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Regulation of Proto-oncogenes Expression in Developmental Epilepsy

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Regulation of Proto-oncogenes Expression in Developmental Epilepsy

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Abstract

Seizures cause selective neuronal cell loss in vulnerable regions such as the hippocampus, cortex, thalamus or substantia nigra (SN) in human or experimental animals. While the HF can propagate seizures, the SN pars reticulata (SNR) regulates the spread of seizures in an age-dependent fashion, and the pars compacta (SNC) affects extrapyramidal motor control. In mature animals, both structures are vulnerable to seizure-induced damage. In prepubescent rats, seizure susceptibility is reduced and CA1 region is primarily affected. The proto-oncogenes, Bcl-2 and Bax, encode specific proteins that inhibit and promote programmed cell death, respectively. In order to determine whether proto-oncogenes are involved in cell death or survival, we examined mRNA and protein expression of Bax and Bcl-2 during several maturational (P13, P20, P30) stages. We used, in situ hybridization and immunohistochemistry following a single episode of KA-induced status epilepticus. At P13, there was no apparent change in the expression levels of Bax or Bcl-2 mRNA or protein within the hippocampus, 48h after status epilepticus. In contrast, Bcl-2 protein markedly declined within the SN sub-regions at P20 and P30, stages that do not exhibit SN damage at the times examined. Similar observations were found in the hippocampus at P20 and P30, except that their expression decreased in areas of cell injury or neuronal loss. Adjacent sections showed that tyrosine hydroxylase (TH) and glutamic
acid decarboxylase GAD levels were undisturbed in the SN. These results suggest that proto-oncogene regulation is not always associated with cell death or survival. Instead, changes in oncogenes expression may regulate the function of major neurotransmitters that provide homeostatic balance between excitation and inhibition, or inhibition of these oncogenes may play a critical role in the age-specific effects that the SNR and selective midbrain nuclei have on seizures.
Introduction

What is Epilepsy?

Definition: The phenomenon of recurrent nervous system seizures is termed epilepsy. The term derives from the Greek word epilambanein, and shares the same root as the verb 'to seize' or 'to attack'.

Previously epilepsy was thought of as a demoniac disease and gradually acquired a particular meaning as an epileptic seizure (Temkin et al., 1971). Seizures are initiated when small clusters of brain cells emit rapid, synchronized, highly rhythmic and repetitive electrical discharges. The dysregulation, first localized to a small area expands within seconds to involve the entire brain and can range from a brief episode to a massive, prolonged, life-threatening convulsion. Patients experience strange sensations, altered emotions, muscle spasms, and loss of consciousness (Hesdorffer et al., 1998). When seizures last more than 30 minutes without interruption, patients are considered to be in status epilepticus (DeLorenzo et al., 1992). Mortality from status epilepticus depends, aside from duration, on etiology, age, gender, race, and history of epilepsy (Towne et al., 1994).

Approximately 2.3 million Americans of all ages are affected by epilepsy and 181,000 new cases are diagnosed with epilepsy and experience seizures each year (Hausef WA, 1998). Approximately 10% of Americans will experience seizures in their life times and 3% by the age of 75 develop epilepsy (Begley, et
al 1998). Death rates are also elevated in individuals suffering from epilepsy, especially when seizures are not controlled (Langan, 2000). The severity of epilepsy varies among individuals. Most seizures are of a brief duration and generally subside without treatment. In contrast, some seizures of longer duration are very difficult to stop even with aggressive drug regiments (Begley et al., 2001).

**Temporal Lobe Epilepsy in the Mature Brain**

Complex partial seizures are one of the most common seizure types encountered in adults (Deonna et al., 1986). The epileptic activity propagates through vulnerable regions such as the hippocampus (HF), cortex, thalamus and substantia nigra (SN). Damage is later found in all of these limbic brain structures. However, hippocampal sclerosis is the hallmark of the disease. The HF is located in the midbrain and consists of two regions, dorsal and ventral (Alaike et al., 1998). Several studies indicate that the HF and amygdala have close anatomic and physiologic interactions, and initiation of the limbic seizure is likely a result of interaction between these structures (Alaike et al., 2001). In patients with intractable TLE the HF undergoes about 50-70% damage and the amygdala about 30-60% (Bruton, 1988; Milier et al., 1994). Since the HF is affected and is known to be involved in learning and memory, many studies have established that temporal lobe epilepsy (TLE) may impair memory abilities.
Temporal Lobe Epilepsy in Children

In humans, temporal lobe epilepsy (TLE) is common in adults but also prevalent to a lesser extent in children (Hauser et al., 1975). However, maturation reduces the amount of tonic and myoclonic components and frequency of epileptic spasms (Fogarasi et al., 2002). Temporal lobe epilepsy (TLE) in children is found within the anterior temporal areas (Jambaque et al., 2001). Seizures occur mostly in young children, especially in the first year of the life (Fogarasi et al., 2002).

