Published in final edited form as:

Epilepsy Res. 2007 January ; 73(1): 53–64.

Irradiation exacerbates cortical cytopathology in the Eker rat model of Tuberous Sclerosis Complex, but does not induce hyperexcitability

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Abstract

Tuberous Sclerosis Complex (TSC) is an autosomal dominant disorder characterized by multi-organ pathologies. Most TSC patients exhibit seizures, usually starting in early childhood. The neuropathological hallmarks of the disease - cortical tubers, containing cytopathological neuronal and glial cell types – appear to be the source of seizure initiation. However, the contribution of these aberrant cell populations to TSC-associated epilepsies is not fully understood. To gain further insight, investigators have attempted to generate animal models with TSC-like brain abnormalities. In the current study, we focused on the Eker rat, in which there is a spontaneous mutation of the TSC2 gene (TSC2+/-). We attempted to exacerbate TSC-like brain pathologies with a “second-hit” strategy - exposing young pups to ionizing irradiation of different intensities, and at different developmental timepoints (between E18 and P6). We found that the frequency of occurrence of dysmorphic neurons and giant astrocytes was strongly dependent on irradiation dose, and weakly dependent on timing of irradiation – in Eker rats, but not in irradiated normal controls. The frequency of TSC-like pathology was progressive; there were many more abnormal cells at 3 months compared to 1 month post-irradiation. Measures of seizure propensity (flurothyl seizure latency) and brain excitability (paired-pulse and post-tetanic stimulation studies *in vitro*), however, showed no functional changes associated with the appearance of TSC-like cellular abnormalities in irradiated Eker rats.

Keywords

tuberous sclerosis; Eker rat; brain pathology; dysmorphic neurons; seizures; hyperexcitability

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1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder that affects many systems in the body, with multiple lesions in the brain, lungs, kidney and heart (Roach et al., 1998). TSC has been shown to be caused by mutations in either of TSC1 or TSC2 genes (Young and Povey, 1998; Yeung, 2003). Tuberin and hamartin, the protein products of TSC2 and TSC1, respectively, play an important role in mTOR (mammalian target of rapamycin) signaling; mutations in these genes lead to aberrant cell development (Mathern et al., 2000; Gao and Pan, 2001; White et al., 2001; Kwiatkowski, 2003a,b; Narayanan, 2003).

The vast majority of the TSC patients suffer from seizures (Gomez et al., 1999), and indeed seizures may be the earliest clinical expression of TSC in children. In the central nervous system, structural abnormalities - tubers – appear in the cerebral cortex; some tubers are reported to be highly epileptogenic (Seri et al., 1991; Koh et al., 2000). Within the tubers, and scattered throughout the cortical tissue, are dysmorphic neurons, giant astrocytes, and hamartomas (Mathern et al., 2000; White et al., 2001). Surgical resections of “active” tubers provides seizure relief in some patients (Guerreiro et al., 1998), and studies of such tissue have provided valuable information about the cellular and molecular processes that might link the TSC mutations to brain hyperexcitability (Mathern et al., 2000; Crino et al., 2001; White et al., 2001; Cepeda et al., 2005). Another approach to this question of TSC-associated epileptogenicity has been to generate and study animal models in which the TSC genes have been modified. A conditional “knockout” of TSC1 has been described in mouse (Uhlmann et al., 2002; Wong et al., 2003; Tavazoie et al., 2005), and a spontaneous mutation of TSC2 has been described in rat (Eker, 1954; Eker et al., 1981). Despite the availability of these models, and the characterization of cellular morphological and physiological abnormalities in various cell populations (Uhlmann et al., 2002; Jansen et al., 2005; Tavazoie et al., 2005), the pathogenic pathways leading to the epileptogenic phenotype remain elusive.

TSC2 heterozygous mutant Eker rat exhibit some histopathologic abnormalities (Mizuguchi and Takashima, 2001; Takahashi et al., 2004), but they don't develop tubers, nor do they exhibit spontaneous seizure activity. Some studies, however, have suggested that these rats do show altered learning and alterations in synaptic plasticity. Eker rats have been shown to exhibit better maze performance and enhanced responses to chemically-induced kindling compared to normal controls (Waltereit et al., 2006). In electrophysiological experiments on hippocampal slice preparations, Eker rats had increased paired-pulse plasticity at hippocampal synapses (von der Brélie et al., 2006). Finally, introducing a “second-hit” – an attempt to affect the remaining intact TSC2 gene or induce other genetic abnormalities - has been shown to exacerbate Eker brain pathology (Takahashi et al., 2004; Wenzel et al., 2004). Interestingly, even in the dysmorphic cells found in Eker brains, there was evidence for tuberin expression (Mizuguchi et al., 2004; Wenzel et al., 2004).

In previous work from our laboratory (Wenzel et al., 2004), we demonstrated that the brain pathology in Eker rats could be exacerbated by exposing young pups to ionizing irradiation. Irradiated Eker rats with the heterozygous TSC2 mutation showed increased numbers of abnormal cells compared to non-irradiated Eker rats or irradiated controls. In the current study, we investigated the dose-dependency of this response to irradiation, and explored some functional consequences of this procedure. We found that cellular abnormalities increased as a function of irradiation dose (between 400 and 750 cGy), and that the manifestation of these abnormalities occurred relatively slowly over brain development. We also found that irradiation treatments increased seizure propensity in rats – but irrespective of the genetic background. Our *in vitro* functional analysis of cortical slices revealed no hyperexcitable changes in irradiated Eker tissue that correlated with increased frequency of cytopathological features.

2. Material and Methods

2.1. Animals and Irradiation

All procedures were consistent with NIH guidelines, and were approved by the UC Davis Institutional Animal Care and Use Committee.

