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Characterization of Migrating Cells from Ventricular Zone (VZ) to Distant Limbic Structures after Multiple Neonatal Seizures

Melissa Corcia
Seton Hall University

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by

Melissa Corcia

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Approval Page

Dr. Linda Friedman
Mentor

Dr. Sulie L. Chang
Co-mentor and Committee Member

Dr. Jane Ko
Committee Member

Dr Sulie L. Chang
Chairperson, Department of Biology

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Table of Contents

Tables

Table of Figures

Abstract

Abnormal migration of developing neurons or neuronal precursors may contribute to aberrant anatomical connections and increased incidence of seizures with maturation. The retrograde tracer carbocyanine dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI) and 4-chloromethyl benzoyl amino tetramethyl rhodamine (CMTMR) were used to determine whether proliferating precursor cells disperse to distant limbic locations in the postnatal brain with increasing number of perinatal seizures. Immunohistochemistry with precursor neuronal and non-neuronal markers was used to identify the cell types affected. Kainic acid (KA) was administered intraperitoneally (i.p.) three times (3xKA), once on postnatal (P) days P6, P9, and P13. DiI or CMTMR was stereotaxically injected bilaterally into the dorsal ventricular zone (VZ) of P13 pups three hours after the third KA-induced seizure. Animals were sacrificed after 5 days or 45 days. In short and long-term controls, diffuse labeling was observed only near the infusion site and few DiI labeled cells co-localized with nestin, an undifferentiated neural stem cell marker, at 5 and 45 days; however a few precursor-like cells lined the third ventricle at both times. In contrast, after 3xKA and 5 days and even more so after 45 days many cells migrated from the injection site and formed a chain notably into ventral cortico-amygdala and ventral hypothalamic structures; a striking overabundance of cell proliferation was observed within the third ventricle that increased with maturation. Nestin and NeuN co-expressed with many immature cells labeled with DiI or CMTMR near the VZ and in distant thalamic and amygdala structures suggesting that these precursors originate from the VZ and may eventually differentiate into neurons

in distant locations to alter appropriate target innervations and induce latent epileptic disease. Co-localization of the third ventricle with GFAP was observed and suggests that early life seizures increase ventricular astrocytosis during the course of development. Co-localization was also observed with vimentin, A2B5, and RYP antibodies, oligodendrocyte and non-neuronal cell precursor markers, in either VZ, corpus collosum, thalamic or limbic structures suggesting that early seizures simultaneously increase proliferating oligodendrocytes and non-neuronal cell types to contribute to previously unrecognized long-term perinatal-induced seizure pathology.

Introduction

Defining Epilepsy

Epilepsy is a chronic brain disorder that is characterized by recurrent unprovoked seizures. It is derived from the Greek word epilepsia, which means "a taking hold of" or "to seize" (Scott, 1969). An epileptic seizure is a state caused by an abnormal excessive neuronal discharge within the central nervous system. Among the most common manifestations of symptoms are episodes of strange sensations, muscle spasms, altered behaviors and emotions, and loss of consciousness (Hesdorffer et al., 1998). Status epilepticus is defined as a seizure lasting more than 30 minutes (Lowenstein and Alldredge, 1998). The incidence of status epilepticus is highest in the first year of life and in people over the age of 60. Approximately 2.5 million people in the United States are affected by epilepsy with an estimated 125,000 new cases each year, 30% of this group comprising children less than 18 years old (Lowenstein and Alldredge, 1998; Holmes, 2002). There is controversy as to whether early seizures are harmful (Camfield, 1997). There has been evidence shown from several rodent models indicating that seizures occurring in the neonatal period do not produce damage, but can have permanent morphological and functional effects (Meldrum, 2001).

Classification

The classification of epileptic seizures is based on clinical events and the characteristics of electroencephalogram (EEG). According to the classification, epileptic seizures are divided into *partial* and *generalized* seizures. Partial seizures are indicated by initial activation of a system of neurons limited to an individual region of one cerebral

hemisphere (Vessal et al., 2004). Partial seizures are distinguished as *simple* or *complex*, when consciousness is retained and when consciousness is impaired, respectively. Simple and complex partial seizures can further develop into *secondarily generalized seizures* with characteristic tonic and clonic motor manifestations (Commission on Classification and Terminology of the International League Against Epilepsy (ILAE), 1981). Generalized seizures are indicated by bilateral EEG patterns, reflecting neuronal discharge which is spread throughout both cerebral hemispheres of the brain (Glaser, 1997). Generalized seizures are divided into tonic-clonic, absence, myoclonic and atonic seizures (ILAE, 1981).

GENERALIZED SEIZURES	**SYMPTOMS**
Tonic-Clonic or "Grand Mal"	Unconsciousness, convulsions, muscle rigidity
Absence or "Petit Mal"	Brief loss of consciousness
Myoclonic	Jerking movements, loss of consciousness
Atonic	Loss of posture or tone in limbs

Table 1: The symptoms caused by generalized seizures (ILAE, 1981).

PARTIAL SEIZURES	**SYMPTOMS**
Simple	No loss of consciousness Muscle rigidity, jerking, spasms Unusual tastes or sensations Memory or emotional disturbances
Complex	Impairment of consciousness Automatisms such as, lip smacking, chewing, fidgeting, and other repetitive movements
Secondary generalized	Symptoms associated with a preservation of consciousness that then evolves into a loss of consciousness and convulsions.

Table 2: The symptoms caused by partial seizures (ILAE, 1981).

Temporal Lobe Epilepsy

The origin of recurrent complex partial seizes is most common in the temporal lobe. Over 60% of seizure cases originate in the temporal lobe, classified as temporal lobe epilepsy (TLE). Other parts of the brain considered to have a low threshold and high susceptibility to seizure activity are the motor cortex, the limbic structures, and regions that play a role in autonomic function (Hauser, 1991). The temporal lobe (TL), including its deeper limbic nuclear aggregates, the amygdala and the hippocampus, are predominantly involved in seizure development. Consequently, the hippocampal formation and amygdala are highly sensitive to seizure-induced neurodegeneration (Zhang et al., 2002). The vascularity of these structures is vulnerable to compression, and the tissues have a high sensitivity to biochemical disturbances produced by hypoxia, metabolic agents, viruses, and genetic states (Glaser, 1997; Hauser, 1991).

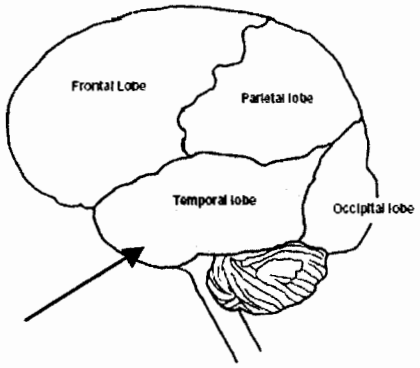

FIGURE 1: The Human Brain is composed of four lobes: frontal, parietal, occipital, and the temporal lobe. The TL is the region primarily affected in epilepsy.

The incidence of epilepsy is increased in a number of postnatal insults such as; head trauma, central nervous system (CNS) infections, cerebrovascular disease, and brain tumors (Annegers, 1996; French et al., 1993). TLE has been described as a disorder secondary to lesions such as; hippocampal sclerosis, vascular malformations, and

neuronal migration disorders (Bruton, 1988; Vadlamudi et al., 2003). The occurrence of febrile convulsions can lead to TLE (French et al., 1993). Approximately 4% of all children will have one or more febrile seizures (Camfield, 1997). Studies have shown that recurrent episodes of febrile seizures are associated with an increased risk of epilepsy later in life. The ages of the rats used in our present study correspond to ages when humans have febrile seizures (Verity, 1998).

Kainic Acid: An Analogue of Glutamate

Kainic Acid Glutamate

FIGURE 2: Stereochemistry of kainic acid and glutamate.

Glutamate is the main excitatory neurotransmitter in the CNS. Glutamate receptors are involved in learning and memory and mediate the excitotoxic damage thought to play a role in seizure disorders (Glaser, 1997; Dingledine et al., 1990). Kanic acid (KA) and various analogues of glutamate, known as excitotoxins, are toxic due to their ability to activate glutamate receptors on neuronal surfaces, resulting in prolonged depolarization, neuronal swelling, and death (Olney et al., 1974). This is followed by delayed selective hippocampal damage and synaptic reorganization that resembles human TLE (Hauser et al., 1991; Sperber et al., 1999) Several parallels between KA-induced status epilepticus in adult rats and TLE in humans include: KA-induced status epilepticus produces partial

seizures with secondary generalization involving limbic structures; the pattern of hippocampal damage resembles TLE following the initial episode of KA-induced status epilepticus; barbiturates and benzodiazepines have anticonvulsant action in both rats and humans (Sutula et al., 1989).

Developmental Epilepsy

Epilepsy is a neurological condition that is more common in infancy or childhood than later in life (Hauser and Kurland, 1975). The occurrence of seizures at this time in development may have devastating effects on developmental outcome, depending on the number and severity of seizures. Animal research has studied the effects of status epilepticus or of a single severe seizure on the developing animal. Seizure occurrence during development, whether prolonged or recurrent, can lead to an increase in sensitivity to various stimuli of the adult animal. This suggests that the onset of seizures during development triggers a change in critical neuronal networks (Szot, et.al., 2001). These effects are correlated with impaired learning and memory of the animal in adulthood.

The development of epilepsy in childhood varies in an age-dependent fashion. Seizures in neonates, infants, and toddlers frequently result from perinatal brain injury, congenital central nervous system malformations, and metabolic derangements (Holmes, 1991). Epilepsy in children can result from anything that interferes with brain development or function. Some babies suffer a lack of oxygen to the brain before or during birth. This oxygen deprivation can cause cerebral palsy and epilepsy. Some children have bleeding in the brain as a result of prematurity or abnormal blood vessels in

the brain (arteriovenous malformations). The bleeding can then result in seizures (Szot, et.al., 2001).

Epilepsy can also be caused by genetic changes. Some children are born with "epilepsy genes" that cause them to have seizures. These seizures may occur early in life or start later, even as late as the third decade of life (Haut et al., 2004). Usually, genetically caused seizures are generalized. In all cases, the brain of a child is more prone to seizures than is the brain of an adult.

Role of the Ventricular Zone in Brain Development

The two germinal regions known as the ventricular zone (VZ) and the subventricular zone (SVZ) generate majority of the neurons and glial cells in the mammalian central nervous system where they migrate to reach their final destinations to differentiate and integrate into brain circuitry (Brazel et al., 2003). The embryonic VZ of the cerebral cortex is a pseudostratified neuroepithelium that contains migrating neurons, radial glial cells, and progenitor cells that give rise to neurons (Noctor et al., 2002).

When a stem cell divides to produce an early progenitor cell it is said to differentiate. Differentiation means that the new cell is more specialized in its form and function. In vitro studies have shown that early progenitor cells taken from the adult forebrain SVZ can proliferate, self-renew. One type will give rise to astrocytes, the other will produce oligodendrocytes or neurons (Lois and Alvarez-Buylla, 1993 also see Figure 3).

