24 hrs (I/R + C. oil 24hrs) rat sections. Magnification: 40X (Bar 20µm)

7.2.3 C. oil promotes migration from proliferating region to the site of damage

After 24hrs of Ischemia a robust increase in the number of newborn BrdU+ cells were observed in the SVZ, the corpus callosum and in the peri-infarct area. About 70% of these BrdU+ cells colabeled with NeuN marker. Fewer BrdU+ cells were observed in the cortical sub region in ischemic animals at 24 hours post reperfusion (Fig.2). BrdU+ cells were more numerous in C. oil treated animals than ischemic controls 24hrs postischemia. The number of BrdU+ cells in the ipsilateral SVZ remained suppressed. This suggests that labeled cells were depleted from the injury-proximal SVZ by proliferative dilution of the BrdU+ label or migration away from the SVZ. This increment was larger in C. oil treated rats (Fig.2).
Fig. 2: Photomicrographs (representative of 5 photomicrographs) showing neurogenesis. BrdU immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1 hr of transient ischemia followed by 24 hrs of reflow in I/R, and I/R-C.oil treated groups. (Five animals per group were used). Sections were immunolabeled with a polyclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, K & N) and neuron specific marker NeuN shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L & O. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 24 h (I/R-24h); (G, H & I), (J, K & L) represents I/R-C.oil group at 24 h (C.oil 24h), rat sections. Double-labeled newly generated migrating neuron: arrow. Magnification: 40X (Bar 20μm).

With C.oil treatment, the proliferative response observed in the peri-infarct cortex was increased (Fig. 3). These results show that cell proliferation occurs in the injured cortex and suggest that most newborn cells with a defined phenotype in the cortex originate in the SVZ. BrdU⁺ neurons in the CA1 region were labeled with NeuN, demonstrating that C.oil promotes
generation of new neurons. By 24 hrs after the insult, some BrdU+ cells migrate to the damaged CA1 region of the hippocampus and express mature neuronal markers, including NeuN (Fig.3)
Fig. 3: Effect of C. oil treatment on neurogenesis at 24 hrs. BrdU labeling (in brain cryosections) of C. oil treated I/R-24 hrs. (Five animals per group were used). 7 consecutive 1-µm confocal images in z-dimension showing NeuN, BrdU immunoreactivity separately or as merged image. Arrows indicate double-labeled cells. BrdU labeling (in brain cryosections) of C. oil treated I/R-24hrs. Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence middle panel and neuron specific marker NeuN shown as red fluorescence upper panel. The merger is shown in lower panel. Double-labeled newly generated neuron: arrows. Magnification: 40X (Bar 20 µm)
7.2.4 C.oil increases expression of phenotypic markers: Glial cell migration

Low-GFAP regions were also examined; no significant differences were observed between groups in the contralateral hemispheres, but the density of BrdU⁺ cells was increased in the ischemic hemisphere in C.oil treated and non-treated MCAo animals at 24hrs (Fig.4). BrdU-labeled cells with high GFAP was observed in the regions of striatum and cortex. Smaller increases in BrdU⁺/GFAP⁺ cells were seen in striatum region 24hrs after stroke. With C.oil treatment after 24 hrs of reflow the BrdU⁺/NeuN⁺ cells and GFAP cells were not out numbered than MCAo untreated rats.

Fig.4: Photomicrographs (representative of 5 photomicrographs) showing GFAP immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24hrs of reflow in I/R, and I/R-C.oil treated groups. (Five animals per group were used). Sections were
immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, K & N) and Glial cell marker GFAP shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L& O. (A, B & C) represents sham; (D, E & F) as ischemia/reflow at 24 hrs (I/R-24hrs); (G, H & I), (J, K & L) represents I/R-C.oil group at 24 hrs (C.oil 24hrs), rat sections. Double-labeled newly generated GFAP" cell: arrows. Magnification: 40X (Bar 20μm)

7.2.5 C.oil accelerates stroke induced angiogenesis

VEGF expression was analyzed because this secreted protein promotes vascular development and may also act as a neural survival signal. The majority of BrdU+ cells co-expressed VEGF (Fig.5). The number of BrdU+ VEGF cells increased in the ischemic and C.oil group almost 2-fold (Fig.5).

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Fig.5: Photomicrographs (representative of 5 photomicrographs) showing VEGF immunoreactivity in brain cryosections from sham group (A-C) cerebral tissue subjected to 1hr of transient ischemia followed by 24hrs of reflow in I/R, and I/R-C.oil treated groups. (Five animals per group were used). Sections were immunolabeled with a monoclonal anti-BrdU antibody, which was observed as green fluorescence (B, E, H, K & N) and VEGF shown as red fluorescence (A, D, G, J & M). The merger is shown in C, F, I, L& O. (A, B & C) represents sham; (D, E & F) as ischemia reflow at 24 hrs (I/R-24hrs); (G, H & I), (J, K & L) represents I/R-C.oil group at 24 hrs (C.oil 24hrs), rat sections. Double-labeled cells: newly generated VEGE+ cells with healthy nucleus: arrows. Magnification: 40X (Bar 20μm)
7.3 Discussion

Proliferation after ischemia is advantageous only if the new cells ultimately repopulate brain regions depleted of cells as a consequence of stroke. The adult vertebrate brain retains the capacity for neurogenesis, which resides largely in selected regions: the subventricular zone (SVZ), especially that portion adjacent to the most rostral parts of the lateral ventricles (Lois and Alvarez-Buylla, 1993; Kirschenbaum and Goldman, 1995), and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Altman, 1963). Some reports suggest that additional regions, such as the cerebral neocortex, may also generate new neurons in the adult, but this is disputed (Rakic, 2002). To study cells born after 24hrs following MCAo a daily injections of BrdU was given for 7 days post stroke. The data presented here suggest that: newborn cells are first found in the SVZ, subsequently migrate through white matter, and accumulate in the cortex and subcortical white matter areas surrounding the infarct forming a regenerative zone with many primitive neural precursor cells immediately adjacent to the injured brain tissue.