Eker rats (TSC2 +/- mutation on a Fischer 344 background) were bred in our animal colony. [Note: TSC2-/- animals are embryonic lethal (Rennebeck et al., 1998).] Pups from TSC2+/- x TSC2+/- matings were genotyped at weaning (Yeung et al., 1995); PCR genotyping was carried out by the UC Davis Murine Genetics Laboratory with primer sequences provided by R.S. Yeung, University of Washington. To exacerbate TSC-like pathologies, we followed a “second hit” protocol (the “first hit” being the gene mutation) as described previously (Wenzel, et al., 2004). On postnatal day P0, P1, P2, P3, P4, P5, or P6, half the pups in a given litter were subjected to full body irradiation (between 400 and 750cGy) using a Gamma Cell 40 Irradiator (Cesium¹³⁷, Shepherd Inc.); the remaining animals in the litter served as non-irradiated controls. In addition, pregnant rats were irradiated on E18. Pups were left with their dams until weaning. From these pups we generated four experimental groups: 1) wildtype non-irradiated; 2) wildtype irradiated; 3) Eker non-irradiated; and 4) Eker irradiated. A subset of animals in each group was tested for seizure threshold at 1 month (n=159) and at 3 months (n=93). Morphological analyses (n=57 rats) were also carried out at 1 and 3 months post-irradiation. Seventy-nine animals (irradiated and non-irradiated Eker and wildtype rats), between 3 and 4 months of age, were used for electrophysiological studies.

2.2. Observations of Seizure Behavior and EEG monitoring

Animals were examined for spontaneous behavioral seizures during daily observation (approximately 10 minutes each morning) of the rats in their cages. Five Eker rats (3 irradiated at P3 750cGy and 2 non-irradiated) were monitored more intensively with video/EEG recording. Three month old animals were implanted (sterile surgical procedures) with single-channel telemetric transmitters (Data Sciences, MN). The transmitter was placed in a subcutaneous “pouch” on the back, just behind the neck. Leads from the transmitter were tunneled beneath the skin to the skull, where they were connected to two skull screws – one located at AP-3/L2.0 (“active” lead) and the other located just behind lambda (“reference” lead). Video/EEG monitoring started the day following surgery and continued for 6 days, with 2 hour daily monitoring sessions. Video/EEG data acquisition and analysis were carried out using Stellate System (Montreal Quebec, Canada) software; this software also provided capability for automatic detection of “spikes” and “seizures” – which could then be assessed visually and compared to the simultaneously-recorded video signal.

2.3. Fluorothyl –induced seizure threshold test

Seizure threshold was tested in 114 animals, measuring latencies to flurothyl-induced seizure activity. Rats were placed in an enclosed Plexiglas chamber (7 × 8 × 14 inches), and fluid flurothyl (Bis(2,2,2-trifluoroethyl) ether, Sigma Aldrich, MO) was dripped onto filter paper at a controlled rate of 20µl/min. Vaporized flurothyl was inhaled by the rats, causing seizures in all animals (Rho et al., 1999). Latencies to generalized tonic-clonic seizures were compared for animals exposed to same irradiation dosage (750cGy) at same developmental day (P3). Statistical differences were assessed between a) irradiated wildtype and irradiated Eker rats; b) non-irradiated Eker and non-irradiated wildtype rats; c) irradiated and non-irradiated Eker rats; and d) irradiated and non-irradiated wildtype rats. Comparisons were made for latencies at 1 month post-irradiation, for 3 months post-irradiation, and between 1 and 3 month post-irradiation timepoints.

2.4. Tissue Preparation for Histological Analysis

Animals were anesthetized with sodium pentobarbital (100 mg/kg, i.p.), then perfused with isotonic saline with heparin (1000 units/ml saline), followed by a solution of 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB) (pH 7.4). The brains were immediately removed and placed in the same fixative for 4 hours at 4°C. After postfixation, the brains were rinsed in PB, cryoprotected in 10% sucrose in 0.1M PB for 1 hour followed by 30% sucrose in 0.1M PB for 48 hours at 4°C, then frozen on dry ice. Thirty μ m transverse serial sections were cut on a sliding microtome equipped with a freezing stage, collected in sets of every fifth section, and then selected for further processing. For general quantitative histological evaluation, sections were stained with cresyl violet (CV) or hematoxylin and eosin (H&E). One set of coronal sections representing different anterior-posterior levels of neocortex was mounted on gelatin-coated slides, air-dried, rehydrated and then stained, followed by differentiation through a series of alcohols, cleared and coverslipped.

The remaining sets of sections were used for immunocytochemistry (ICC), in order to characterize/quantify the various cell types (e.g., dysmorphic neurons, giant cells, balloon-like cells) in the neocortex of Eker rats at one and three months postnatally. Subsets of alternating sections were processed for glial and neuronal markers, using a modification of the avidin-biotin complex (ABC)-peroxidase technique (Hsu et al., 1981), as previously described by Wenzel et al. (2004). Sections were rinsed in 0.1M PB; endogenous peroxidases were then inactivated by treatment with 0.5-1 % H₂O₂ in PB for 2 hours. Sections were treated with 3% bovine serum albumin (BSA) (Boehringer Mannheim, Indianapolis, IN), 3% goat or horse serum (Sigma, St. Louis, MO) and 0.25% Triton X-100 (TX) in 0.05M PB, 0.15M NaCl, pH 7.4 (PBS) for 1 hour to reduce nonspecific staining. Sections were rinsed in PBS for 30 minutes and incubated for 24 hours at 4°C in the various antisera [anti-neuron specific nuclear protein (NeuN), (Chemicon, Temecula, CA), 1:1000; anti-glial fibrillary acidic protein (GFAP), (DAKO Corporation, Carpinteria, CA), 1:4000; anti-glutamate decarboxylase 67 (GAD67), (Chemicon), 1:2000; anti-neurofilament protein (Chemicon), 1:2000; anti-phospho-S6 ribosomal protein (PS6), (courtesy of Dr. Peter Crino, Cell Signaling, New England Biolabs, Beverly, MA), 1:1000] in PBS containing 1% goat or horse serum, 2% BSA and 0.25% TX. Following rinses for 2 hours in PBS, sections were incubated in biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), diluted 1:500 for 24 hours at 4°C, rinsed 2 hours in PBS and then incubated in ABC (Elite ABC Kit, Vector Laboratories), diluted 1:500 in 1% goat or horse serum, 2% BSA, 0.25% TX and PBS for 24 hours at 4°C. Sections were rinsed thoroughly in Tris-HCL buffer (TB), pH 7.4 and 7.6, respectively, and then incubated for 15 minutes in 0.025% 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO) in TB, pH 7.6. After reacting for 5-10 minutes in fresh DAB with 0.003% H₂O₂, sections were rinsed in TB, followed by PB. Specificity of the immunostaining was evaluated by omitting primary antibodies from the regular staining sequence or pre-absorption of the antibody to the peptide. Sections were mounted on slides, dehydrated, cleared, and coverslipped with Permount.