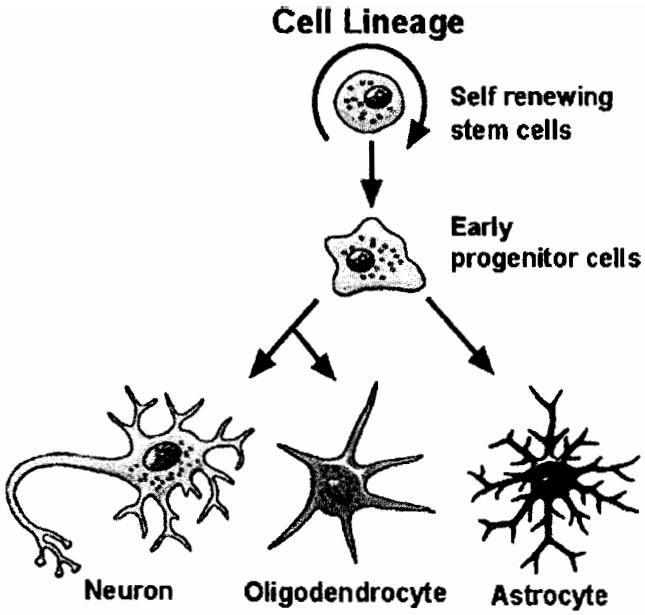

Figure 3: Cell differentiation in CNS.

Cell Migration during Neuronal Development

Cell migration plays an essential role in the formation of the CNS. It is the process in which a neuronal cell body displaces from its last cell division in the proliferative zone to its final destination in the mature brain. The ability of immature neuronal precursor cells to relocate before establishing there final position and permanent synaptic relationships is one of the hallmarks of cortical development (Rakic, 1990).

Two types of neuronal migration have been identified in the developing brain: radial migration and tangential migration (Lois and Alvarez-Buylla, 1994). Radially migrating neurons originate in the ventricular zone of the cortex and migrate perpendicularly to the ventricular surface by using radial glial fibres as guides (Lois and Alvarez-Buylla, 1996). Tangentially migrating neurons arise outside of the cortex in a

region of the basal telencephalon called the medial ganglionic eminence (MGE) and migrate over long distances to the cortex (Marin and Rubenstein, 2003). The SVZ precursors migrate along this tangential pathway in the lateral wall of the lateral ventricle, converging into the rostral migratory stream (RMS) (Kirschenbaum et al., 1999). Cell migration can occur by taking either one of these paths during development; however, one pathway can predominate over the other depending on the region and state of maturation (Brittis et al., 1995). Defects in cell migration at this time in development can lead to mental retardation, epilepsy, and learning disabilities (Marin and Rubenstein, 2003).

Neuronal Tracers

The use of neuronal tracers is an effective technique in identifying neuronal pathways and functions and their anatomical relationships between groups of cells or individual cells. Normal projection or migratory patterns may be enhanced, altered, or disrupted in any number of physical or genetic manipulations. Neuronal tracers are primarily used to either retrogradely label neurons or to anterogradely label the processes of neurons in one anatomical brain region to another. Retrograde labeling is defined as the process to "back-fill" neurons with projections to a particular area. (Molecular Probes Handbook of Fluorescent Probes and Research Products).

Carbocyanine dyes are particularly used in studies aimed to follow the migration of cells over long distances. The retrograde tracer 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) is a lipophilic, carbocyanine membrane stain that diffuses laterally to stain the cell in its entirety. It labels cell membranes by

inserting its two long (C_{18} carbon) hydrocarbon chains into the lipid bilayers. However, it is weakly fluorescent until it is fully incorporated into cellular membranes. DiI is able to travel from one cell to another, making it possible to monitor retrograde transport to distant locations. This red-orange fluorescent dye is often used as a long-term tracer for neuronal cells (Alifragis et al., 2002).

In addition, Cell Tracker Orange CMTMR [4-chloromethyl benzoyl amino tetramethyl rhodamine] labeling is a novel and efficient method of tracing neuronal migration in the developing brain. In contrast to DiI, CMTMR belongs to a group of chloromethyl cell trackers that freely permeate cell membranes and react with intracellular thiol groups to yield a fluorescent product. Thiols are active in processes such as proliferation, movement, and transport of cells. They also play a significant role in the synthesis of DNA. It has been demonstrated that labeling with CMTMR can overcome the technical limitations of membrane-binding tracers such as DiI (Alifragis, et.al., 2002). The advantage of using CMTMR is that it is a fluorescent dye that permeates and interlocks into the cell and does not travel from one cell to another. Therefore, cellular pathways can be identified and confirmed. For the purpose of this study, retrograde neuronal tracers were used in order to map the migratory pathways of the cells affected by seizure activity in the developing central nervous system (Molecular Probes Handbook of Fluorescent Probes and Research Products).

The specific aim of the present study was based on the following rationale: The highest incidence of seizures is in development. Early life seizures often go untreated due to unknown long-term consequences. The determination of long-term effects in

regards to cell migration and proliferation after seizures will contribute to a better understanding of the epileptic process and the care of patients having spontaneous seizures later in life. Our objectives were the following: (1) To determine whether induced perinatal seizures cause an increase in migration and proliferation of precursor cells originating from the ventricular zone; (2) To characterize over-proliferating precursor cells which migrate to distant limbic locations after perinatal seizures.

Experimental Design

Sprague- Dawley rats, of both sexes, were obtained from Taconic Laboratories Inc. (Germantown, NY) to breed rat pups, which were used for the experimental procedure. At the start of each experiment, animals were randomly grouped as naïve controls or experimental subjects. On postnatal (P) days 7 or 7, 9, and 13 the animals were weighed and then given an intraperitoneal (i.p.) injection of KA to induce seizures. A single injection of KA on P7, P9, and P13 was represented as 3 x KA. Seizures lasted approximately 3-5 hours, and the severity of seizures was scored. KA exhibited the following responses: squealing, scratching, wet-dog-shakes, falling over, forelimb clonus, stiff tails, head and body tremors and salivation. Next, the animals were anesthetized with intramuscular injections of ketamine and xylazine mixture. Once the animal was unresponsive, which was determined by toe or tail pinch, it's head was shaved and then put in a stereotaxic frame, stabilized by ear and incisor bars. In reference to bregma, stereotaxic coordinates were used for injection into the left and right ventricles. The ventricular zone was chosen as the injection site because of the role it plays as the origin of newborn cells. After surgery, the animals were placed under a heated lamp until they recovered and gained complete consciousness, and then returned to their lactating mother. We analyzed our animals at two time points. The animals were either sacrificed 5 days post-dye injection, representing our short term study, or 45 days post-dye injection, representing our long-term study, which allows us to study the long-term effects of seizures on the developing brain. The cells were characterized using immunohistochemistry on both coronal and sagital sections of the brain.

Methods

Induction of KA Status Epilepticus

Sprague-Dawley Albino rats of either sex were used and status epilepticus was induced by administration of KA at postnatal ages of 6 days (P6, 2mg/kg, s.c.), 9 days (P9, 2mg/kg, s.c.), and 13 days (P13, 2.5mg/kg, i.p.). Pups were kept on a 12 hr light:dark cycle at room temperature (55% humidity) and remained with the lactating mother.

Stereotaxic dye injections

Sprague-Dawley rat pups were anesthetized with intramuscular injections of 0.2ml of ketamine and xylazine mixture according to the following regimen: 4 ml of ketamine (50 mg/ml) + 1 ml of xylazine (20mg/ml). The rat's head was shaved and positioned in a stereotaxic apparatus. A 1 ml micro syringe needle was filled with a solution of DiI (50ug/0.95ml PBS) (Sigma Aldrich) and then lowered towards the injection site. Stereotaxic coordinates for the lateral ventricle in mm with respect to bregma of P13 Pups are: AP: -3.2; L: 2.6; D: -2.5 (from the skull) (see figure 4). The pups received an injection volume of 0.25µl. DiI was stereotaxically injected bilaterally into the dorsal ventricular zone. DiI was also substituted with Ethidium Bromide (EtBr) (Sigma Aldrich) (10ml/990ml of PBS) to test for specificity of dye. Additionally, CMTMR (1mg dissolved in 0.18 ml of DMSO) (Molecular Probes) was also injected using the same method as the DiI and EtBr dyes.

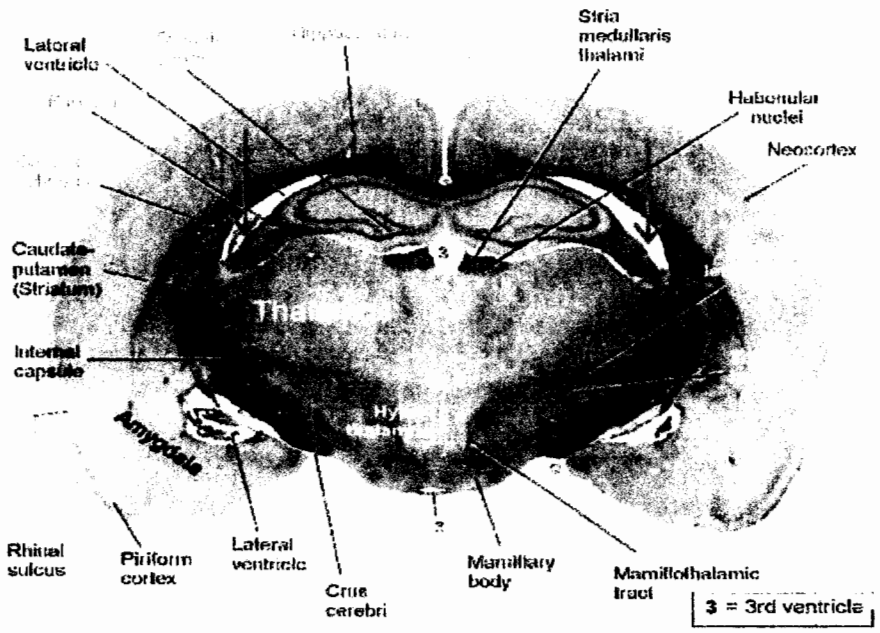

Figure 4: Coronal section of rat brain with anatomical structures identified and also illustrating stereotaxic injection site in left and right lateral ventricles, indicated by red arrows.

Immunohistochemistry

A number of antibodies were used to determine whether proliferating cells contain neuronal or non-neuronal markers. First, animals were deeply anesthetized with a lethal dose of sodium pentobarbital and perfused intra-aortically with 20ml of ice cold 0.9% saline followed by 30 ml 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative for 12 h, and cryoprotected in 0.1 M PB containing 20% sucrose for another 24 h. The brains were frozen in methylbutane (-50°C) and 30-μm-thick sections were cut using a cryostat at -19°C and collected using gelatinized slides. Additionally, 50-μm-thick sections were collected in 0.1 M Tris-buffered saline for

immunohistochemistry. Sections were then incubated for 1hr in a solution containing 5% normal goat serum or 5% normal horse serum in 0.1% BSA/PBS for minimizing non-specific staining. Primary monoclonal antibodies developed from mouse (anti-neuN 1:100; Chemicon, anti-PSA-NCam 1:50; Chemicon, anti-GFAP, 1:1000; Chemicon, anti-A2B5, 1:100; Chemicon, RYP 1:3, generously donated by Steve Levinson) and polyclonal antibodies developed from rabbit (anti-vimentin 1:500; AbCam, anti-nestin, 1:100; Chemicon) and goat (doublecortin, 1:100; Santa Cruz) were added (3-5 μg/ml) in 0.1% BSA/PBS for 24 hrs shaking at 4°C. Sections were rinsed with PBS and incubated for 2 hrs with secondary fluorescent antibodies: biotynilated goat anti-rabbit IgG (1:100), biotynilated rabbit anti-goat (1:50), or biotynilated horse anti-mouse (1:100) and then washed with 0.1% Triton X in PBS. Sections were examined under a fluorescent microscope and photographs were taken.