Focal seizures of temporal lobe origin in infants may seem generalized clinically. The abnormalities associated with seizures of temporal lobe origin in young children consist of dysplasias, migrational disorders, and low-grade tumors (Harvey et al., 1997). TLE in childhood can produce mental retardation, language disorders, attention and memory disturbances, neurobehavioral problems, and learning disabilities (Stafstrom et al., 1993; Holmes et al., 1997). The early stages of development are a critical time in which seizures and SE may have more detrimental effects than in the mature brain. Neonatal seizures are particularly important since they are frequently associated with high mortality and morbidity, and may often be the only sign of central nervous system abnormalities (Hermann et al., 1992), auditory perception processing (Kester et al., 1991) and global visual processing (Doyon et al., 1991).
Kainic acid (KA) is a potent neurotoxin and analogue of glutamate that induces limbic seizures in adult and newborn experimental animals, but with different intensity, behavioral phenotype and neuropathological consequences. In adults the damage closely resembles human temporal lobe epilepsy (Corsellis and Bruton, 1988; Sutuia et al., 1989; Ben-Ari, et al., 1981). Other parallels between KA-induced status epilepticus in adult rats and TLE in humans include: partial seizures with secondary generalization and blockade with antagonists (e. g. benzodiazepine, pentobarbitai). Two main patterns of neurodegeneration described in rats following an i. p. KA injection include: (i) acute damage to extra-hippocampal structures such as the amygdale, entorhinal and piriform cortex, and thalamus that occurs between 6-24h following the onset of status epilepticus (Ben Ari, 1985; Friedman et al., 1994; Gramsbergen and Van den Berg, 1994). Damage in the temporal lobe typically precedes the appearance of seizures in several models (Mello et al., 1993; Nissinen et al., 2000). However, damage in the HF is associated with prolonged seizures (Jackson et al., 1998). The kindling model of TLE also suggests that repeated seizures cause selective loss of neurons in the HF (Cavazos and
Sutula, 1990) and amygdala (Tuunanen et al., 1997) in adults.

**Age-dependent Seizures and Damage**

Development of spontaneous seizures is age-dependent. Clinical studies and experimental models of epilepsy demonstrate that the immature brain develops and propagates seizures more readily than the mature brain (Moshe et al., 1983; Albala et al., 1984; Cavalheiro et al., 1987; Hauser et al., 1996). Hippocampal volume reduction is often associated with partial complex seizures at young ages (Saga and Oxbury, 1987; Lawson et al., 2000).

In rodents, the period of maximal seizure susceptibility appears in the 2nd and 3rd weeks of postnatal life (Albala et al., 1984; Cavalheiro et al., 1987). Despite a higher propensity towards seizures, the immature brain is relatively resistant to neurodegeneration in response to a single episode of KA-induced status epilepticus (Nitecka et al., 1984; Holmes et al., 1988; Friedman et al., 1997; Sperber et al., 1999). In contrast, KA induced seizure in peripuberal rats leads to damage in CA1 that follows this resistant period (Sankar et al., 1998). Rats younger than 30 days of age rarely develop spontaneous seizures (Stafstrom et al., 1992). Molecular mechanisms underlying delayed selective neuronal cell death associated with epilepsy remain unknown. In adult animals, molecular studies have shown that many genes may be altered following induction of seizures in various epilepsy models.
The Role of the Substantia Nigra in Age-dependent Seizures

The SN is a small nucleus located in the ventral midbrain. This nucleus contains dopaminergic (DOPA) neurons, which are detected by the presence of the dark-colored pigment called neuromelanin. SN consists anatomically of two main parts: the substantia pars compacta (SNC), which is composed of a sheet of DOPA neurons whose dendrites varicose project into the second part, the pars reticulata (SNR) which consists of inhibitory neurons with γ-aminobutyric acid (GABA) as their primary neurotransmitter (Fallon et al., 1995). In the SNR, DA released from the dendrites of DOPA neurons plays an essential role in the basal ganglia outflow; finally, the SNC acts as a center for the propagation of the seizure, and the SNR plays an important role as the center of the inhibitory system of status epilepticus (Fan et al., 2000).

Programmed Cell Death

Programmed cell death acts to shape the final complement and arrangement of the neurons in all neocortical regions (Chun and Schatz 1999). Programmed cell death during development has traditionally been thought to result after a competition for a limited supply of trophic support by differentiating neurons leaves some cells deprived of essential growth promoting factors (Oppenheim, 1991). This process has generally been thought to occur after
birth with the majority of cell death taking place postnatally. More recently, cell death has been found to occur extensively during embryonic development, possibly occurring even during stages of cell proliferation (Kuida et al., 1998).