2.5. Quantitative Analysis of Eker-related Pathologies and Irradiation Effects

Animals of each experimental condition were examined for occurrence of pathological cell features related to the Eker mutation and different irradiation doses at one and three months post irradiation. From each animal, 30 coronal brain sections (one section from each series of five) stained with CV, GFAP or neurofilament, were selected for the counting of dysmorphic neurons, giant astrocytes, and hamartomas within neocortex and hippocampus; sections spanned the brain from the level of the rostral caudate nucleus-putamen to the dorsal/middle hippocampus. Neurons were defined to be dysmorphic based on the following criteria: cell body diameter 2-3 times larger than that of large pyramidal cells in lamina V neocortex; heterotopic position - occasionally in clusters - within the neocortical laminae or hippocampus;

and disoriented processes (visualized with neurofilament staining). Large GFAP-positive cells, exhibiting giant cell bodies (greater than 20-50µm in diameter) and extended and thick processes (up to several hundred microns in length) which formed vascular end-feed, were defined as giant astrocytes. Collections of GFAP-positive cells, embedded in a fiber-rich matrix, located near the ventricles or within the corpus callosum/striatum and/or the deeper neocortex, were defined as hamartoma-like lesions. In addition to their GFAP-positivity, the large cells in these lesions were eosinophilic (H&E staining). In the graphs below, the quantified occurrence of these various pathologies is given as the average numbers of each pathological feature per 30 sections, from 2-3 animals for each experimental condition.

2.6. Electrophysiology

Irradiated wildtype and Eker rats (irradiated at P3 with 750cGy) and non-irradiated animals of each genotype were used for *in vitro* slice electrophysiology experiments. Animals were briefly anesthetized (Halothane, Sigma Aldrich, MO), decapitated, the brain quickly removed and placed in ice cold artificial cerebro-spinal fluid (ACSF; consisting of 124 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 1.2 mM MgSO₄, 26 mM NaCO₃, 2 mM CaCl₂ and 10 mM dextrose). The brain was then blocked and glued onto the stage of the Vibratome slicer (Vibratome 3000; Vibratome Inc, St. Louis, MO, USA). 400µm thick brain slices were cut in transverse or sagittal orientation in 1°C oxygenated ACSF. Slices were stored in oxygenated ACSF at room temperature for at least 1 hour prior to experiments. Slices were transferred to an interface recording chamber (Fine Science Tools, Foster City, CA, USA) and perfused with ACSF. Slices were equilibrated in the chamber at 37°C for at least 20 min before starting the experiments. Extracellular recording electrodes were pulled from borosilicate glass pipettes (1mm OD, Sutter Instruments, Novato, CA, USA) and filled with 2M NaCl (1-5MΩ). Bipolar stimulating electrodes were placed in layer VI of neocortex (at the border of the white matter), directly below the recording site in layer II/III of primary somatosensory cortex. Field responses were evoked by 40 µsec current pulses, between 0.6 and 1.0 mA in amplitude. To determine the stimulus intensity for testing in a given slice, the stimulus amplitude which elicited a population spike (i.e., “threshold” = T) was determined; stimuli at 0.5 × T were then used for eliciting field excitatory postsynaptic potentials (fEPSPs). Field EPSPs were recorded (Axoclamp-2A, Molecular Devices, CA) as negative shifts from baseline, and fEPSP amplitude was calculated as the difference between baseline and the peak negative deflection. Field responses were evoked at 0.05 Hz; a fEPSP amplitude was calculated each minute as the average of 3 responses. Responses were monitored for at least 15 minutes, to establish a stable fEPSP baseline, before additional stimulation parameters (below) were imposed. Field potential data were digitized and processed on computer; all slices used for electrophysiology were subsequently fixed in 4% paraformaldehyde for later histological evaluation.

A paired-pulse paradigm was used to study the strength of excitatory and inhibitory influences in response to stimulation. Pairs of stimuli (same amplitude) were delivered at varying interpulse intervals - from 5 to 400msec - at 0.05 Hz. The ratio of the second fEPSP amplitude to the first fEPSP amplitude was calculated, and plotted for different intervals. Statistical comparisons were made across groups for each interpulse interval (pair-wise t-tests).

On a different set of slices, we applied a tetanic stimulation protocol. A stable field amplitude was established, and then the tetanic stimulus was applied -- 5 bursts of 25 stimuli/burst, at 100Hz (Kirkwood et al., 1993; Walcott and Langdon, 2002); bursts were separated by 20 seconds and stimulus amplitude during the tetanus was increased to threshold (T) for the population spike. Post-tetanus responses were recorded for 40 minutes, with stimuli delivered at 0.05 Hz and stimulus amplitude at 0.5 T. Average fEPSP amplitude for each minute was normalized to the average baseline response. Statistical analysis (pairwise t-tests) was performed at each timepoint across groups (i.e., between: a) irradiated wildtype and irradiated

Eker, b) non-irradiated wildtype and non-irradiated Eker, c) irradiated wildtype and non-irradiated wildtype and d) non-irradiated and irradiated Eker). In addition, each post-tetanus point was compared to the pretetanus within-group changes (post-tetanic changes in fEPSP amplitude compared to baseline).