Cell Type	**Marker**	**Dilution**	**Description**
Neurons:			
Mature	NeuN	1:100	Vertebrate neuron-specific nuclear protein
Immature	DCX	1:100	Migrating neuroblast marker
Precursor cells	Nestin	1:100	Progenitor stem cell marker
	PSA-NCAM	1:200	Migrating neuroblast marker
Astrocytes	GFAP	1:1,000	Glial fibrillary acidic protein Intermediate-filament
Microglia	Vimentin	1:200	Intermediate filament protein
Oligodendrocytes	A2B5	1:100	Neuronal cell surface antigen
	RYP	1:3	Oligodendrocyte marker

Table 3: Immunohistological markers used to characterize DiI and CMTMR labeled cells based on there cellular morphology and affinitity.

Histology

To identify the anatomical structures affected by perinatal seizures, hematoxylin/eosin and thionin staining were carried out on all sections that were processed for DiI and CMTMR labeling. Air-dried sections from all brains were also processed for immunohistochemistry in order to simultaneously monitor hyperbasophilia, morphology and cell migration.

Results

Histological Staining Demonstrates Change in Cell Morphology

Histological staining was used to determine whether there was an alteration in cell morphology in limbic structures after perinatal-induced status epilepticus. After three kainate-induced seizures, a change in morphology was noted. For example, in a subset of neurons, hyberbasaphilic staining was observed in extrahippocampal limbic structures such as the amygdala and entorhinal cortex (Fig. 5). These cells appeared shrunken and lacked orientation. Histological findings suggested there was a local change in cells that already reached maturity or that these cells may have migrated from a distant location. Therefore, to determine the potential pathway leading to these distant limbic structures within the CNS we used DiI and CMTMR to trace the migration of cells.

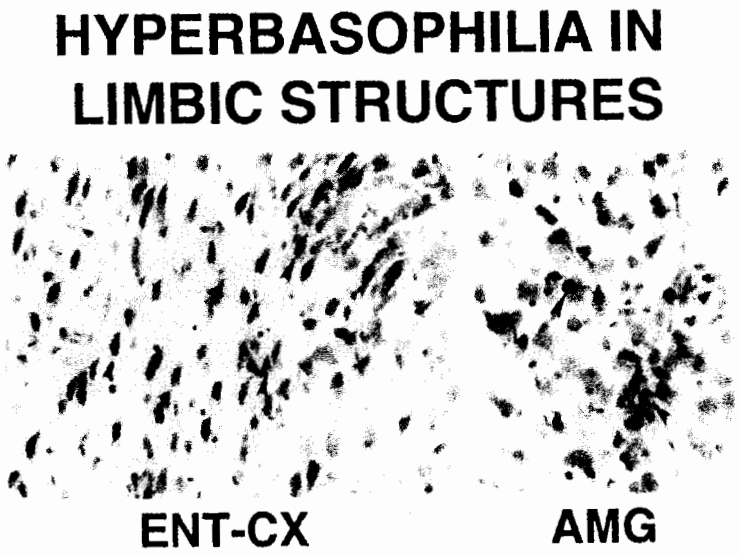

Figure 5: Histological staining of entorhinal cortex and amygdala after kainite-induced seizures shows hyperbasophilia in a subset of neurons (arrow heads).

Ethidium Bromide Shows Non-Specific Labeling in Ventricular Zone

In order to control for diffusion artifacts, a non-specific DNA binding dye, EtBr, was used as a control marker (Omar et al., 1999). After injecting EtBr into the lateral ventricle, typical non-specific staining was observed (Figure 6). Occasionally a cell picked up the dye, but widespread diffusion also occurred around the cell (Figure 6C).

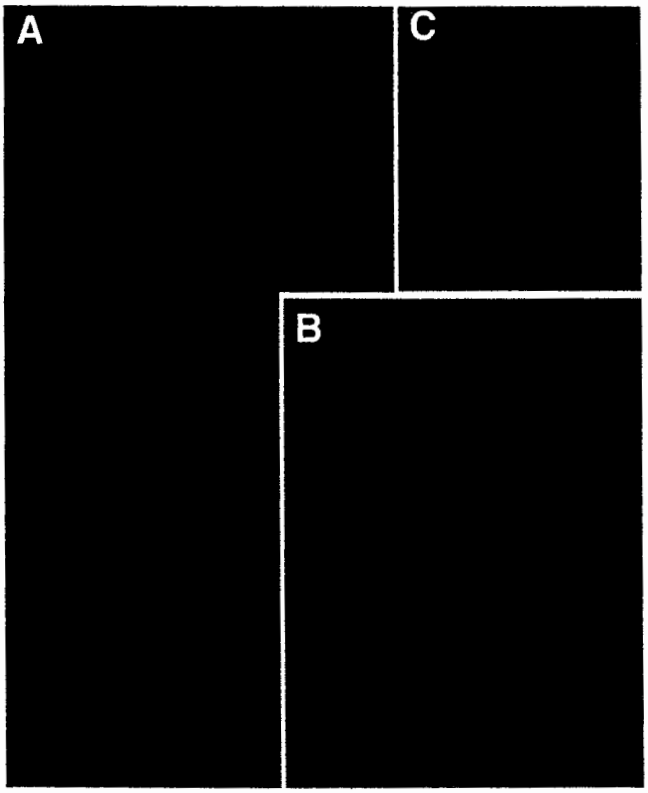

Figure 6: Ethidium Bromide shows non-specific labeling observed in the ventricular system in naïve animal after 5 days shown at low magnification (A) and at higher magnifications (B and C).

DiI Labeling of VZ in Naïve Controls Shows Non-Specific Labeling and Low Amounts of Specific Labeling

In control animals injected with DiI, non-specific and limited labeling in the VZ was revealed. The dye accumulated predominantly within the regions of injection (Figure 7A and D). Minor labeling was observed in the third ventricle (3V) (Figure 7B,

C, E, and F) and a few cells formed a chain and appeared to follow radial glial guides (Figure 7F and Table 3). The lateral ventricle (LV) also expressed DiI labeled cells (Figure 7D); however, most of the labeling was non-specific within this region (Figure 7A and D).

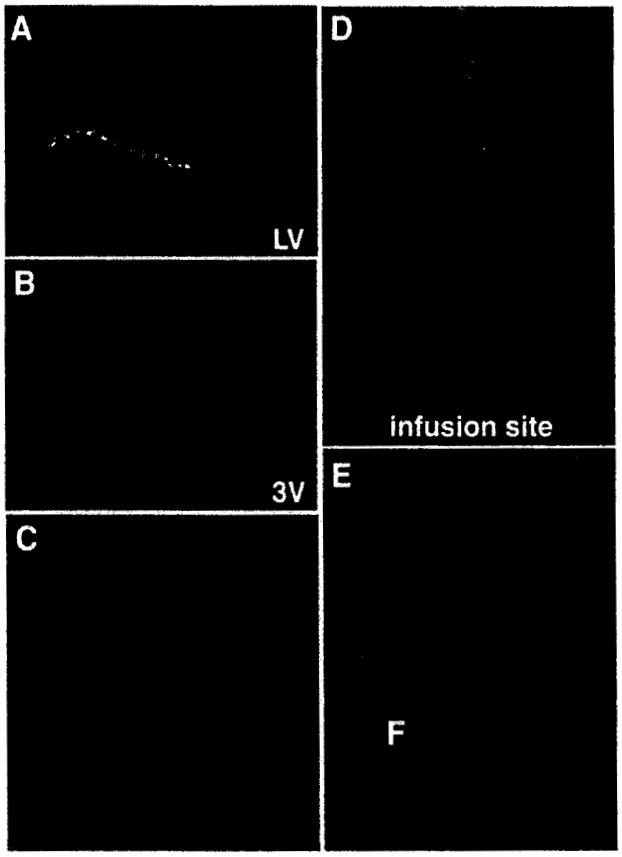

Figure 7: In control animal 5 days post-DiI injection, there was diffuse dye accumulation in the LV (A) and the injection site (D). There was minimal cell labeling in the 3V shown at low (B, C, and E) and at high magnifications (F).

DiI Labels Cells in Distant Limbic Structures after 3xKA and 5 Days

We administered kainic acid i.p. to induce status epilepticus during the first and second weeks of development, after the third and final KA seizure subsided. DiI or CMTMR was stereotaxically injected bilaterally into the dorsal ventricular zone (Figure

8). As a result of injecting into the LV, the fluorescent dyes were transported throughout the entire VZ, including the LV, third and fourth ventricles; the choroid plexus and blood brain barrier (BBB) were also prominently labeled (Figure 8). DiI labeled cells were traced to distant limbic locations within the CNS, such as the cells of the ventral thalamus and in the amygdala (Figure 8A). High magnification of the cells showed enhanced specificity of the fluorescent dye tracer DiI compared to control injected animals (Figure 8B-C).

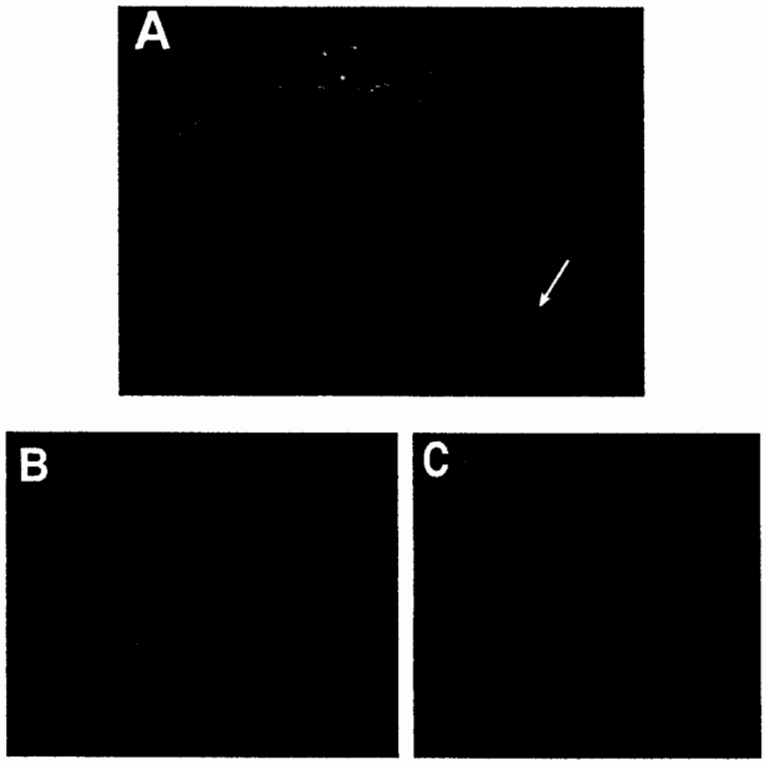

Figure 8: Coronal section showing a global view of entire rat brain in which DiI not only diffuses locally, but is transported throughout the VZ to distant locations, indicated by white arrow, after 3xKA and 5 days post-dye injection (A). The cells migrating to distant structures within the brain are shown at high magnification (B) and higher magnification showed punctate labeling of a single cell (C).