During development of the nervous system, approximately 50% of the neurons that originally formed are eliminated (Oppenheim et al., 1991). The extent of neuronal survival is dependent on the production of neurotrophic molecules, which are produced by the target tissue. Only those neurons receiving sufficient neurotrophic factor from the target tissue continue to proliferate and differentiate, whereas others are lost through programmed cell death, a process called apoptosis (Metzstein et al., 1998; Sastry and Subba Rao, 2000). Apoptosis may play a role in neurodegenerative diseases and aging (Sastry and Subba Rao, 2000).

**Bcl-2 Family of Proto-oncogenes**

The Bcl-2 family of proto-oncogenes is recognized as a common regulator of multiple apoptotic pathways (Reed et al., 1994). The Bcl-2 family can be separated into two functionally distinct groups, the anti-apoptotic Bcl-2, Bcl-x, and Bcl-w, and the pro-apoptotic Bak and Bik proteins (Akao et al., 1994; Tsujimoto et al., 1987). These proteins have been identified as repressor or effector proteins, in different models of apoptotic neurodegeneration. Bcl-2 is a 25kDa membrane-bound protein (Akao et al. 1994; Hockenberry et al., 1990;
and Tsujimoto et al. 1986) that can block neuronal apoptosis in the developing brain (Allsopp et al. 1993; Garcia et al., 1992). Bcl-2 is localized in the mitochondrial membrane, in the nuclear envelope and in the endoplasmic reticulum (Hockenberry et al., 1990). Bax, another member of the Bcl-2 family, accelerates apoptotic cell death (Oltvai et al., 1993; Vekrellis et al., 1997). However, the level of activation of the Bax protein can be post-translationally regulated by Bcl-2, thus suggesting the existence of a feedback mechanism that may help to maintain the ratio of Bcl-2 to Bax protein in a physiologically appropriate range (Oltvai et al., 1993; Sadelack et al., 1995).

There is growing evidence that the Bcl-2 family of proteins controls the activation of apoptotic effectors such as cytochrome c and Apaf-1 in the caspase pathway in developmental models exhibiting neurodegeneration. In the caspase pathway, members of the "BH3-domain-only" subset of the Bcl-2 family of proteins connect proximal death signals to the core apoptotic pathway (Green, 2000; Kroemer, et al. 1999; Adams et al. 1998; Gross et al. 1999 and Huang et al. 2000). After activation of CD95 (Fas) or TNFR1 death receptors, BID is cleaved and activated as p15 tBID (Gross et al 1999). Activation of Bax has been proposed to initiate release of cytochrome c, and activation of Apaf-1. Caspases-9 apoptosome and additional downstream effector caspases (Li et al., 1998, figure 1).

Bcl-2 homodimerizes with itself and forms heterodimers with Bax in
regulating certain forms of apoptotic cell death. The ratio between anti-apoptotic and pro-apoptotic members is important for determining whether cell death is initiated or not (Sato et al., 1994; Oltvai et al., 1993; sedlack et al., 1995). Decrease in the basal expression of Bcl-2 might in this case lead to inappropriate cell death and might contribute to the development of degenerative diseases. Bcl-2 mRNA is expressed in the SN, which contains the dopaminergic neurons that degenerate in Parkinson's disease.

In the normal nervous system of the rat, during development and adulthood, the Bcl-2 mRNA is localized in neurons and the level of the Bcl-2 protein is higher in the developing brain than the adult brain (Frankowski et al., 1995). Bcl-x and Bax genes are also expressed in rat brains. High levels of Bax are observed in the adult brain in contrast to Bcl-2 (Krajewski et al. 1994; Green, 2000; Kroemer, et al. 1999). Bcl-2, Bcl-x and Bax mRNA and protein are all expressed in the CA1, CA2, and CA3 of the HF and in the ischemic transient infant rat brain (Ferrer et al. 1997).

In the current study, we examined Bcl-2 and Bax expression following KA administration of convulsant doses to young rats. Immunohistochemistry and double-staining immunofluorescence with antibodies to these proteins were used in combination with in situ hybridization for the corresponding mRNA to assess Bcl-2 and Bax expression at times and ages associated with cell death and survival. The exact localization, expression and distribution of Bcl-2 and
Bax is a key piece of information for understanding the role of these proto-oncogenes following seizure induced excitotoxicity. However, dissociation between neurotoxicity and proto-oncogene expression was observed in our study. For example, we found that both Bcl-2 and Bax proteins and mRNA were sustained after KA induction in the HF except in areas of cell injury and they were reduced in brain areas that survive the seizure at the ages examined. Many studies have shown that mRNAs encoding a variety of genes are up regulated in KA resistant regions such as in the dentate gyrus. KA-induced status epilepticus in adult rats does not produce changes in Bax and Bcl-2 expression that correlate with the delayed pattern of neurodegeneration. Expression of proto-oncogenes in developmental epilepsy as a potential neuroprotective mechanism has not been previously explored.
Figure 1. The Caspase pathway.
Experimental Design.