3. Results

3.1. Seizures and seizure threshold

None of the animals exhibited abnormal behavior during routine daily observations. None of the rats implanted for video/EEG monitoring (three irradiated (P3, 750cGy) and two non-irradiated Eker rats), monitored at 3 months post-irradiation, displayed abnormal EEG or behavioral patterns.

Twenty wildtype rats (10 non-treated and 10 irradiated at P3 at 750cGy) were compared with forty-four Eker rats (22 non-treated and 22 irradiated at P3 at 750cGy) for seizure threshold. Testing was carried out when the animals were 1 month of age. In addition, twenty wild-type rats (10 non-treated and 10 irradiated) and thirty Eker rats (15 non-treated and 15 irradiated) were tested at the 3 months of age. All tests were done at the same time of day, at room temperature. In the 1 month old group, we found no statistically significant differences between wildtype and Eker rats in the non-treated group, and between wildtype and Eker rats in the irradiated groups (Fig. 1A). There was a trend, but no significant difference in seizure latency between irradiated and non-irradiated wildtype rats. The difference between non-treated and irradiated Eker rats, however, reached statistical significance ($p < 0.05$, one way ANOVA); irradiated Eker rats showed an almost 10% reduction in the latency to generalized seizure compared to non-irradiated Eker rats (Fig. 1A). In animals at 3 months of age, there were no statistically significant differences between wildtype and Eker rats (Fig. 1B) in both non-treated and irradiated groups. However, we again found differences between non-treated and irradiated wildtype ($p < 0.005$) and between non-treated and irradiated Eker rats ($p < 0.001$). In both cases, the seizure latencies of irradiated animals were reduced by 17% (compared to latencies of non-treated animals of the same genotype). Finally, there were no significant differences when comparing latencies within each group at 1 month and 3 months (compare Fig. 1 A and B for each group).

3.2. Irradiation effects on the brain pathology

Irradiation had a general effect on all animals, causing significant reductions particularly in those brain regions in which neurons are born relatively late – i.e., the dentate region of the hippocampus and the granule cell layer of the cerebellum. As previously reported, cytopathological brain features were never observed in non-treated and irradiated wildtype rats, and were rare in non-irradiated Eker rats.

In sections from from irradiated Eker (TSC2+/-) rat brains, histological examination confirmed and expanded our previous observations (Wenzel, et al., 2004), as summarized in Table 1. In general, irradiated Eker (TSC2+/-) rats exhibited cytopathological features that included dysmorphic neurons (Fig. 2A,B,G) and giant astrocytes (Fig. 2H-J), pathologies commonly observed in dysplastic cortex of TSC brains. In addition, hamartoma-like lesions (Fig. 2C,D) with large “balloon-like” cells (Fig. 2E,F) were seen in older animals. Although clusters of 10-20 dysmorphic neurons were observed in some animals, no cortical tubers were found. Further, irradiation induced no obvious changes in the pattern of GABA (presumably inhibitory) neurons and terminals – as reflected in GAD67 immunocytochemical staining (data not shown). Quantification of pathological cell features showed that the occurrence and frequency of abnormal cell features was only weakly dependent on the timepoint of irradiation,

but strongly dependent on irradiation dose, and on the duration of post-irradiation survival (Table 1).

Our previous study showed that tuberlin was still expressed in the dysmorphic and giant cells of irradiated Eker cortex (Wenzel et al., 2004). In the current study, we also examined the expression of phospho-S6 ribosomal protein (PS6). In theory, if formation of the TSC complex is disrupted (e.g., by loss of normal tuberlin), there should be an upregulation of the mTOR/S6 pathway (Baybis et al., 2004), resulting in enhanced expression of PS6. Indeed, the giant/dysmorphic cells of irradiated Eker cortex showed high expression of PS6, as did the cells of the hamartomatous lesions seen in these animals (Fig. 3B, C). However, high levels of PS6 were also seen in large pyramidal cells of normal/control rat cortex (Fig. 3A).

3.2.1. Effects of irradiation timing—Early postnatal irradiation (P3-P4) led to a reduction in size of the dentate gyrus and cerebellar cortex in both irradiated wildtype- and Eker (TSC2 +/-) rats. Earlier (P1) γ -irradiation resulted in slightly greater reduction of brain weight (<10%) than later irradiation (P4). Irradiation at E18 resulted in obviously microcephalic brains, associated with disorganization of the neocortex and appearance of hippocampal heterotopia.

Twenty-eight animals were used to study the effect of the timing on the brain pathology. Four hundred cGy or 750cGy irradiation was used at different pre- and postnatal days (Fig. 4A). The 400cGy irradiation dose was applied from P18 to P6, but the 750cGy irradiation dose was used only from P1 to P4 since doses greater than 400cGy at E18 were lethal in all rats. When tissue pathology was evaluated at 1 month post-irradiation, cell counts (for dysmorphic neurons) from neither 400cGy- nor 750cGy-irradiated animals showed any significant dependency on the day of irradiation (Fig. 4A, left). Similarly, when tissue was examined at 3 months post-irradiation, there was little apparent effect of day of irradiation for the 400cGy group (Fig. 3A, right). However, for 750cGy dose, P4-irradiated animals showed a greater number of dysmorphic neurons than the P1-irradiated group (Fig. 4A, right).