Kainate-induced SE increases proliferating cell numbers in the 3V, amygdala and ventral thalamic nuclei

The distribution of cells in different regions throughout the brain was assessed by using the fluorescent dye tracer DiI. In control animals, a few cells labeled in the 3V (Figure 9A). There was no labeling in distant structures such as the ventral thalamus and amygdala (Figure 9C-E). In contrast, three episodes of perinatal kainate-induced seizures showed rapid effects. There was an increase in the number of cells labeled with DiI (Figure 11) and CMTMR (Figure 12) in the 3V (Figure 9B, D-F). Quantification showed that the increase in the number of labeled cells was significant and was also observed in the amygdala and the thalamus (Figures 9 and 14).

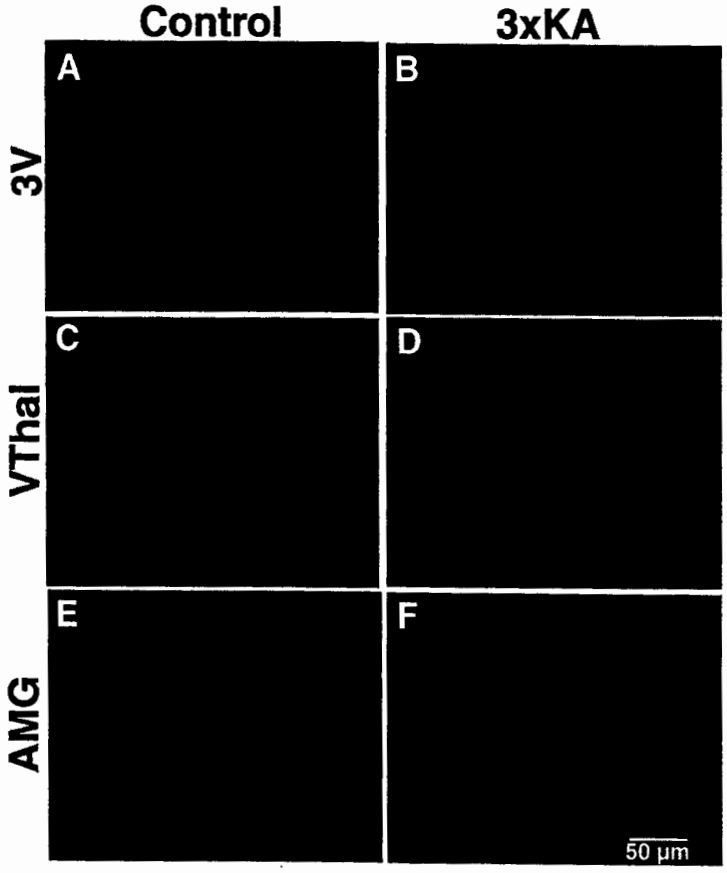

Figure 9: After 3xKA and 5 days post-dye injection, there was an increase in cell labeling in the third ventricle (B), the ventral thalamic nuclei (D) and the amygdala (F). The control coronal sections within these same regions showed some specific labeling in the third ventricle (A), but virtually no labeling was seen in the ventral thalamus (C) and amygdala (E).

Long-Term accumulation of DiI in Control after 45 days

In order to determine whether cell proliferation and cell migration continue to progress in the developing animal to adulthood, animals were also analyzed after 45 days. The long-term study showed post-dye injection that in naïve animals some specific labeling was observed in the ventricles, but much of the labeling appeared rather diffuse (Figure 10). Quantification of the number of cells labeled in local and distant regions was compared at 45 days (Figure 14).

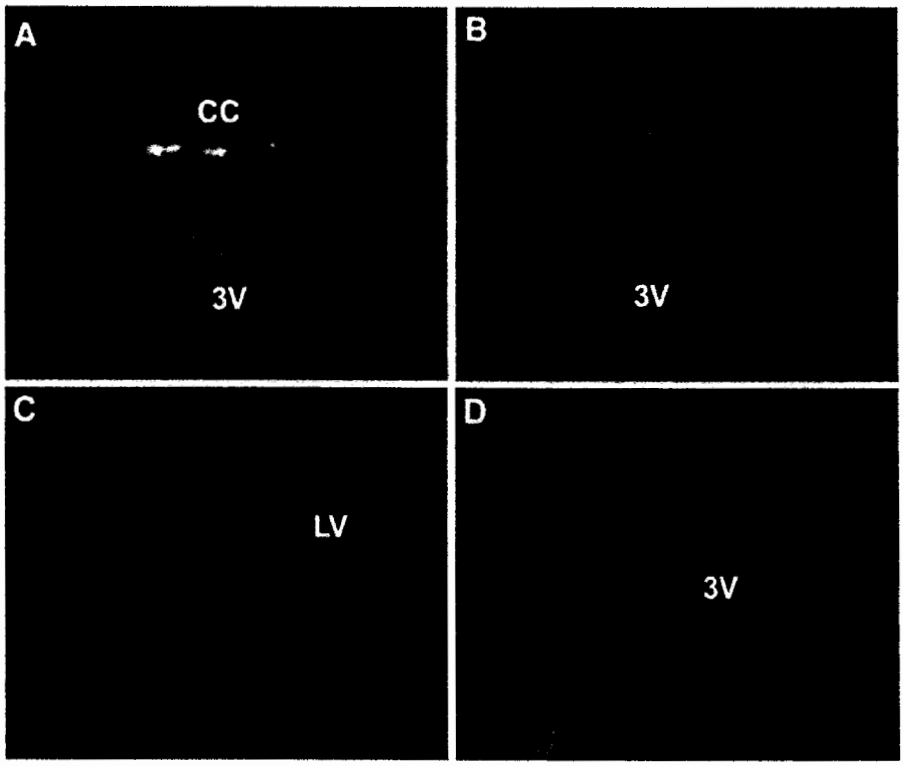

Figure 10: Long-term accumulation of DiI in control animal in third ventricle, viewed at low magnification (A) and high magnification (B). DiI labeled cells observed in lateral ventricle (C) and posterior to third ventricle (D).

Long-term Study Shows Increase in DiI Labeling Throughout VZ after 3xKA

After KA-induced seizures and 45 days, marked increases in DiI labeling were observed throughout the ventricular system. Labeling was prominent and punctate. Specific labeling patterns in seizure animals reflected further enhancement of cell proliferation of the SVZ over time. Increased punctate labeling was also observed in the third ventricle and the corpus collosum (Figure 11B-D). Therefore, DiI labeled cells were plentiful throughout the ventricular system and in distant limbic locations. Our results show evidence of continued increases in cell proliferation with maturation.

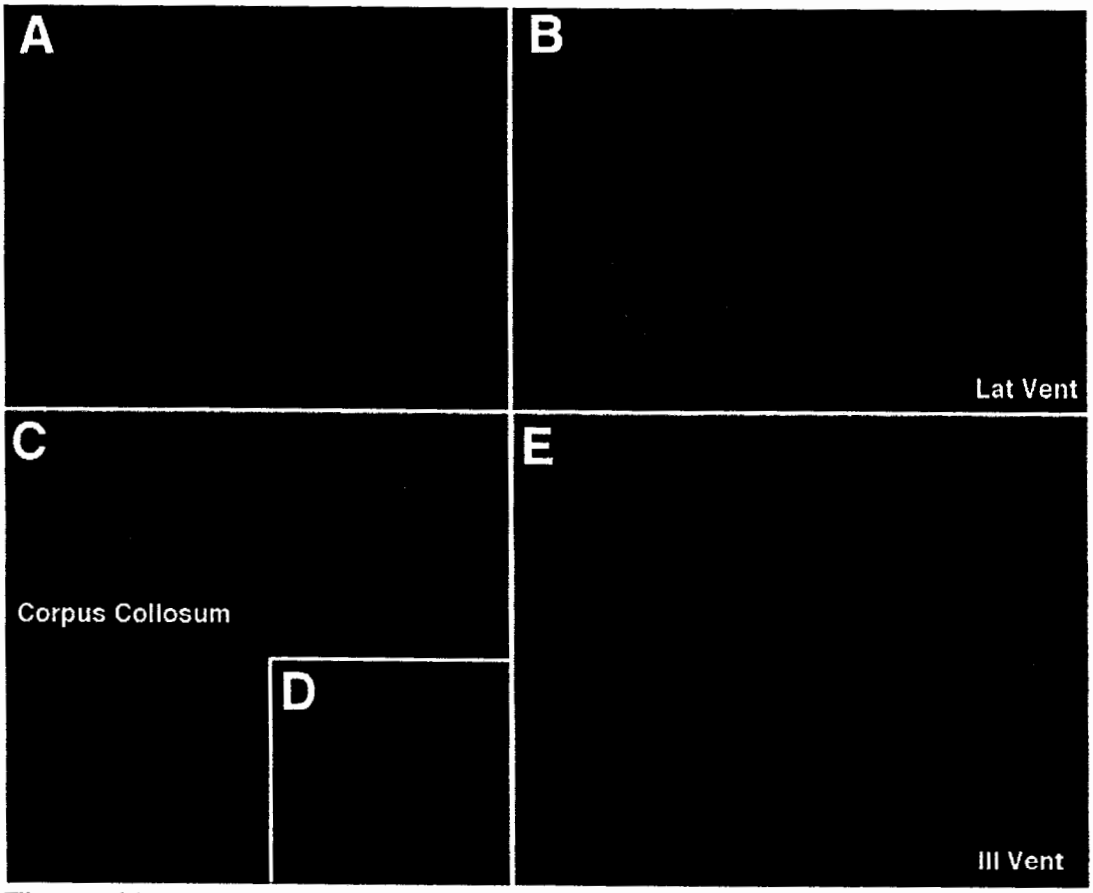

Figure 11: Increased cell proliferation throughout VZ after kainate-induced SE (A).Sagital sections through the rostral forebrain SVZ show increased labeling in the lateral ventricles (B), the corpus collosum (C) and high magnification of cells from panel C (D), and the dorsal third ventricle (E).

CMTMR Pathways after 3xKA and 45 Days

In contrast to DiI, a second dye tracer CMTMR that readily permeates cell membranes, revealed the morphology of cells at higher resolution; labeling was less diffuse. Overall the labeling patterns were similar in that CMTMR labeling was observed throughout the ventricular system, along the pathway to the olfactory bulb, within the third ventricle, the thalamus, and amygdala nuclei (Figures 12 and 13). The cells in the thalamus were disorganized and lacked orientation similiar to our histological observations (Figure 12D). Interestingly, proliferating cells also dispersed from the hippocampal formation into the cortex (Figure 13A). Notice many of the cells appeared in pairs and follow a specific pathway.