Young Sprague-Dawley rats were obtained from Taconic Laboratories Inc. German town, NY) at least 5 days prior to any experimental procedures. The rats were weighed at the start of the experiments and randomly grouped for experimental (n=7) and control (n=4). At postnatal (P) day 13 (P13), the animals experienced seizures by receiving an intraperitoneal injection of 2.5 mg/Kg of kainic acid. At P20, they received 7mg/kg and at P30 they received 12-mg/kg injections. The control animals received intraperitoneal injections of PBS. KA injections produced age dependent effects in the experimental group. P13 pups exhibited squealing, wet dog shakes, stiff tails, head and body tremors, and shivers. Response to KA changed with increasing in age. At P20 and P30 response to KA included: wet-dog-shakes, falling over, head nods, occasional torelimb clonus, jumping and salivation and tonic-clonic convulsions.

Only rats exhibiting increased locomotor activity about 30 minutes after the injection of the KA followed by rapid “wet-dog” shakes and recurrent tonic-clonic convulsions were used in these studies. At the completion of the experiments at 48 and 72 h after KA administration, rats were sacrificed by injection of pentobarbital followed by perfusion through the heart with pre-chilled 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The brains were rapidly removed and fixed in 4% PFA in PBS for 6-8 hours, then transferred to cold PBS for at least 24 hours prior to processing for
immunohistochemical analysis.

**Immunohistochemistry**

Immunohistochemistry was used to determine the effect of seizure behavior on expression of Bax or Bcl-2 that may affect cell death or survival. After the brains were fixed, the Vibratome was used to cut the HF and SN into 40 µm sections. Sections were used for polyclonal and monoclonal antibody labeling. The rabbit polyclonal Bcl-2 antibodies (N-19, sc-492 Santa Cruz Biotechnology) were specific for epitopes corresponding to amino acids 4-21, mapping at the amino terminus of human Bcl-2 (identical to the corresponding domain of mouse Bcl-2). This antibody does not cross react with Bcl-x or Bax. The rabbit polyclonal Bax antibodies (N-20, sc-493, Santa Cruz Biotechnology) were specific for epitopes corresponding to amino acids 43-61 of mouse Bax. This antibody is specific for Bax and does not cross-react with Bcl-2 or Bcl-x. The antibodies were used at dilutions of 1:100 and 1:200, for Bcl-2 and Bax, respectively.

Histological sections were quenched, incubated in 0.1% hydrogen peroxide in phosphate-buffered saline PBS, (to remove endogenous peroxidase activity) for 30 min, and then washed with PBS. Blocking was performed with 5% normal goat serum (NGS) and 0.1% TritonX-100 in PBS for 1 hour. Sections were then incubated with primary antibody to Bcl-2 (1:100), Bax (1:
Sections were washed three times with PBS and incubated with secondary antibody (Biotinylated anti rabbit IgG) for two hours. The sections were processed with Vectastain ABC kit (Vector lab Inc) at a dilution of 1:100 for 45 min. Sections were visualized by diaminobenzidine (Vector Lab, SK-4100) as a chromogen. Sections were then heat dried onto gelatinized slides dehydrated, cleared, and covered with cover slips. Haematoxylin-Eosin staining was used for histology.

**Double fluorescent labeling**

**Tyrosine hydroxylase**

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of dopamine and neuroepinephrine (Serratti et al., 1998). The neurons in the SN are exclusively dopaminergic neurons. Since these neurons are the source of striatal DA, a major neurotransmitter responsible for motor functions, their loss underlies the appearance of clinical motor disabilities (Dunnett et al., 1999).

**GABA receptor**

The amino acid GABA has long been considered the major inhibitory neurotransmitter in the adult cortex (Connors et al., 1988; Knjovic and Schwartz, 1967). GABA exerts its effects by activation of ionotropic GABA\(_\text{A}\) and metabotropic GABA\(_\text{B}\) receptors (Bormann, 1988; Connors et al., 1988; Kaila,
1994). Glutamic acid decarboxylase Gad is a unique enzyme for GABAergic neurons. GAD was used as a marker to identify GABAergic neurons that co-express Bax and Bcl-2.
Methods

Double immunofluorescence labeling

For sequential double labeling, sections were dissected in Vibratome (same as single labeling). To remove endogenous peroxidase activity, sections were incubated for 15 min in 0.1% hydrogen peroxide. Sections were subsequently washed and non-specific binding was blocked with 5% normal goat serum. Primary rabbit antibodies to Bcl-2 or Bax were used in dilutions of 1:50 and 1:100 respectively. Mouse monoclonal TH (Chemicon, MAB318) in dilution of 1:500 was used with the primary antibody. The sections that were incubated overnight at 4°C were washed three times in PBS. Sections were then incubated for two hours at room temperature with a 1:100 dilution of biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA) and FITC goat anti-mouse to detect TH. Sections were then washed three times in PBS and incubated for 1 h at room temperature in a 1:200 dilution of streptavidin Texas Red (TX, Vector Labs). Sections were washed several times in PBS and after mounting were checked by immunofluorescence microscope.