3.2.2. Effects of irradiation dose—We exposed P3 wildtype and Eker rats to full body irradiation at 400, 500, 650, 700 and 750cGy (Fig. 4B). Irradiation at 750cGy was the upper limit for animal viability; irradiation at 800cGy was lethal for all animals. Morphological assessments made at 1 month revealed a slightly larger number of dysmorphic cells at higher doses than at lower doses (Fig. 4B), but the dose-dependency was most dramatic at 3 months post-irradiation. Cell counts performed on 3 month-old Eker rats showed almost twice the number of dysmorphic cells in the brain of animals irradiated with higher doses (700/750cGy) than with lower doses (400/500cGy) (Fig. 4B). The dose-dependency was also seen (although not quite as dramatically) in counts of cells with abnormal glial phenotype; GFAP ICC revealed increased numbers of giant astrocytes in brains irradiated at higher dosages than at lower doses (Fig. 4C: compare 750cGy vs. 400cGy).

We did not find hamartomas in the brains of irradiated Eker rats examined at 1 month post-irradiation, for any irradiation dose. At 3 months, hamartoma occurrence was generally independent of the irradiation dose (Fig. 4C).

3.2.3. Progression of changes over time—The occurrence of pathological cell features were rare in irradiated Eker rats at 1 month post-irradiation (Fig. 4D; Table 1), but showed dramatic progression when compared to abnormalities at 3 months post-irradiation. For all irradiation doses, when the consequences of irradiation were examined at 3 months post-irradiation, the frequencies of both dysmorphic neurons and giant astrocytes were 2-4 times the frequency seen at 1 month post-irradiation (Fig. 4A,C,D). Further, hamartoma-like lesions were seen only at 3 months post-irradiation. These results strongly support the occurrence of

a progression of pathology over time (and certainly between 1 and 3 months). It should be noted that this progressive pathology was restricted to Eker rats.

3.3. Electrophysiology

Seventy-nine animals were used for electrophysiological slice experiments. All animals in the irradiated group were exposed to 750cGy irradiation at P3, and slices were made from animals at 3–4 months postnatal – i.e., at a time when pathological features were clearly apparent. Field potential recordings were obtained from the layer II/III in the primary somatosensory neocortex. Electrical stimulation at the border between deep neocortex and corpus callosum elicited negative shifts in the field potential corresponding to membrane depolarization of single neurons (intracellular recordings with sharp electrodes). Forty seven rats (13 non-treated and 10 irradiated wildtype, 11 non-treated and 13 irradiated Eker rats) were examined using a paired-pulse stimulation protocol. The plots of facilitation/inhibition vs. paired-pulse interval (Fig. 5A) showed no statistically significant differences between wildtype and Eker rats, in both non-treated and irradiated groups. We studied the irradiation effect on changes in synaptic strength in brain slices from wildtype and Eker rats (8 animals in each group, non-treated and irradiated wildtype and Eker rats) following tetanic stimulation. Five 100Hz, 1 second long stimulation trains elicited no significant long-term changes in slices from non-treated animals (Fig. 5B, left). In slices from irradiated wildtype rats, the same tetanic stimulation protocol induced an increase in fEPSP amplitude starting 20 min after tetanus (Fig. 5B, right); however, the slices from irradiated Eker rats showed no significant change in the fEPSP amplitude after the high-frequency stimulation trains (Fig. 5B, right). Despite this apparent difference between irradiated wildtype and Eker rats, statistical comparison between these two groups showed that differences in posttetanic fEPSP amplitude were not significant.

4. Discussion

In the current study, we have shown that the central nervous system manifestation of TSC-like pathology can be exacerbated in Eker rats by exposing the animals to a “second” hit insult, ionizing irradiation. Our data indicate that the degree of pathology depends on irradiation dose and, to a lesser degree, on the developmental timepoint of the irradiation “treatment.” For any given treatment, in genetically predisposed Eker (TSC2+/-) rats, the number of abnormal cells/lesions increased over time (1 month vs. 3 months), showing a developmental progression of brain pathology. Despite the occurrence of TSC-like neuropathological features in irradiated Eker rat neocortex, our study failed to demonstrate any significant seizure-related differences between these animals and normal rats. In particular, evoked seizure thresholds did not differentiate irradiated Eker rats and irradiated controls, and EEG monitoring failed to reveal spontaneous seizures or electrical epileptiform discharge patterns. It should be noted, however, that our EEG sampling was limited (small sample number, relatively short monitoring times), so that these negative EEG findings may reflect an inability to identify subtle and/or infrequent seizure-related events.

4.1. Irradiation and brain pathology

Ionizing irradiation has been shown to affect developing organisms, causing a variety of pathological conditions. Actively dividing and immature cells are most affected by irradiation (Hicks et al., 1959; Altman et al., 1968), where the treatment often results in cell death or injury. Prenatal (Hicks et al., 1959; Roper et al., 1997a; Marin-Padilla et al., 2003) and early postnatal irradiation treatments interfere with the normal development of neuronal precursor cells, leading to long-lasting effects on the brain (Tofilon and Fike, 2000; Jyothi Lakshmi et al., 2002; Limoli et al., 2004). Irradiation has also been shown to injure radial glia (Roper et al., 1997a), resulting in abnormal migration of later-born neurons. While irradiation timing largely determines the *type* of the brain pathologies (dyslamination, heterotopia, cortical thinning, etc),

the irradiation dose determines the *extent* of damage (Hicks et al., 1959; Altman et al., 1968; Setkowicz et al., 2003). Thus, the earlier and more intense the irradiation insult, the more pronounced is the brain pathology. In our study, the earliest time-point of irradiation was E 18, and the resultant brain pathology from E18 irradiation was similar to that reported by Roper and colleagues (Roper et al., 1997a; Roper, 1998) who applied their irradiation insult at E17; we found disorganized and thinned neocortex, and heterotopic cell clusters in the hippocampus. The extent of the pathology was greater in our studies, probably due to the higher irradiation dosage (400cGy vs. 225cGy).