There are reports of an endogenous neurogenic response restricted to the striatum after a large focal ischemia in the forebrain (Parent et al., 2002; Thored et al., 2006). A slow endogenous repair process in the hippocampus after global ischemia is very well documented (Nakatomi et al., 2002). Cellular regeneration in the striatum and hippocampus might be a consequence of their known proximity to proliferative zones that are known to generate neurons in the adult. However, the presence of cortical regeneration is still debatable (Arvidsson et al., 2002). Although cortical neurogenesis is controversial, much localized lesions of neurons in this area have been shown to generate cell replacement with newly generated neurons surviving for long periods and extending axons to appropriate distant target sites. (Magavi et al., 2000) Occluding the MCA at the pial surface results in ischemic injury restricted to the cerebral cortex, rapid activation of proliferation in the SVZ and migration of newborn cells into the parenchyma (Gotts and Chesselet, 2005a, b). Newborn cells were recently shown to continuously form after ischemic injury involving the striatum (Thored et al., 2006). The present study BrdU+/DCX+ double-positive cells were found to be formed. The SVZ was found to be of increased size over time and DCX+ cells were observed. Our study is limited because we did not perform a long time evaluation (more than week). Future experiments with labeling GFP and tracking these cells at the cortex from the SVZ or whether at least some emerge from other locations including the cortex, where they may reside in a quiescent form. The two recent articles that failed to detect newborn neurons at the
immediate infarct border (Komitova and Eriksson, 2004; Gotts and Chesselet, 2005b). A contradicting observation was made that newborn progenitors were constantly present in the area close to the lesion. It is possible that factors such as inflammation, metabolic stress or local inhibitory components within the white matter surrounding the infarct prevent terminal differentiation of newborn cells into neurons. Importantly, newborn cells expressing neuronal antigens could be seen far more often in the group treated with C.oil.

Does the mere increase in the numbers of newborn cells is associated with better functional outcomes after cortical ischemia? The better neurological score, spatial memory and latency time studies support this notion (Chapter 3). Furthermore, VEGF-induced angiogenesis in the peri-infarct area, free radicals, may also contribute to improved metabolism and function of surviving neurons. C.oil acts as potent antioxidant (Chapter 5).

Importantly, C.oil treatment resulted in increased numbers of progenitors in the cortex after ischemia. This implies that factors related to the presence of ischemic injury are necessary to further increase neurogenesis and induce migration of newborn cells to the cortex.

Recent reports that the inducible form of nitric oxide synthase (iNOS) linking to neurogenesis after ischemic injury (Zhu et al., 2003). MCAo in adult rats induces iNOS at high levels in the ipsilateral DG. Inhibition of iNOS activity without altering its expression attenuates the level of BrdU incorporation after ischemia. The same is true in iNOS knockout mice; however, infarct volume is also significantly decreased in these animals. C.oil in MCAo could attenuate the iNOS expression (Chapter 6). This makes it difficult to dissociate the decrease in cell proliferation observed with a lack of iNOS activity from a lesser extent of injury. Nevertheless, these data provide yet another potential mechanism that may be responsible for the cell proliferation observed after ischemic damage.

Taken together, our results suggest that in ischemic lesions restricted to the cortex, probably long-term stimulation with C.oil can stimulate regenerative processes that induce partial behavioral recovery. In the present study we observed BrdU+ neurons in the CA1 region were labeled with NeuN, demonstrating that C.oil promotes generation of new neurons. Ischemia has a deleterious effect on learning in a Morris water maze, a hippocampus-dependent learning task. The C.oil treated rats performed significantly better compared with I/R after the insult (Chapter 4).
These preliminary data encourage studies to determine the regenerative consequences of these long-term cellular responses to further define appropriate ligands that can promote recovery. The number of BrdU$^+$ cells in high-GFAP regions of cortex and striatum of C.oil treated animals increased significantly above ischemic and sham animals, and this increase was maintained for 24hrs (Fig.4). Recovery of DCX cells supported the conclusion that these cells were generated in the SVZ and migrated toward the area of injury in the cortical subregion umbra. Thus, many of the newborn cells observed in the infarct penumbra may have originated in the SVZ.

In the present study, slight increases in DCX$^+$, and GFAP$^+$/BrdU$^+$ regions in curcumin treated animals was observed. Many cells expressed NeuN. Immature DCX$^+$ cells may eventually mature into NeuN$^+$ neurons, but the severely challenged penumbra may not provide adequate cues to allow more complete differentiation. Although some degree of spontaneous recovery after stroke is normal, the molecular and cellular mechanisms underlying recovery are poorly understood and existing treatment for stroke is generally applicable to only a small percentage of cases, or is limited in efficacy. Endogenous mechanisms of neuroprotection from ischemia, including mechanisms of neurogenesis, may hold clues for the development of improved therapy.