In Situ Hybridization

In situ hybridization was used to examine Bcl-2 and Bax mRNA expression at the level of HF and SN after KA injection. For in situ studies the rats were decapitated after 24 and 48 hours of SE and the brains frozen in pre-
chilled 2-methylbutain and stored at -70 °C until use.

**In Situ Hybridization Histochemistry**

Twenty-micron thick sections of frozen brains were used for *in situ* hybridization. Sections were prepared from control and kainic acid treated rats at 24 h and 48 h after KA administration. Sectioned tissues were transferred to poly-L-lysine-subbed slides (Poly-L-lysine, Sigma, p-1524). After the slides were air-dried, they were post fixed in 4% buffered PFA (para formaldehyde, Sigma p-6148) in PBS for 30 minutes. Sections were then acetylated in acetylation solution for 10 minutes at RT and washed twice in 2X SSC for 10 minutes. The sections dehydrated in 50%, 75%, and were stored in 95% ETOH at 4°C until used.

**In Situ Hybridization Molecular Biology**

**Plasmid Preparation**

Standard bacterial culture media Luria-Bertani (LB) broth and agar plates were prepared as described by Sam brook *et al* (1989). Ampicillin was added to LB 100µg/ml. Overnight cultures of Bcl-2, Bcl-x and Bax were diluted to 1:50 in LB broth plus ampicillin, and the subcultures were incubated with agitation at 37°C. The cells were harvested by centrifugation, digested and plasmids were collected by Qiagen Plasmid Mini Kit (Qiagen, 12123).
Linearization of Template with Restriction Endonuclease

Bcl-2 (full-length rat Bcl-2 cDNA is 708 bp, with the first and last 15 bp derived from mouse, Cell 1987 49:455-463) was linearized with EcoRI (Promega, R6017) and transcribed with T7 (promega, p2075) for the sense part. The anti sense part was linearized with Hind III (Promega, and transcribed with T3 (Promega, p2083). Bax (rat Bax cDNA is 446bp, and corresponds to bases 85-53 of the human Bax cDNA, Endocrinology 1995 136:232-619) linearized with EcoRV (promega, R6355) and transcribed using SP6 (promega, p1085) RNA polymerase.

Probe Synthesis (cDNA)

The 50 µl reaction mixture was prepared by mixing: 5µl of 5X transcription buffer, 1.5µl rATP 10mM, 1.5µl rCTP 10mM , 1.5µl rGTPmM, 1.5µl RNAsa block II, 1.5µl DTT0.75M, 5µl,35[S]-rUTP (1250Ci/mmol), 0.8 µg template, 2µl RNA Polymerase and DEPC water. The mixture was incubated for 1 h at 37°C for T3, T7 and Sp6 at 40°C.

Probe Purification

The probe was purified by adding 75µl of DEPC water and 100µl of chloroform, then centrifuged at 13000rpm for 5 min. The aqueous (upper) phase was collected and 100µl DEPC water was added to the remaining phenol phase and centrifuged for 5 minutes. The upper phase was then collected and combined with the original water phase. A 24:1 mixture of
chloroform/isoamyl alcohol was added to the combined water phase and centrifuged for 2 min. The upper phase (200\(\mu\)l) was collected and combined with 40\(\mu\)l of 10M NH\(_4\) acetate, and 900\(\mu\)l of ice-cold 100% ETOH. The probe was precipitated by incubation at -70°C over night and centrifuged at 12000 rpm for 45 minutes. The supernatant was carefully removed and the pellet air dried for 1-2 hours and resuspended in 40\(\mu\)l DEPC water.

**Pre-hybridization Solution**

The slides with brain sections were laid on a clean pad to dry. A ring of rubber or glue was made around the section. The humidified chambers (Nalgene plastic boxes) were laid with filter paper and soaked with 50% formalin in DEPC water. The pre-hybridization solution was made with herring sperm 150\(\mu\)g/ml DNA, 2.5X Denhardt's, 0.6M NaCl, 10mM Tris, 1mM EDTA, 0.05% SDS, 50% deionized formamide, 0.05 mg/ml total yeast RNA and DEPC water. Slides were placed into a humidified chamber and covered with pre-boiled hybridization solution. Slides were incubated at 49°C overnight. The next day the solution was removed by aspiration. The hybridization solution was mixed with probes calculated to ±10^6 cpm/section and boiled for 5 minutes. The sections were hybridized overnight at 42°C in a buffer containing herring sperm 150\(\mu\)g/ml DNA, 2.5X Denhardt's, 0.6M NaCl, 10mM Tris, 1mM EDTA, 0.05% SDS, 50% deionized formamide, 0.05mg/ml total yeast RNA, 10mM DTT and 10% Dextran sulfate. After hybridization the sections were washed briefly in
1X SSC for 30 min in a washing solution (50% formamide (SIGMA F-7503), 1X SSC, 10mM DTT, and 44% water). The sections were treated with 20µg/ml RNAse buffer at RT for 20 min and washed twice with 0.2X SSC for 1 h. They were then dehydrated in 50%, 70%, and 95% ETOH. The slides exposed on Kodak X-Omat AR at -70 for 4-5 days.
Results

