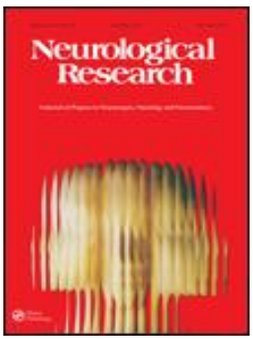

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The effects of continuous and single-dose radiation on choline uptake in organotypic tissue slice cultures of rabbit hippocampus

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The objective of the present study was to determine the time-dependent course of choline uptake in mature organotypic slice cultures of rabbit hippocampal formation and to assess the effects of continuous and single high-dose irradiation on choline uptake in cultivated slices in vitro. *Transverse slices of hippocampus were dynamically incubated in a cerebrospinal fluid-like culture medium for 72 h. To study the changes in choline uptake longitudinally, the slice cultures were processed with 0.1 μM [^{3}H]-choline, and tritium accumulation was counted. Two different gamma irradiation sources (^{125}I seeds and a clinical ^{60}Co source) were used as representative models of interstitial radiosurgery and other radiosurgical techniques. A total dose of approximately 6000 cGy was delivered to the brain slices in one session or in a continuous, relatively low-dose rate fashion, and their effects on high-affinity choline uptake were examined. In another set of experiments with ^{125}I, 5 μM hemicholinium-3 was used in choline uptake procedures as a competitive high-affinity choline uptake inhibitor. The results can be summarized as follows: (1) in the control group of the hippocampal tissue culture, there was a significant increase in tritium accumulation values from 0 to 48 h and a decrease thereafter; (2) continuous ^{125}I irradiation caused a highly significant depression of the accumulation of tritium compared to that observed in the control group throughout its application for 72 h; (3) there was no significant change in the accumulation of tritium in the slices after single high-dose rate irradiation with a ^{60}Co source; and (4) 5 μM hemicholinium significantly depressed the accumulation of tritium in both the control and the ^{125}I-irradiated groups, and there was no longer a difference between ^{125}I-irradiated and control groups when both groups were treated with hemicholinium. These results demonstrate that the delivery of continuous but relatively low-dose rate gamma irradiation is more efficacious than single high-dose external irradiation on high-affinity choline uptake in hippocampal nervous tissue. The results also indicate that continuous irradiation specifically affected the high-affinity energy-dependent choline uptake mechanism, whereas nonspecific choline uptake did not seem to be disturbed.* [Neurol Res 2001; **23**: 669–675]

Keywords: Choline uptake; hippocampus; organotypic cultures; radiosurgery; radiation; epilepsy; acetylcholine

INTRODUCTION

The use of radioisotopes and other sources of radiation for medical and scientific purposes has created a need for precise knowledge of the biological effects of irradiation. Relatively little research has been done on the effect of ionizing radiation on neuronal function, e.g., on neurotransmitter synthesis, storage, release and uptake. Important data are still lacking about brain tolerance and neurobiological effects of irradiation[1]. This deficiency is related in part to the lack of appropriate animal models to answer these questions.

Choline transport is an important aspect of functioning cholinergic synapses as well as of cell membrane integrity, and the capacity of high-affinity choline uptake correlates with changes in cholinergic neuronal activity[2,3]. Cultivation of nervous tissue provides access to individual neural structures for morphological, physiological and pharmacological studies. Among presently available *in vitro* preparations, slice cultures of brain tissue are used increasingly, mainly because cultured brain regions keep their 'organotypic' characteristics under these culture conditions. Therefore, it is likely that changes in the capacity of choline uptake in organotypic tissue slice cultures may be a representative model of the neurochemical changes inflicted on the human brain by radiosurgical interventions[4–8].