Recent studies on human fetal TSC brain tissue have shown that features characteristic of TSC brain (nodular clusters of cells suggestive early tubers, subependymal hamartomas, heterotopic neurons) appear by the 19th or 20th week of gestational age (Park et al., 1997; Wei et al., 2002). Given these observations, it is possible that the abnormalities that give rise to cytopathological TSC-like features (dysmorphic neurons, giant astrocytes, etc.) occur much earlier than our irradiation delivery, and that the lack of more robust neuropathological effects (and associated dysfunction) simply reflects a poor choice of irradiation timing. It is possible that a “second hit” delivered earlier than the irradiation window in our experiments may eventually be shown to be effective in producing significant TSC-like brain pathology. However, observations on human tissue (and particularly on tissue from fetuses that failed to come to term) are difficult to relate to model development in rodents. As indicated above, our decision to study P0-P5 time points was based on preliminary experiments that not only revealed an optimal window for irradiation, but also showed that earlier insult led to significant general brain damage that was not consistent with the goals of our modeling.

Finally, it is important to note that previous irradiation studies (Roper et al., 1993; Roper et al., 1997a; Marin-Padilla et al., 2003) were carried out on “normal” (wildtype) rats, and did not report induction of cytologically abnormal cell types such as dysmorphic neurons, giant astrocytes, and “balloon cells” in hamartoma-like clusters. In contrast, we previously showed that full body irradiation of Eker rats, with 400cGy γ -irradiation, produced cytopathology (Wenzel et al., 2004). The development of morphologically abnormal cell types, with pathologies that resembles those found in brains of TSC patients (Mizuguchi et al., 2000; Takahashi et al., 2004), is most likely a function of the interaction between irradiation and the genetic defect in Eker rats. Untreated Eker rats have also been shown to exhibit abnormal cortical development if they are allowed to survive to old age (1.5-2 years for rats - see Mizuguchi et al., 2000); however, even in these older animals, the cytopathology appears to be relatively rare.

Do the dysmorphic/giant cells seen in Eker cortex actually “model” the giant cells in human TSC? Histologically, the correspondence is striking. In the current study, we also used PS6 immunocytochemistry to assess the possibility that the mTOR pathway was dysfunctional in Eker dysmorphic cells, as would be predicted if these cells were deficient in producing adequate tuberlin to form the TSC complex that inhibits PS6 formation. Indeed, the large cells of irradiated Eker cortex showed dense immunopositivity for PS6 (giant neurons and astrocytes, as well as cells in hamartomas). While this result is consistent with a tuberlin deficiency, we are hesitant to point to this result with much confidence, since we also found significant PS6 immunopositivity in large pyramidal cells in normal rat cortex. We do not believe that we can draw useful conclusions from these observations.

Our data suggest that irradiation can exacerbate the development of cytopathology in Eker rats, with the degree of pathology directly related to the irradiation dose and timing. Clearly, however, given the relatively small numbers of abnormal cells found per irradiated brain, the irradiation causes abnormal cell development in only a sub-population of the developing cells. Consistent with our previous report (Wenzel et al., 2004), the maturation of that pathology

appears to be progressive; we saw dramatically more abnormalities in Eker rats 3 months after irradiation than at 1 month, irrespective of time-point and dose of the irradiation treatment.

4.2. Irradiation-induced neuropathology and brain excitability

Animal models of cortical dysplasia have provided important insights into possible mechanisms of generation and propagation of epileptiform activity associated with such structural abnormalities. The freeze lesion model of cortical microgyria, and the MAM-model of cortical dysplasia, have shown that developmental cortical abnormalities can lead to abnormal cortical function (Baraban and Schwartzkroin, 1995; Baraban and Schwartzkroin, 1996; Luhmann et al., 1998; Jacobs et al., 1999a,b; Baraban et al., 2000; DeFazio and Hablitz, 2000; Peters et al., 2004; Redecker et al., 2005; Tschuluun et al., 2005). Irradiation-induced abnormalities in brain development also lead to abnormal function. Electrophysiological studies on brain slices from irradiated rats exhibit hyperexcitability and decreased inhibition (Roper et al., 1997b; Smith et al., 1999a,b; Zhu and Roper, 2000; Chen and Roper, 2003). *In vivo* studies have demonstrated increased seizure-susceptibility in rats following prenatal irradiation (Werboff et al., 1961; Vernadakis and Timiras, 1963; Cooke et al., 1964; Roper et al., 1995; Setkowicz et al., 2003; Setkowicz et al., 2005). Moreover, frequent spontaneous seizures have been reported in rats exposed to low-to-medium dose irradiation early in development (100-145 irradiation at E16-17) (Kondo et al., 2001; Kellinghaus et al., 2004). None of the animals examined in our study exhibited spontaneous seizures or abnormal EEG patterns, but irradiation did reduce seizure latency in both wildtype and Eker rats. The major differences between our experimental design and those of previous studies were: a) the irradiation dose (400-750cGy vs. 100-225cGy); b) the timing of treatment (E18-P5/6 vs. E16-E17); and c) rat strain (Fisher vs. Sprague-Dawley).

In contrast to our results, recent studies have reported functional and electrophysiological abnormalities in Eker rats. Waltereit et al. (2006) showed radial maze abnormalities and enhanced chemical kindling, and von der Brélie et al. (2006) described enhanced paired pulse plasticity and reduced LTP and LTD in hippocampus. The basis for the apparent differences between their studies and ours is unclear, but there are a number of possibilities. First, the behaviors studied by the other authors are primarily hippocampally-mediated – whereas we examined only neocortical electrophysiology and generalized seizure sensitivity. Second, (and perhaps more likely), there may be significant strain differences that interact with the TSC2 mutation; our background strain was Fisher, whereas these other investigators used Long Evans rats. A comparison of rat strain-dependent seizure propensity has been reported for a variety of epileptogenic treatments (Loscher et al., 1998), but we are unaware of any studies on strain influences with respect to irradiation-induced pathology and/or excitability.