Immunohistochemistry of Proto-oncogenes

In our studies, KA was injected into P13, P20 and P30 rats to determine the effects of SE on Bax and Bcl-2 gene expression in the HF and SN. Immunohistochemistry was used to determine the patterns of Bax and Bcl-2 proteins in the HF and SN following KA-induced status epilepticus at three ages (P13, P20 and P30). In control animals at the three ages examined, the immunoreactivity of Bcl-2 and Bax were dense and prominently expressed throughout the HF. Labeling was localized in the cytoplasm and nucleus of neuronal cells as a fine granular precipitate within both the HF (CA1, CA2, CA3), and SN (pars reticulata and pars compacta).

Age-dependent Seizure-Induced Neurodegeneration

Age dependent damage was generated at the pyramidal cell layer of the HF at CA3. In P13, no damage was detected at 48-72 h after status epilepticus (figure 2, A and B). In P20 pups as well, no damage was seen (figure 2, C and D). In contrast, at P30 lesions were seen 48-72 h after seizure termination (figure 2, E and F). This damage was detected in the pyramidal cell layer of the HF mostly in CA1 and some in the CA3 region (figures 2, E and F).
Figure 2. Immunohistochemistry of Bax (left panel) and Bcl-2 (right panel) 72 h after KA treatment.

The immunoreactivity of the HF cell layers are preserved and condensed when compared with control animals at P13 (A-B) and P20 (C-D). In contrast, at P30 lesions were seen 48-72 h after seizure termination. This damage was detected in the pyramidal cell layer of the HF, mostly in CA3. The decrease in immunoreactivity seen with Bcl-2 and Bax were associated with cell loss or injury (E-F).
In the SN, Bax and Bcl-2 immunoreactivity in control animals was uniform and condensed. The strongest expression of Bax and Bcl-2 protein was observed within the SNC, which contains dopaminergic neurons. In P30 animals, depletion of Bax immunoreactivity was observed in the SN mostly in the SNC and pars reticulata at 48-72h post-kainic acid administration (figure 3). At the time examined, these age groups do not yet exhibit obvious cell loss or injury to the SN after status epilepticus.
Figure 3. Immunohistochemistry of Bax 72h after status epilepticus in P30. A) Bax immunoreactivity in SN and parts of the mid brain in control animals was robust, shown at low magnification. B) High density of Bax immunolocalization is seen in dopaminergic neurons of the SNC. C-D) After seizure termination, there is a large-scale depletion of Bax expression in the SN and parts of the midbrain.
Bcl-2 protein stains uniformly and intensely in control animals, and is prominently expressed throughout the SN (figure 4A). The Bcl-2 immunoreactivity was greatly reduced in the SN, mainly in the SNR and SNC, following seizure termination at different time points of the study in P30 animals, when compared with control animal at the same age (figure 4B). Haematoxylin-eosin staining of adjacent sections in the SN showed the intensity of the neuronal cell layer of the SN, SNC and SNR. There was no visible modification in the cell layer of the SN, and cells were uniform and unchanged. Hence, there was no apparent cell loss in this structure at this age (figure 4C). Down regulation of Bcl-2 accelerates programmed cell death in vitro, although decrease in Bcl-2 does not seem to induce apoptosis per se.
Figure 4. Photograph of Bcl-2 in SN at P30.

A) The schematic on the left shows the Bcl-2 immunoreactivity of the SNC in the SN (with the high density of dopaminergic neurons in the SNC) in control animals. B) Bcl-2 protein is depleted 48h after seizure termination at P30. C) Haematoxylin-Eosin staining in adjacent sections of the SN shows that there was no apparent cell loss in this structure at this age.
In situ hybridization