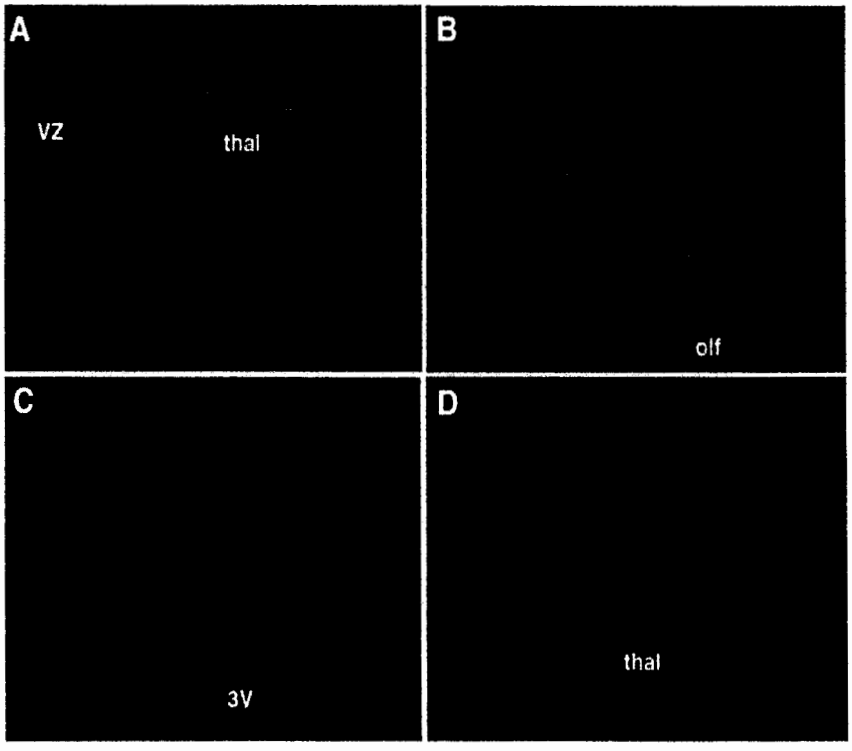

Figure 12: CMTMR labeled cells after three episodes of kainite-induced seizures and 45 days post-dye injection observed in VZ (A), pathway to olfactory bulb (B), the third ventricle (C), and the thalamus (D).

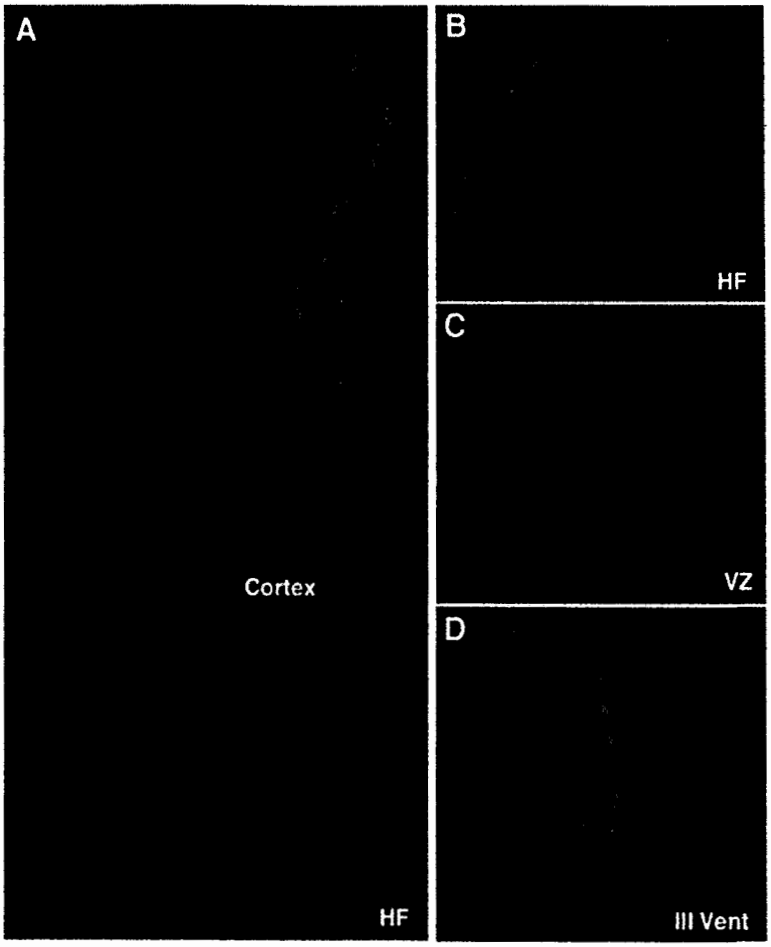

Figure 13: CMTMR labeled cells after kainate-induced seizures in our long-term study were observed in the cortex (A), the hippocampal formation (HF) (B), the VZ, and the third ventricle.

The number of cells migrating to distant locations throughout the brain was quantified in the control and experimental animals in the short and long-term studies. Counting the number of cells showed there was a significant increase in the number of cells labeled after 3xKA and 5 days post-dye injection. An even greater increase was observed after 3xKA and 45 days, particularly in the 3V and the LV.

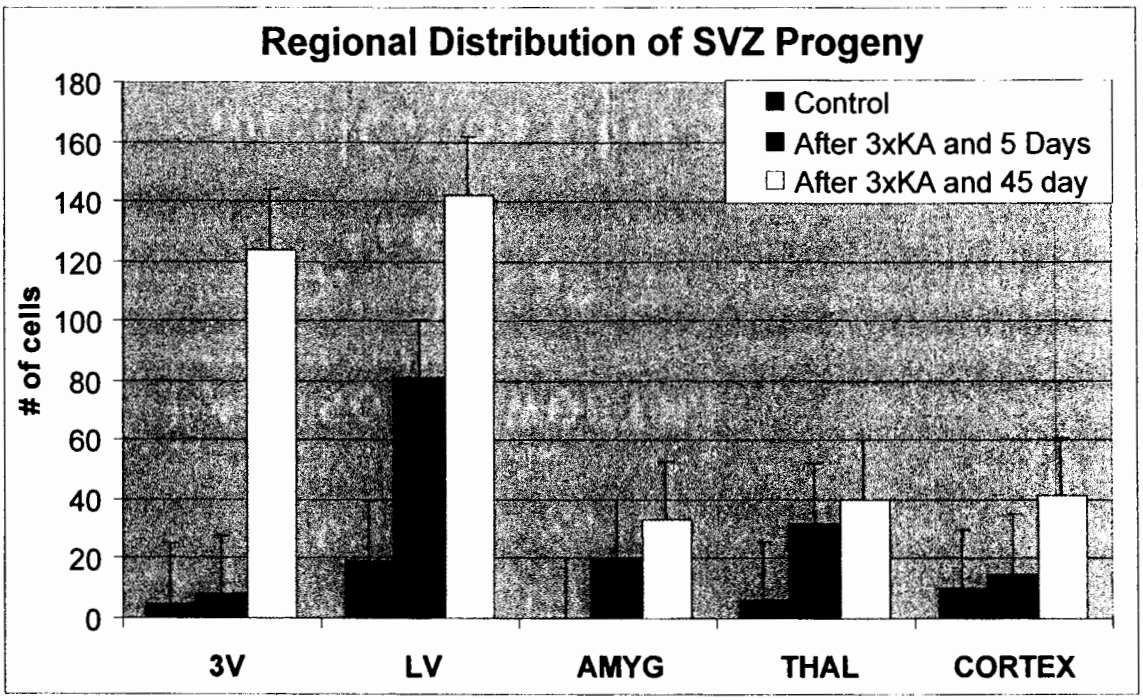

Figure 14: Quantification of number of cells in 3V, LV, amygdale (amyg), thalamus (thal), and cortex after kainate-induced seizures in our short term and long term study compared to our control.

Long term Characterization of Migrating Cells

Co-localization of NeuN in Neuronal Precursor Cells

With the use of seven independent cell markers, precursor, migrating cells were characterized induced by three perinatal seizures. NeuN, a well known antibody for labeling neuronal cells, co-labeled with DiI in the VZ after KA-induced seizures and 45 days post-dye injection (Figure 15A-B). NeuN marked the pyramidal cell layer; however, NeuN did not co-label within this region or in the white matter associated with the VZ (Figure 15C-D). NeuN was also co-localized with DiI in the cortico-amygdala

(Figure 15E-F). However, based on the morphology, these were mature pyramidal cells indicating that there was an increase in retrograde transport from SVZ to this structure.

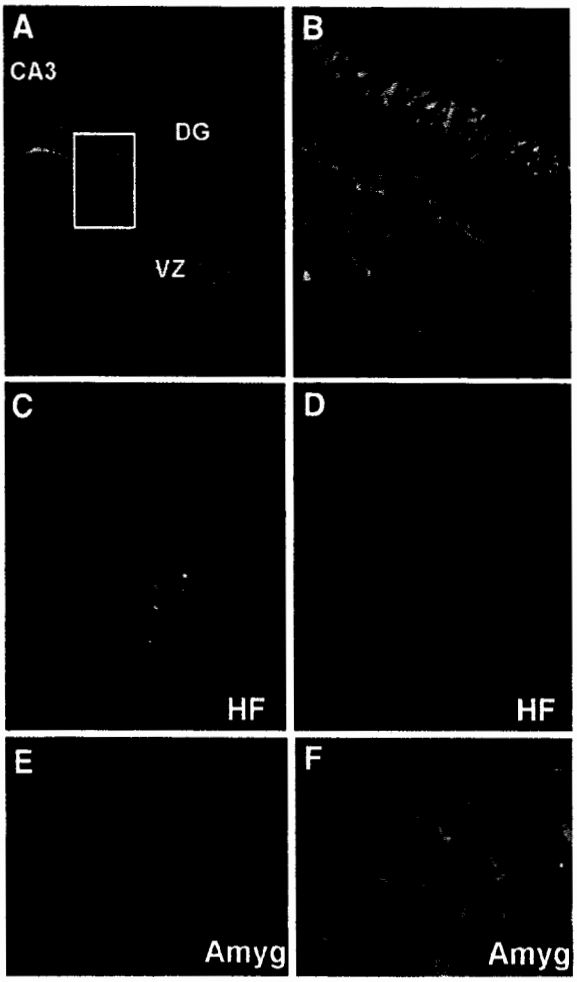

Figure 15: NeuN co-labels with DiI in VZ after 3xKA in our long term study, observed at both low (A) and high(B) magnification. NeuN labeled cells (C) did not co-localize with DiI (D)in the hippocampal formation (HF), associated with the VZ. However, DiI cells in the amygdala (E) did co-localize with neuronal marker NeuN in the distant limbic structure, the amygdala (F).