A goal of the current study was to address the relationship between degree of brain pathology (i.e., number of the abnormal cells) and hyperexcitability in the Eker model of TSC. While we found that the number of cytologically abnormal cells increased in Eker rats (but not TSC2^{+/-} + controls) with increasing irradiation dose, we found no correlated measure of excitability. Seizure threshold decreased following irradiation, but this effect was seen in both TSC2^{+/-} and TSC2^{+/+} rats, indicating that irradiation alone was the salient variable. We found no measure of functional hyperexcitability in our slice electrophysiological studies on neocortical tissue. There are several considerations that may be important in explaining these perhaps surprising results. First, it is possible that the high irradiation doses used here caused such significant neuronal damage in Eker rats that any tendency toward hyperexcitable was offset by a general depression due to reduction of the neuronal population. This explanation is consistent with our somewhat paradoxical results from the tetanic stimulation study, in which irradiated wildtype – but not Eker – tissue showed facilitation. Interestingly, Kellinghaus et al. (2004) showed that low-to-medium -- but not high -- irradiation doses were effective in

inducing spontaneous seizures in rats. A similar finding was reported by Kondo et al. (2001), where low-dose (145cGy), early (E17) irradiation was effective. Lower dose irradiation may cause different types of neuronal injury (and/or changes in the extra-neuronal milieu) (Tofilon and Fike, 2000; Limoli et al., 2004), causing hyperexcitability where higher doses simply eliminate neuronal populations. Another possible explanation for the apparent discordance between our results and those of previous studies is the different types of brain abnormalities caused by early vs. later irradiation. The earlier irradiation protocol (E16-17) led to pronounced heterotopic cell clusters in the hippocampus and cortex (Kondo et al., 2001; Kellinghaus et al., 2004), whereas our protocol generated only scattered dysmorphic neurons and glia – and at an admittedly low frequency.

Cytologically abnormal cell types examined in tissue from TSC- related surgeries have been shown to exhibit functional abnormalities, including changes in membrane physiology and altered receptor expression (White et al., 2001; Uhlmann et al., 2002; Wong et al., 2003; Jansen et al., 2005; Tavazoie et al., 2005). Similar studies in other animal models of TSC have revealed cell properties consistent with seizure propensity (Uhlmann et al., 2002; Wong et al., 2003; Jansen et al., 2005). Why, then, were we unable to demonstrate hyperexcitability in tissue from irradiated Eker rats? First, it should be noted that we did not examine the single cell properties of cytologically abnormal neurons or glia. And it is at least plausible that development of scattered/isolated abnormal brain cells is not sufficient to induce general tissue (cortical) hyperexcitability. We did show that high irradiation doses (750cGy at P3) led to development of relatively large hamartomatous structures (>500µm) in the Eker brain. In addition, clusters of abnormal cell types were found in the neocortex of irradiated Eker rats (Fig. 2A). We were not able to select such regions of abnormality for our *in vitro* studies, however (the abnormalities occurred at too low a frequency to detect without a specific marker that is visible in viable tissue), and thus carried out most of these electrophysiological analyses on cortical slices with relatively normal cytological features. We would predict that hamartomas and clusters of abnormal neurons would lead, at least locally, to rearrangement of the surrounding cortical circuitry, but evidence for functional reorganization was absent in our experiments; neither the paired-pulse stimulation paradigm nor the tetanic stimulation protocol revealed an altered balance between synaptic excitation and inhibition in irradiated Eker brain. Finally, we restricted our quantitative analysis to cellular abnormalities exclusively in neocortex, and focused on neocortex for our slice studies of hyperexcitability. The involvement of other brain regions that might be affected by the irradiation, and that might contribute to general seizure propensity, was not evaluated in our electrophysiology experiments.

Our studies of the Eker model of TSC have provided interesting data regarding potential “second hit” influences on TSC-like cellular pathology, and especially highlighting the progressive nature of these cytopathological features during postnatal brain development. The studies are disappointing, however, in the relative absence of correlated functional (hyperexcitability) correlates. While many additional variations on “second hits” and neurophysiological analyses could be performed, we are pessimistic about the usefulness of the Eker model for studying the relationship between cortical pathology and seizure propensity.

Acknowledgements

This study was funded by an Epilepsy Foundation Postdoctoral Research Training Fellowship (NT) and NIH/NINDS grant NS18895 (PAS). We are grateful to K. Katleba and A. Altangerel for excellent technical support, and to Dr. Peter Crino for supplying the phospho-S6 antibody.

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Figure 1.

Effects of γ -irradiation on latency to generalized seizures (seizure threshold) induced by flurothyl exposure. Seizure thresholds were measured at one month (**A**) and three months (**B**) following irradiation. Irradiated and non-irradiated wildtype (wt) rats were compared to age matched irradiated and non-irradiated Eker (het) rats. Irradiation produced a decrease in seizure threshold at 1 month and 3 months for both wildtype and Eker rats, indicating that irradiation per se, and not genetic background, was the key variable.

Figure 2.
Histopathological features of neocortex from Eker (TSC2^{+/-}) rats irradiated early in postnatal development.

A. Low power photomicrograph (parietal neocortex – layers labeled I–VI) from a cresyl violet-stained coronal brain section of a 3 month old Eker rat irradiated at P3 (750cGy). Arrows indicate large, dysmorphic neurons.

B. Higher magnification of the cell body of a dysmorphic neuron (arrow), located at the border between layers IV and V.

C. Low power photomicrograph of a large hamartoma-like lesion (arrow) located at the interface between corpus callosum (CC) and deep neocortex. This section, from a 3 month old Eker rat (irradiated at P3 with 500cGy), was immunoreacted for GFAP; the hamartoma shows intense GFAP immunoreactivity (IR) in cells and processes (compare with adjacent neocortex).