The probe described above was used to analyze the expression of Bax mRNA in the HF and the SN. The results obtained with in situ hybridization revealed expression of the Bcl-2 and Bax mRNA in the HF at CA1, CA2, and CA3. The signal detected by in situ hybridization is specific for Bcl-2 and Bax because hybridization under identical conditions with a sense probe showed minimal or no signal. The Bcl-2 mRNA signal was localized over the pyramidal neurons in the CA1-CA3 areas of the HF and the granule neuron in the dentate gyrus. In control animals the Bax mRNA signal in HF was found to be uniform and prominently expressed in CA1-CA3 and in the dentate gyrus. Modification of the Bax mRNA signal in the HF was not observed in P13 animals at 24-48h post kainic acid administration when compared with control animals. However, animals at the same age showed a slight increase in Bax mRNA signal in the dentate gyrus at 24-48h following kainic acid administration (figure 5).
Figure 5. *In situ* hybridization of Bax in the HF at P14 in control and kainic acid treated rats. Bax mRNA was unchanged in control (A) and kainic acid treated (B) within the HF at P14.
The Bcl-2, Bcl-x and Bax mRNA were expressed throughout the SN in control animals. The strongest expression was seen in the SNC, which contains dopaminergic neurons. However, Bcl-2, Bcl-x and Bax mRNA expression detected in the SNR, which contains GABAergic neurons (figure 6). Modifications in the intensity of the Bax mRNA signal were observed at 24-48h after kainic acid administration. Decrease in Bax mRNA was observed in the SNC and to a greater degree in the SNR, which is similar to our previous immunohistochemistry results that showed a strong decrease in Bax protein expression in the SNC and SNR. *In situ* hybridization after generation of the auto radiograph slides were dipped in emulsion and individual cells labeled. The primary results suggest that the cells are affected differentially and that decreases are not uniform. Some cells express low level of Bax and some cells express higher level. Emulsion dipped sections showed that dopaminergic neurons of SNC expressed higher levels of Bax compared to a subset of cells within the SNR. Also, some cells in the SNC showed similar mRNA levels compared with some cells in SNR (figure 7).
Figure 6. In situ hybridization of Bcl-2 (A), Bcl-x (B), Bax(C) in control rats at P20, at the level of the SN. The Bcl-2, Bcl-x and Bax mRNA were expressed throughout the SN in control animals. The strongest expression was seen in the SNC, which contains dopaminergic neurons.
Figure 7: *in situ* hybridization of Bax mRNA showed a decrease in the SN, SNC, and SNR. (A). Emulsion dipped sections showed that dopaminergic neurons of the SNC expressed higher levels of Bax compared to a subset of cells within the SNR. Also some cell in the SNC showed similar mRNA levels compared with some cells in the SNR.
Double immunofluorescence Labeling

Double labeling was used to identify the cell types that would show a decrease or an increase in the expression of Bax or Bcl-2 as a consequence of the seizures. Since dopamine is the primary neurotransmitter of the SNC and GABA is that of the SNR, these were the first candidates to be chosen. An antibody to tyrosine hydroxylase (TH) was used to specifically identify Catecholamine (CAT) neurons in the sliced tissue sections and to provide a morphological marker of their axonal and dendritic processes.

In control animals, the Bcl-2 immunoreactivity was present in both SNC and SNR. The intensity of immunoreactions was higher in SNC than SNR (figure 8A). Decrease in Bcl-2 immunoreactivity was observed at 72h after kainic acid administration, which is in agreement with in situ and immunohistochemistry experiment (figure 8B).

Figure 8C shows the appearance of the SN DA in a 40µ section from a saline-treated control rat. Figure 8D shows the section from rats treated with a single injection of KA (11mg/kg). Comparison of these two figures shows that there was no apparent cell loss in this structure at this age.
Figure 8. Double immunofluorescence staining with Bcl-2 antibody (red) and TH (antibodies for identifying dopaminergic neurons, green) to identify the cell types exhibiting apoptosis.

A) SN in control animal shows high intensity is on SNC. B) SN in KA treated animal showed decrease in expression of Bcl-2. C) Dopaminergic neurons of the SNC in control animals. D) Dopaminergic neurons of the SNC show no significant change.
Figure 9: Co-localization of Bcl-2 and tyrosine hydroxylase. Immunofluorescence double labeling with TH and ATC (green) exhibit that the dopaminergic neurons of the SN are only expressed in the SNC. Bcl-2 antibody labeled with TX (red) was expressed in both the SNC and SNR.

Fig 10: Co-localization of Bcl-2 and tyrosine hydroxylase. The cells that co-express both Bcl-2 and TH at a higher magnification.
Figure 11. Double labeling of Bcl-2 and TH.
A) Double labeling of Bcl-2 and TH shows co-localization with DA neurons. B) The cells that express both Bcl-2 and TH are projecting toward the cells that only express Bcl-2, and there is an interaction between these two types of cells. Therefore, the projecting cells are non-GABAergic.