DiI Labeled Cells Express Astrocytic Marker GFAP in VZ After 3xKA But Not In Control animals

GFAP is a marker for mature astrocytes, the non-neuronal components of the brain. GFAP co-labeled with DiI in the VZ after kainate-induced seizures and 45 days, indicating perinatal seizures have long-term consequences on astrocytosis. GFAP labeling was also observed throughout the ventricular system (Figures 16 and 17). GFAP labeled cells co-localized with DiI in dorsal third ventricle (Figure 16A), the ventral third ventricle (Figure 16F), and the lateral region of third ventricle (Figure 16C). This detailed co-labeling was observed at higher magnification to further illustrate the typical "star-like" morphology of astrocytes (Figure 16B and 17D-F).

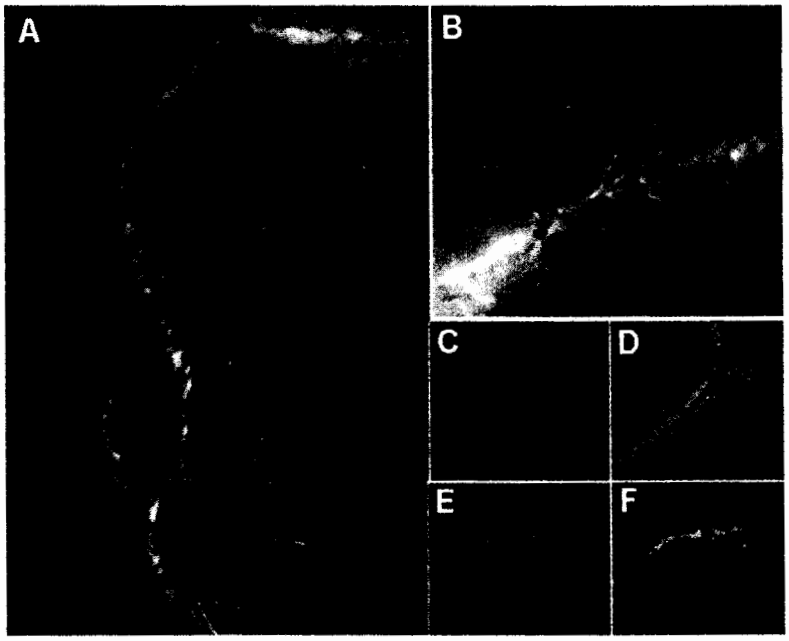

Figure 16: Superposition of GFAP and DiI show co-localization in the dorsal third ventricle, shown at low magnification (A) and at high magnification (B), in which the mature cell type did not co-label with DiI. Co-localization was also observed with DiI labeled cells in the lateral (C and D) and the ventral portions (E and F) of the third ventricle.

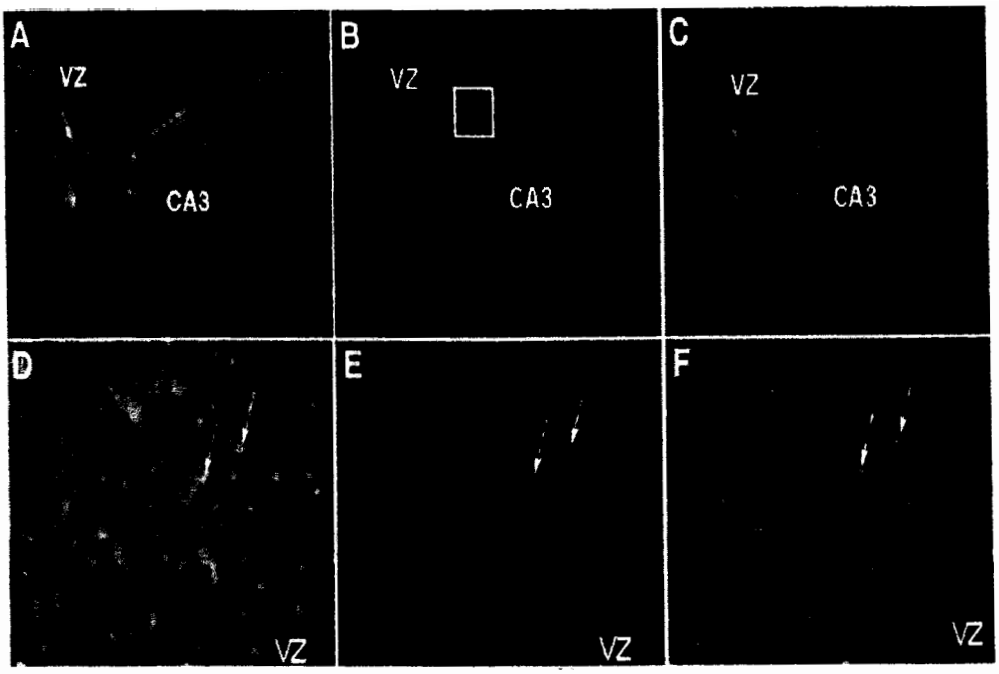

Figure 17: GFAP labeled cells (A and D) co-localized with DiI (B and E) after 3xKA and 45 days at the level of VZ. Superposition of images of GFAP and DiI show co-localization of cells observed at high magnification (F).

Nestin Co-labels with DiI in VZ and Amygdala after 3xKA and 45 Days

Nestin is an intermediate filament gene used extensively as a marker for CNS progenitor cells in development (Dahlstrand et al., 1995). Nestin co-labeled with DiI in the 3V (Figure 18C) and the amygdala (Figure 18F), but not in the VZ (Figure 18E). Therefore, there is evidence of precursor neuronal cells found in distant limbic structures, such as the amygdala, which may have migrated from the subventricular zone, the site of injection.

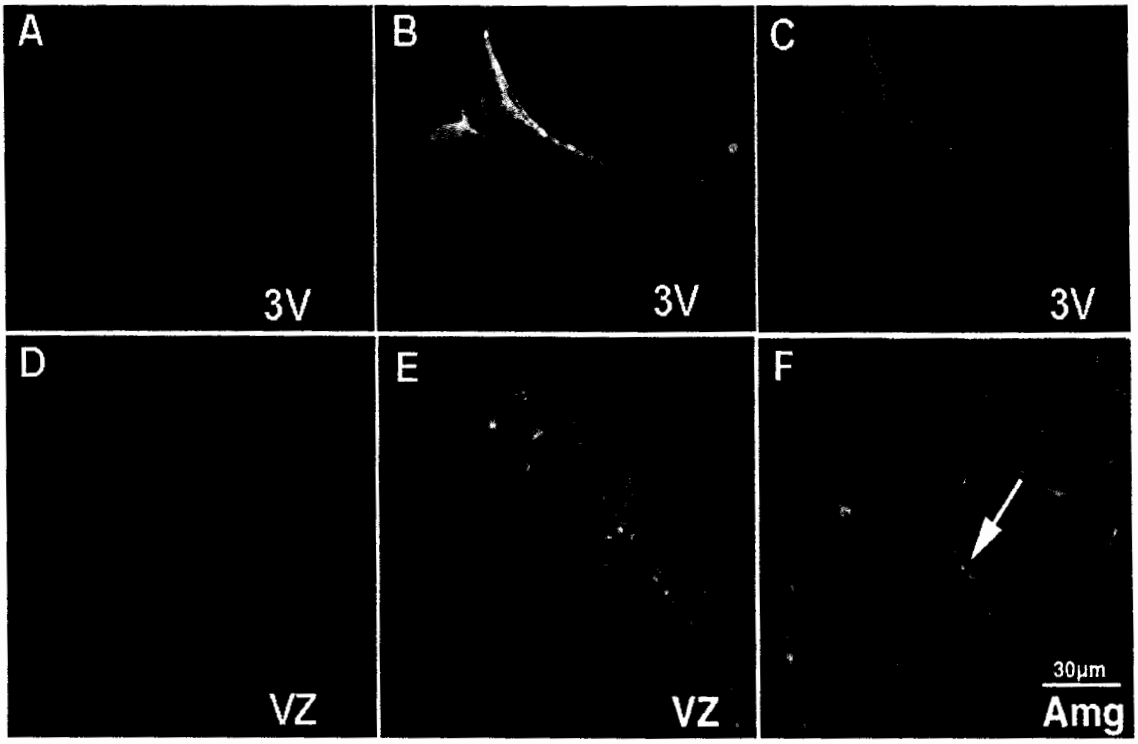

Figure 18: Superposition of DiI labeled cells (A) and nestin marker (B) co-localize in the third ventricle, shown at low magnification (C). Superposition of images of DiI labeled cells (D) with nestin co-localized in the VZ (E). Lastly, superposition of DiI and nestin co-localized in the amygdala (F white arrow).

Evidence of Non-Neuronal cells in the 3V after 3xKA and 45 days

Vimentin is a marker for the non-neuronal cell component of the brain. Vimentin is an antibody specific for immature astrocytes expressed early in development and is replaced by GFAP with further maturation (Stringer,1996). Vimentin co-labeled with CMTMR in the 3V after three kainate-induced seizures in our long term study (Figure 19). Therefore, we have provided evidence that both mature astrocytes and immature astrocytes are present within the third ventricle and continue to increase into adulthood after 45 days.

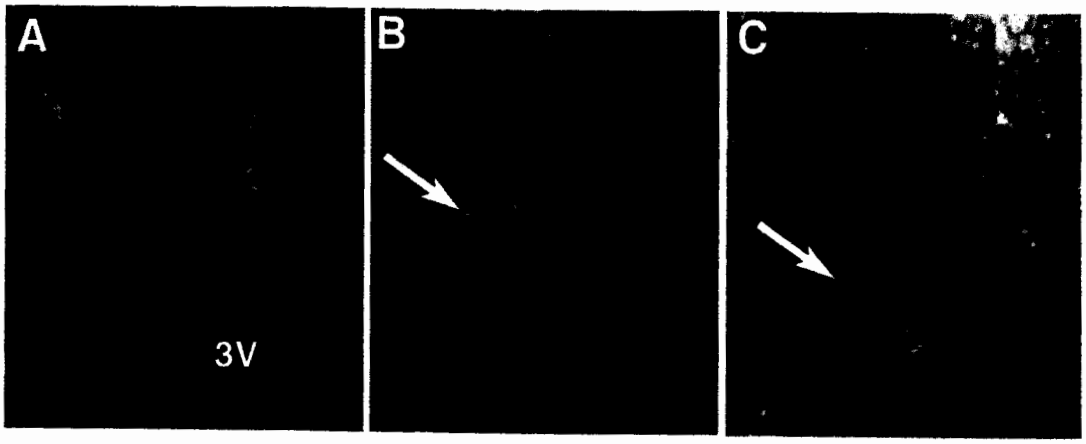

Figure 19: Superposition of CMTMR and vimentin co-localized in the 3V after 3xKA and 45 days (A). Also shown at high (B) and higher magnification (C white arrow).

Neuronal Precursor Antibodies Co-label with CMTMR in Long-term Control

Neuronal precursor progenitors express the polysialylated (PSA) form of the neuronal cell adhesion molecule (NCAM) and were detected in the thalamus of the neonatal rat brain after 45 days post-dye injection (Figure 20A-C). Neuronal precursor marker Nestin was similar to PSA-NCAM in that it co-localized with CMTMR in the thalamus in the long-term control animals. Co-localization with PSA-NCAM and nestin was observed in a few cells within the thalamus near the VZ, the site of injection. There were no cells observed in distant locations. Thus, our long-term study results showed that precursor neuronal cells are nestin and PSA-NCAM immunoreactive in the thalamus and vimentin immunoreactive in the third ventricle.