D. Multiple small hamartomas (arrows) could also be found in irradiated Eker rats (3 750cGy). Again, cells in these hamartomas exhibited high GFAP IR in cell bodies and processes.

E/F: High magnification of a cresyl violet-stained cell (E) and two GFAP-immunoreacted cells (F) within hamartomas. These cells resemble the large eosinophilic (H&E-stained) cells in TSC tubers.

G. A large, dysmorphic neuron (arrow) in neocortical layer IV (3 month old Eker rat irradiated at P4 with 750cGy). The tissue was immunoreacted for neurofilament. Note the intense IR in the soma and proximal processes.

H. Example of a giant astrocyte (GFAP IR) in the neocortex of a 3month-old Eker rat (irradiated at P0 with 400cGy). This astrocyte forms irregularly-shaped and extended processes. Arrowheads indicate normal neocortical astrocytes for comparison.

I. Low power photomicrograph of the dentate gyrus (GC) from a 3 month old Eker rat (irradiated at P4 with 750cGy); several giant astrocytes were found in the hilus (Hi) (boxed area).

J. High magnification of the boxed area in I, showing giant astrocytes (arrows) with irregularly-shaped processes. Arrowhead indicates a normal astrocyte for comparison. Scale bars: 200µm, A, C, D, I; 40µm, B, F-H, J; 20µm, E.

Figure 3.

Immunostaining of phospho-S6 ribosomal protein (PS6) in representative coronal sections of the cortex from a 3 month-old wild-type rat (A), and an Eker rat (B and C) irradiated at postnatal day 3 with 750cGy. **A.** Scattered PS6 immunoreactivity localized to neuronal cell bodies across the cortical layers. Note that some of the large layer V pyramidal cells (arrows) show high levels of protein expression. **B.** High PS6 expression in two dysmorphic neurons (arrows) in irradiated Eker cortex (layers IV and V). There is a lower level of PS6 protein expression in the surrounding cortical neurons. **C.** PS6-positive cells in a subependymal hamartoma-like lesion in the caudate nucleus and infiltrating into the corpus callosum (CC). Abbreviations: VI, V: neocortical layers VI and V; CC: corpus callosum. Scale bars: 50µm in A, B and C.

Figure 4.

Semi-quantitative analysis showing the relationship between irradiation parameters (timing, dose, etc.) and cytopathology in Eker rat neocortex. Each bar represents the average number of abnormalities from 2-3 Eker rat brains; cell counts were made from 30 sections per brain (every 5th section, extending from the level of rostral striatum to the middle of hippocampus). Counts were made in animals at 1 month (white bars) and 3 months (gray bars) post-irradiation. Because the sample number is so small for any given time point or irradiation dose, no error bars have been calculated.

- A.** Irradiation timing effect. The number of dysmorphic cells is plotted as function of the irradiation timepoint, for 400 and 750cGy γ -irradiation doses. The time of 400cGy irradiation had little effect when assessing dysmorphic neurons at 1 or 3 months post-irradiation. At 750cGy, a slightly later irradiation timepoint resulted in greater pathology assessed at 3 months post-irradiation.
- B.** Irradiation dose effect on dysmorphic neurons. All irradiation treatments were given at P3. The number of dysmorphic neurons is plotted as function of the irradiation dose (400-750cGy). For both 1 and 3 month assessment times (but most dramatically for the 3 months time), increasing dose resulted in an increasing number of cytologically abnormal neurons.
- C.** Irradiation dosage effect on giant astrocytes and hamartomas. All irradiation treatments were given at P3. Numbers of giant astrocytes increased significantly following irradiation, but the dose effect was subtle. Hamartomas were not detected at 1 month post-irradiation, but were evident (at low numbers) at 3 months – again, without an obvious irradiation dose effect.
- D.** Schematic illustration of Eker (TSC2+/-) histopathological features at one and three months of age. Animals were irradiated at P3 with 400 or 750cGy. There was only a low frequency of cytopathological features at 1 month post-irradiation. At 3 months post-irradiation, however, the number of dysmorphic neurons, giant astrocytes and hamartomas were significantly increased (for both 400 and 750cGy irradiation doses) as compared to the early timepoint. Indeed, at the 750cGy irradiation dose, there was a 2-3-fold increase in occurrence of dysmorphic neurons and giant astrocytes.

Figure 5.

- A.** Paired-pulse data from neocortical slices. The left graph shows fEPSP ratios vs. inter-pulse interval for non-treated wildtype (wt) and Eker rats. The right graph shows data for experiments on irradiated wildtype and Eker rat cortex. Pair-wise comparisons were made for each interval; there were no significant differences across groups. Inset shows an example of a stimulus-evoked field potential recorded in neocortex. Asterisk indicate stimulus artifact.
- B.** Changes in fEPSP amplitude following tetanic stimulation in neocortical slices. Graphs show fEPSP amplitude (relative to baseline) as a function of time, for non-treated wildtype and Eker rats (left), and irradiated wildtype and Eker rats (right). An average measure was calculated each minute for each slice; error bars show standard error (n=8 rats for each group). Pair-wise comparisons at each timepoint revealed no significant differences across groups, except for an enhanced response in the irradiated wildtype cortex at ~ 20 minutes post-tetanus. .

Table 1**SUMMARY OF HISTOPATHOLOGICAL FEATURES**

Irradiation Dosage/Day		400 cGy		750 cGy	
Pathologies		P1	P3	P1	P3
1 Month	Dysmorphic Neurons	+	+	+	+
	Giant GFAP+ Cells	+	+	+	+
	Hamartoma-like Lesions	None	None	None	None
	Dysmorphic Neurons	+	+	+++	+++
3 Months	Giant GFAP+ Cells	+++	+++	++++	++++
	Hamartoma-like Lesions	+	+	+	+
+ 1-10 abnormal features					
++ 11-30 abnormal features					
+++ 31-60 abnormal features					
++++ >60 abnormal features					