As radiosurgery for various diseases of the brain has become widely used, the present study was designed to investigate and compare *in vitro* continuous and single high-dose ionizing radiation effects on choline uptake,

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Table 1: Time course and the effect of continuous irradiation with ^{125}I, single dose irradiation with ^{60}Co and/or 5 μM hemicholinium on the accumulation of tritium

	Control group		^{125}I group		^{60}Co group		Control group with 5 μM HC		^{125}I group with 5 μM HC	
Hours	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
0	38	12851 ± 422	38	12852 ± 422	38	12852 ± 422	19	8177 ± 154	19	8177 ± 154
14	24	20245 ± 748	11	16665 ± 890	12	18149 ± 778	12	9914 ± 291	11	8776 ± 304
24	24	23842 ± 1109	12	19017 ± 1523	12	24425 ± 1453	12	11552 ± 389	12	10244 ± 499
38	23	30408 ± 1439	12	23345 ± 1230	12	28305 ± 955	12	11481 ± 347	12	10015 ± 410
48	24	33105 ± 1634	9	23325 ± 2019	12	33514 ± 2220	12	12819 ± 447	12	8645 ± 498
62	22	25289 ± 1987	12	19711 ± 1291	12	29432 ± 1636	12	8512 ± 615	11	7897 ± 1256
72	23	20467 ± 1722	10	10840 ± 443	12	18699 ± 1139	12	7348 ± 474	12	4875 ± 371

Each point represents the mean ± SEM of tritium accumulation (dpm mg^{-1} tissue). HC, hemicholinium; *n*, number of slices).

using mature organotypic slice cultures of central nervous system tissue. The objective of this study was to establish basic data for the development of laboratory investigations on the radiobiology of radiosurgery, and to describe the effects of continuous and single-shot high-dose irradiation on choline uptake in hippocampal slice cultures.

MATERIALS AND METHODS

Animals, chemicals and solutions

A total of 17 Watanabe rabbits (2–3.5 kg) of both sexes were obtained from Charles River WIGA GmbH (Sulzberg, Germany) and used for the experiments. The animals were housed one per cage in a vivarium at a constant temperature of 24°C in a well-humidified (50%) atmosphere with 12 h light/dark cycles (lights on at 07:00 hours) for at least three days before the experiments commenced, and were given a standard laboratory diet and water *ad libitum*. The animal studies were approved by the local animal care committee.

Choline chloride (methyl-^3H, 79.2 Ci $mmol^{-1}$) was purchased from NEN-Research Products (Du Pont de Nemours GmbH, Dreieich, Germany); amino acids (alanine, arginine, citrulline, cysteine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, serine, taurine, threonine, tryptophan, tyrosine, valine), bovine serum albumin (Chon V fraction), and hemicholinium-3 from Sigma Chemie GmbH (Deisenhofen, Germany); NaCl, KCl, KH_2PO_4, $NaHCO_3$, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, NaOH, and glucose from E. Merck (Darmstadt, Germany); L-ascorbic acid from Riedel-de Haen (Seelze, Germany); and penicillin/streptomycin from Gibco Laboratories (Grand Island, NY, USA).

Two solutions were prepared freshly at the beginning of each experiment: (1) cerebrospinal fluid-like culture medium[6–8] for the cultivation of brain slices, and (2) modified Krebs–Henseleit buffer for the preparation of the slices and [^3H]-choline uptake procedures. The culture medium and a modified Krebs–Henseleit buffer containing the chemicals in defined concentrations were saturated with 5% CO_2 in O_2 for 30 min; the pH was adjusted to 7.4. Both of the solutions were kept at a temperature of 3°C during the intervals (10–14 h) between usages.