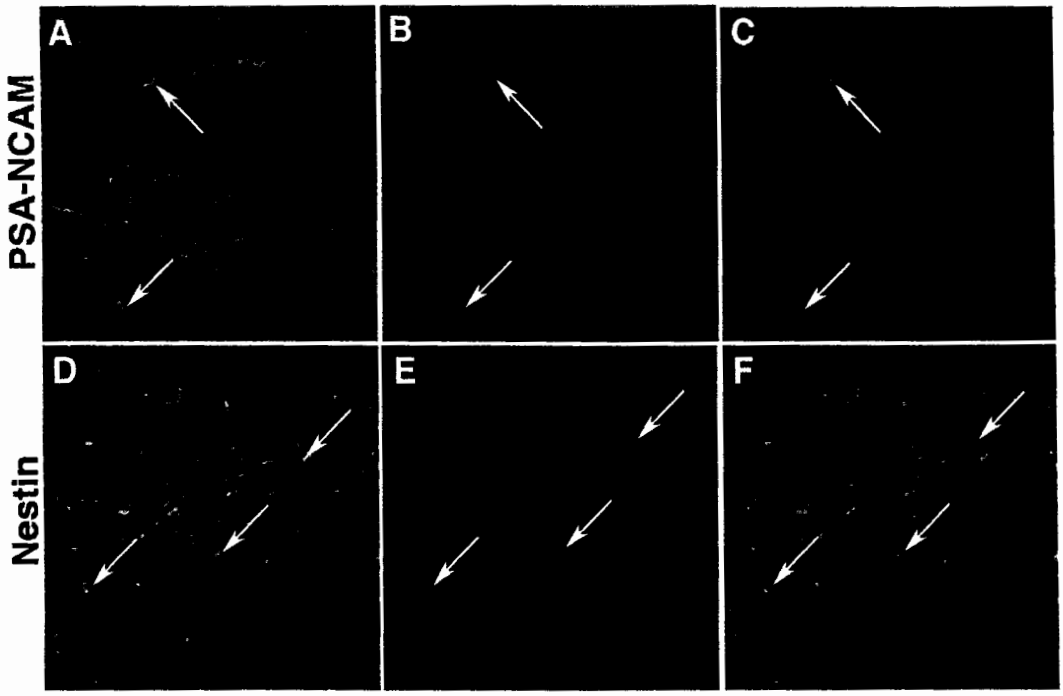

Figure 20: PSA-NCAM labeled cells (A) co-localized with CMTMR (B) afer 45 days post-dye injection in the control animal. Superposition of images of PSA-NCAM and CMTMR show co-localization of cells at the level of the thalamus (C). Nestin labeled cells (D) also co-localized with CMTMR (E) in long term control. Superposition of images of nestin and CMTMR show co-localization of cells in thalamus, near VZ.

Doublecortin co-localizes with CMTMR in the VZ in our control and kainate induced model after 45 days post-dye injection

Doublecortin (DCX) is a microtubule-associated phosphoprotein that plays a critical role in the cytoskeletal reorganization (Molecular Probes Handbook of Fluorescent Probes and Research Products). This protein is expressed by migrating neurons throughout the developing and adult CNS (Lois and Alvarez-Buylla, 1993). In controls, a few cells appeared to migrate out of the SVZ and co-labeled with DCX, a neuroblast marker. After 3xKA and 45 days, some CMTMR cells did not co-label with DCX, whereas many cells migrating out of the VZ (near hippocampal formation)

appeared to express this neuronal precursor marker suggesting that neuroblasts were labeled (Figure 21).

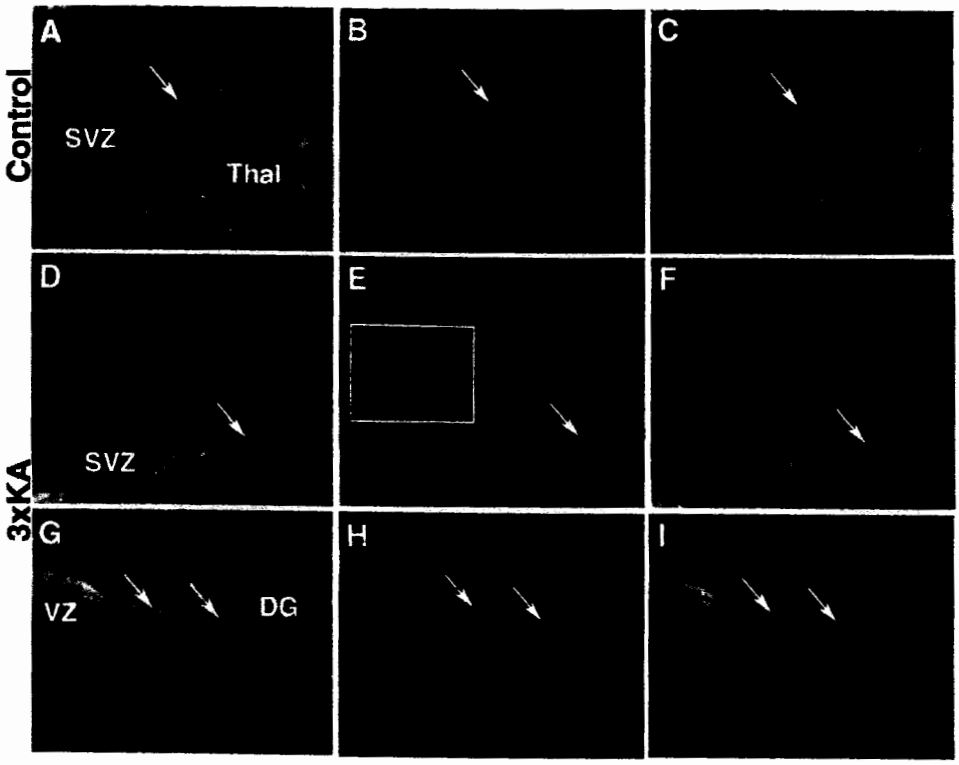

Figure 21: Superposition of DCX (A) and CMTMR labeled cells (B) co-localize in the VZ in our control animal after 45 days post-dye injection (C). DCX (D, and G) and CMTMR labeled cells (E white box and F) did not co-localize with some CMTMR cells, but did colocalize with others (H and I) of the VZ.

A2B5 Marker Co-localizes with CMTMR

A2B5 is non-neuronal cell marker that reacts with neuronal cell surface antigens to express oligodendrocytes in the CNS. Co-localization was observed with A2B5 and CMTMR in the thalamus, but not in the VZ after three episodes of kainate-induceded seizures and 45 days post-dye injection (Figure 22). In summary, our results show evidence of neuronal precursor markers nestin and PSA-NCAM markers within the

thalamus in our control long-term study, and non-neuronal oligodendrocyte marker A2B5, in the thalamus of our kainate-induceded model, also after 45 days.

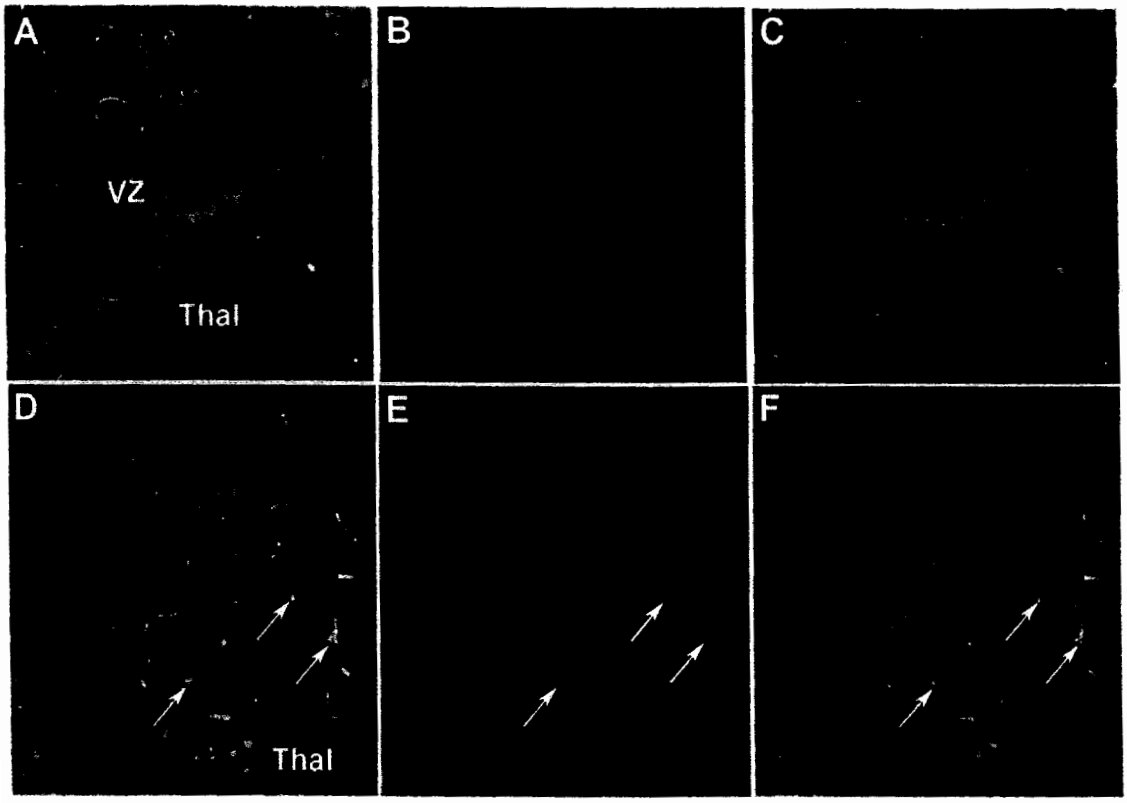

Figure 22: A2B5 labeled cells (A) do not co-localize with CMTMR (B) after 3xKA and 45 days at the level of VZ (C). However, A2B5 cells (D) co-localized with CMTMR (E) in the thalamus. Superposition of images of A2B5 and CMTMR show co-localization of cells (F white arrows).

RYP antibody co-localizes with DiI in our long-term study

In order to provide further evidence for the presence of oligodendrocytes, a second antibody known as RYP was used. After three episodes of kainate-induceded seizures and 45 days post-dye injection, co-localization with DiI and RYP was observed in the corpus collosum (cc), the VZ, and the caudate putanum, also known as the striatum (Figure 23). Therefore, oligodendrocytes were observed in various structures of the CNS

in our kainate-induceded animal model after 45 days, expressed by both A2B5 and RYP antibodies.