Figure 12. Bax and GAD immunofluorescence labeling.
There was a decrease in expression of Bax after KA treatment whereas the adjacent section showed that GAD levels were undisturbed.
Figure 13. Quantification of Bax and Bcl-2 72h after status epilepticus. 72h after status epilepticus, there was a reduction in optical immunodensity. Densitometric analysis of immunolabelling showed an approximately 50% reduction in oncogenes expression.
Discussion and conclusion

Many previous studies have stressed the importance of the HF as well as the SN in limbic seizures. The Bcl-2 family of proto-oncogenes, which encode specific proteins under different physiological and pathological conditions, regulates programmed cell death or apoptosis. Programmed cell death has been hypothesized to be a default pathway, which is kept under control in order to permit cells to continue to exist and proliferate. The Bcl-2 family (Bcl-2, Bax or Bcl-x) is differentially regulated in the SN and HF throughout development. Bcl-2 is one of the anti-apoptotic proteins (a member of Bcl-2 family) that is critical for post-mitotic neuronal cells, and overexpression prevents degeneration of both sympathetic and sensory neurons in vivo (Choi et al., 1995; Collins et al., 1980; Guarnieri et al., 1993) and in vitro (Nakayama et al., 1994). Additionally, Bcl-2 has rescued cells not only from apoptotic but also from necrotic death (Ferrer et al., 1998). Mutant mice with deficient Bcl-2 show degeneration of sensory and sympathetic neurons during postnatal development (Michaelidis et al., 1996). Mice with mutations in the Bcl-x (a member of Bcl-2 family) gene show massive apoptosis (Motoyama et al., 1995), a phenomenon also seen in vitro (Roth et al., 1996).
Bcl-2, Bcl-x and Bax proteins and mRNA are expressed in adult and pups as well as in kainic acid treated rats. We examined our hypothesis about why the immature brain is resistant to seizure-induced damage, by using in situ hybridization for mRNA detection and immunohistochemistry for identifying the Bcl-2 and Bax proteins, after administration of kainic acid. We have demonstrated at P14 that the level of Bax mRNA and protein in HF was unchanged. In contrast at P20, down regulation of Bax mRNA occurred in the SN. But this structure did not show any detectable cell loss at two levels of the SN. Accordingly, we have demonstrated the same results with double immunofluorescence labeling of DA and GABAergic neurons of this region. Interestingly, in P30, changes in Bax and Bcl-2 protein expression are observed 72 h after kainic acid treatment in CA2 and CA3 of the HF, which is associated with cell loss.

Emulsion dipped slides can label individual cells. The RNA data suggest that the cells were affected differentially and that their decrease is not uniform. The dopaminergic neurons of the SNC express higher levels of Bax compared to the subset of cells within the SNR, and some of these cells show similar mRNA levels when compared with those with in the SNC. These may be different cell types like somatostatine cells or other types of cells. It is worth
stressing that Bcl-2 and Bax interactions, as positive and negative regulators of cell death, are conditioned by their cellular localization.

In the SNR, most of the GABA content is associated with GABAergic neurons. GABA content prevents the spread of seizures. We examined with TH enzyme to DA neurons and GAD to GABAergic neurons. We found that the DA content was sustained in the same neurons that down regulate Bax and Bcl-2 genes. In other words, the DA neurons of the SNC and GABAergic neurons of the SNR were undisturbed despite the prolonged seizures. Therefore, decreases in the SN were not malfunction of these cells of their neurotransmitters and indicate the cells were still cell healthy. What remains to be clarified are the structural modifications that can compromise the localization, dimerization and function of Bax with other members of the Bcl-2 family in the nucleus following kainic acid excitotoxicity.

In the present study we show that that down regulation of these proto-oncogenes is not associated with consistent patterns of Bcl-2, Bcl-x and Bax protein or mRNA in sensitive regions such as the HF or SN. In fact, our results suggest that inhibition of Bax and Bcl-2 proto-oncogenes may play a critical role in the age-specific effects that the SNR has on seizures. One hypothesis is that down regulation of proto-oncogenes may cause tolerance by raising the seizure threshold to prevent development of spontaneous seizures.
Inhibition of Bax and Bcl-2 proto-oncogenes may play a critical role in these age-specific effects. In perpubital rats, a simultaneous reduction of Bax and Bcl-2 mRNA and protein expression following KA induction of seizures in the SNC and SNR caused no apparent increase in apoptosis. Taken together, the present data shows that the cells express oncogenes may act as a relay center to regulate excitation and inhibition between neurons of the SN compacta and SN reticulata. Thus, classical activation of Bax to initiate release of cytochrom c, activation of Apaf-1, caspase-9, and other downstream effectors caspases to induce cell death (Li et al., 1997) does not appear to apply in this model. Altered Bcl-2 and Bax protein patterns play a critical role in the age-specific effects that the SNR and selective midbrain nuclei have on seizures by affecting upstream transmitter nuclear systems. Therefore, it is important to further characterize these proto-oncogenes within the DA and GABAergic neurons of the SNC and SNR to elucidate components underlying age-dependent regulation of seizures. Understanding the role of these oncogenes on seizure thresholds during the postnatal period and seizure-induced brain damage that occurs later in life will impact disease management for seizure disorders at different ages.
References


expression in rat hippocampus following kainate-induced status epilepticus. Dev Neurosci, 19, 529-542.