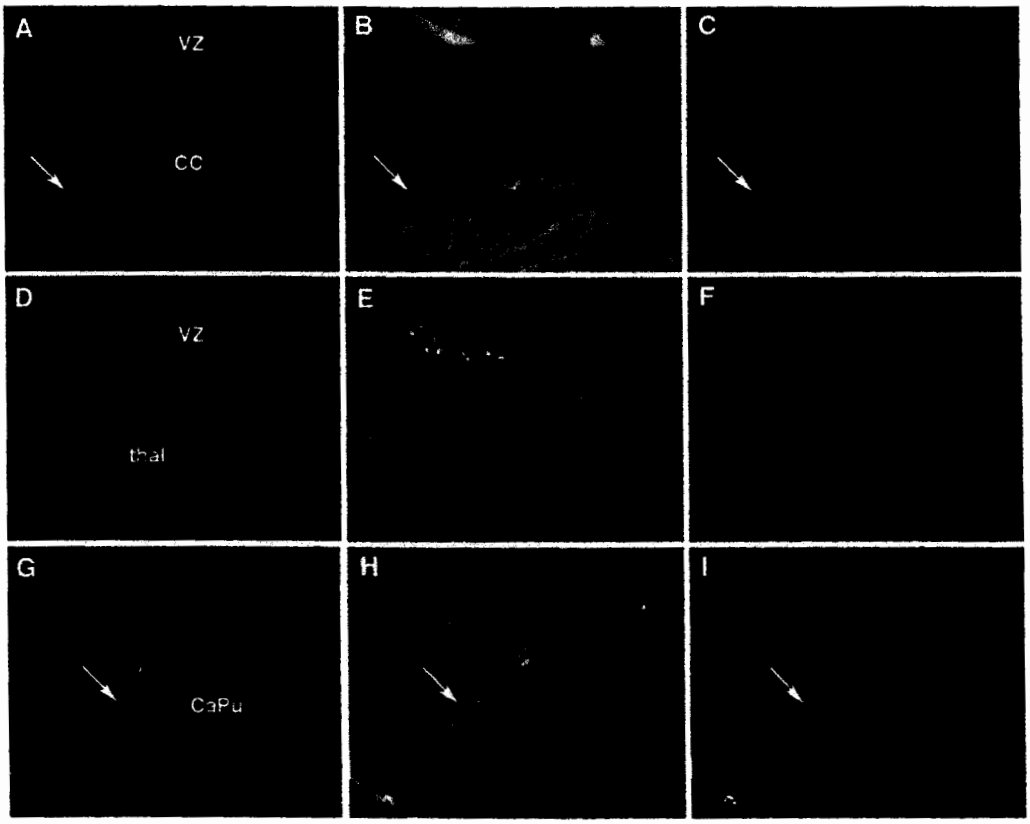

Figure 23: Superposition of DiI labeled cells (A, D, and G) and RYP marker (B, E, and H) co-localize in the corpus collosum (cc) (C), the VZ (F) and the caudate putamen (I) after three episodes of kainate-induced seizures in our long term study.

Discussion

In the present study there were three main findings discovered with two fluorescent dye tracers following three episodes of status epilpeticus induced during the neonatal period: (1) there were increases in precursor cell proliferation throughout the ventricular system; (2) increases in cell migration to distant limbic structures; and (3) increases in retrograde transport to cortico-limbic areas. The upregulation of the total number of proliferating cells in the ventricular system, amygdala nuclei, thalamus, and parietal cortex occurred at early and late times after status epilepticus (5 and 45 days). Thus, the increases observed in development progressed into adulthood. Interestingly, precursor cells continued to appear in an immature state even after animals matured to adults. This suggests that differentiation is delayed and that abnormal migration of developing neurons may cause a latent response to lead to spontaneous seizures in adulthood once cell differentiation occurs and aberrant connections are formed. Moreover, facilitation in retrograde transport to limbic structures such as the amygdala cortex may also indicate a change in neuronal function which may depend on the age of the animal.

Neuronal migration disorders (NMD) are associated with developmental epilepsy

Epilepsy is often associated with cortical malformations, previously referred to as neuronal migration disorders (NMD), which occur as a result of a disturbance in the normal migration and differentiation of cells during development (Thom, 2003). Detailed studies of the dynamic movements of cell migration have suggested that cells undergo distinct phases and that specific cell types take distinct migration paths

(Kriegstein and Noctor, 2004). Cortical malformations are referred to as dysplasias, a term defined as defective tissue developed during embryonic life. From a neuropathological view, cortical dysplasia can be considered a form of NMD (Spreafico et al., 1998). To date, it is not known whether early seizures may affect the normal course of developing neurons and/or glia to produce dysplasias. However, recently heterotopias have been recognized with modern neuroimaging methods within the periventricular, subependymal, and subcortical regions (Thom, 2003). In addition, neuronal precursor cells have been shown to increase in the adult brain following pilocarpine-induced seizures (Parent et. al. 2002). We postulated that these systems are also altered by early life seizures. Our study showed that three episodes of perinatal seizures can rapidly alter the proliferation of precursor cells and certain migration pathways. This suggests that early seizures may lead to abnormal cortical and limbic structure development. Since children with epilepsy are at significant risk for cognitive and behavioral impairments, long-term changes in cell differentiation and migration, observed herein, may underlie some of these abnormalities (Holmes, 1991).

Neuroblast proliferation, differentiation, and migration are age and time-dependent

Clinical and experimental research has studied the effects of status epilepticus and brief seizures on neuronal structure and function in the developing brain; however, the modes of migration and migratory routes are more diverse and complex than previously thought (Haut et al., 2004; Kriegstein and Noctor, 2004). Neurogenesis in the SVZ of postnatal and adult mammalian forebrain has been well researched; however, the mechanisms underlying cell migration and differentiation within this region is not

understood (De Marchis, 2001). It was demonstrated that intrinsic properties of precursor cells may be modified by grafting adult SVZ derived stem cells into the lateral ventricle (Soares and Sotelo, 2004). Because precursor cells continued to appear to over-proliferate and remain in an immature state after perinatal seizures even after animals matured to adults our hypothesis suggests that many of the youngest CNS cells, expressing neuronal precursor markers, may eventually differentiate into neurons with time to cause spontaneous seizures to occur later in life.

Changes in neuronal migration can be considered a type of seizure-induced pathology (Haut et al., 2004; Thom, 2003). The increase in the number of neuroblast precursor, migrating cells (detected with NeuN, DCX, PSA-NCAM, and nestin) was widespread in the ventricular system and this occurred within 5 days post-dye injection and after three early KA-induced seizures. This suggests that early seizures may need to be treated shortly after seizures are presented. Even one episode of status epilepticus resulted in some of these changes (unpublished observation), suggesting that a single perinatal seizure could have long-term consequences. Multiple perinatal seizures are presumed to have more deleterious side effects. Our data showed that there were greater increases in neuronal precursors after three prolonged perinatal seizures that extended and survived into adulthood suggesting that there is a long-lasting enhancement of general cell proliferation and migration with time. These findings are consistent with that of Parent et al, (2002) whereby neuronal precursor cells were found in distant limbic structures after seizures induced with pilocarpine in the adult brain, however, these did not survive in their model. The morphological finding that DiI and CMTMR labeled

cells were organized and formed chains is also suggestive of elevated neuroblasts and is consistent with other studies using PSA-NCAM to identify neuronal precursor cells (Doetsch and Alverez-Bullya, 1996).

In contrast to adults (Gall et al., 1991; Lowenstein et al., 1994; Akbar et al., 2001), immediate early genes (IEGs), nerve growth factors (e.g. NGF, BDNF) and heat shock proteins (HSPs) are not well activated by seizures at young ages such as at P10 or P21 except in the region of the dentate gyrus suggesting that growth factors may not be responsible for the observed increases in precursor proliferation (Dugich-Djordjevic et al., 1992; Nehlig and Periera de Vasconcelos, 1996; Dube et al., 1998). However, seizure activity has been shown to increase the expression of certain neural growth factors such as bFGF (Humpel et al., 1993) and EGF-like molecules (Opanashuk et al., 1999). Moreover, BDNF was reported to increase in the hippocampal CA3 of the limbic system as early as P7 (Kornblum et al., 1997). Therefore, it is also possible that certain growth factors are differentially regulated in development and may support mitogenesis and cell differentiation. Persistent elevations in certain growth and neurotrophin factors (not yet identified) that may be stimulated by early seizures could lead to a permanent change in the production of precursors of the SVZ.

Changes in precursor non-neuronal cells after prolonged developmental seizures.

Astrocytes express two different intermediate filaments during development: glial fibrillary acidic protein (GFAP) and vimentin. The activation of astrocytes is indicated by an increase in expression of GFAP caused by a physical insult (Stringer, 1996). Injury-induced gliosis has been regarded as an age-dependent repair process with

epileptogenic effects (Setkowicz and Janeczko, 2003; Kálmán and Ajtai, 2001). Stringer demonstrated that reactive gliosis, including both an increase in GFAP immunohistochemistry and the appearance of immunoreactivity for vimentin, was present 2-7 days after repeated seizures and that vimentin-immunoreactivity appears only in cells undergoing cell division (Stringer, 1996).

Our study also showed enhanced proliferation of non-neuronal cells (detected with GFAP, Vimentin, A2B5, and Ryp antibodies) particularly within the ventricular zone after early life seizures suggesting an increase in cell proliferation of the non-neuronal cell type also occurs after seizures and lasts for a long time. These results are consistent with kindled seizures, which also increase astrocytosis of the SVZ (Miyazaki et al., 2003). Oligodendrocytes were also co-labeled with CMTMR, that readily permeates cell membranes, in distant structures suggesting that they too are reaching incorrect targets and may contribute to pathological changes after perinatal seizures. Therefore, early life seizures can expand the SVZ neuroblast and non-neuronal populations and subsequently alter cell differentiation and cell migration in the adult rat brain to perpetuate epileptic disease. An increase was also noted in retrograde transport since NeuN co-localized with CMTMR in the cortico-amygdala within the pyramidal cell layer indicating there may be long-lasting increases in metabolic pathways of the limbic system. Therefore, antigenic markers have verified at early and long-term stages, that multiple phenotypes of precursor cells continue to proliferate and migrate within the CNS depending on the region and stage of development. Persistent germinative zones of the

immature brain into adulthood may provide a potential source of abnormal endogenous precursor populations to produce dysplasias and latent epilepsy.

Conclusion

In the short term study, results indicate that alterations in precursor cell proliferation and migration of immature cells occurs over long distances and rapidly. In the long-term study after three kainate-induced seizures, three types of unrecognized pathologies were found: (1) an increase in cell proliferation of the neuronal component of the VZ, verified with neuronal marker NeuN (2) increases in cell proliferation of the non-neuronal cell component in the white matter, an area assosiated with the VZ, and (3) an increase in the mature neuronal cell type in the amygdala. DiI labeled cells co-localized with neuronal precursor markers Nestin and NeuN in ventral thalamus and amygdala, which provides evidence for potential cell differentiation into neurons at distant locations. Because differentiation is delayed, it suggests that abnormal migration of developing neurons may cause a latent response which may lead to spontaneous seizures in adulthood once cell differentiation occurs and aberrant connections are formed. Co-localization of the third ventricle with GFAP, suggests that early life seizures increase ventricular astrocytosis which may also contribute to abnormal neuronal activity. Co-localization with Vimentin, A2B5, and RYP suggests that early life seizures increase proliferating precursor cells and oligodendrocytes which may further differentiate later in life and contribute to epileptic disease.

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