Hippocampal slice cultures and incubation

After decapitating the animal with a guillotine, the brain was rapidly removed and put into ice-cold modified Krebs–Henseleit buffer to protect the tissues from hypoxic damage. Dissection and removal of the hippocampal formation was made on an ice-cold aluminum plate. The brain was dissected by making a sagittal section between the hemispheres, and following the ventricular system the hippocampal formation was identified, reversed and removed. The two hippocampi, moistened with Krebs buffer, were transferred to the cutting table of a McIlwain tissue chopper (Fa. Bachofer, Reutlingen, Germany) on which a layer of moistened round filter paper was placed (Rundfilter Nr 591-50 mm, Schleicher & Schüll, Kassel, Germany), and then cut into 350 μm transverse slices which maintained the typical lamellar structure of the hippocampus. About 10 min passed from the time of killing the animal until the hippocampi were sliced. Hippocampal slices which appeared to be in good condition (350 μm thick, 4–7 mm diameter, 4–7 mg wet weight) were selected and transferred into well-illuminated, sterile, 35 mm-diameter, plastic Petri dishes containing 2 ml of culture medium. For each experiment, two sets of three dishes (13 slices per dish) were used. *Table 1* shows the total number of slices (*n*) used in each set of the experiments.

All dishes (separating irradiated and control groups) were transferred into the incubation chamber, and were kept at a temperature of 22°C and gassed continuously with a humidified atmosphere of 95% O_2–5% CO_2. The incubation chamber was rotated slowly (16 revolutions/min) and intermittently (for 4 sec, 4 times per min)[9] to avoid stasis, to maintain the exchange of metabolites and O_2/CO_2 during incubation, and to facilitate the free-random movements of the slices with regard to the radiation source in order to assure homogeneous distribution of the slices within the irradiated target volume. In the incubation chamber, the irradiated and control groups were separated from each other by a lead

wall. Each of the two compartments of the dual chamber was supplied by the humidified gas mixture. The culture medium was changed twice per day (14, 24, 38, 48, 62, and 72 h after the onset of incubation) and incubation was maintained for 72 h.

Irradiation

These experiments involved the use of two gamma irradiation methods, one for continuous high-dose rate irradiation, and a second for single high-dose exposures. ^{125}I seeds (Model 6702) were obtained from Amersham Buchler GmbH (Braunschweig, Germany) and used as gamma irradiation sources. Before the experiments, each seed was calibrated in an ionization chamber (Curiementor, PTW, Freiburg, Germany). The beta-emission of the ^{125}I is totally irrelevant due to the titanium cover of the seeds. After calibration, two seeds were fixed in a teflon catheter and sterilized. Then, at the beginning of the incubation, three catheters (with six seeds) were left in the centers of the dishes. Seed activities (total activity of two seeds) ranged from 34.20 to 40.75 mCi. The accumulated dose at 8 mm distance was calculated by assuming a point source. Thus, the dose rate constant was 76–90 cGy h^{-1} at 8 mm in air. The doses at this distance ranged from 5397 to 6430 cGy (mean 5783 cGy) after 72 h.

Single high-dose irradiation (229 cGy min^{-1} for 26 min; total dose 6000 cGy) was delivered from a clinical ^{60}Co source (Telecobalt, Philips Medical Systems, Best, The Netherlands). A vertical beam was used (gantry 180°, field 20×20, accommodation time 20 sec, high voltage 660 V, quality factor 1.000). After irradiation, the slices were transferred into the incubation chamber.

[^{3}H]-choline uptake procedures

The uptake procedures were performed twice per day (at the beginning, and after 14, 24, 38, 48, 62, and 72 h). After rinsing once with Krebs buffer, the cultured slices (six per group) were selected and transferred into tubes containing 0.1 μM choline-chloride-[methyl-^{3}H] (79.2 Ci/mmol-15.4 μl) in the modified Krebs–Henseleit buffer (2 ml) at the specified time points, and then the tubes were layered in a water-bath at a temperature of 37°C. The solution was gassed continuously for 30 min, i.e., the bubbling was adjusted to move the slices gently at the bottom of the tube. After the uptake procedure, the slices were washed twice with 2 ml of modified Krebs–Henseleit buffer over a period of 20 min at 37°C in the tubes layered into the uptake procedure bath, by gassing with 95% O_2 + 5% CO_2. Hemicholinium-3 as a competitive inhibitor of high-affinity choline uptake was used in another set of experiments. High affinity choline uptake was determined as the difference in the tritium accumulation in the absence and presence of hemicholinium-3. The inhibition of choline uptake was determined by adding hemicholinium-3 to the same tubes used for [^{3}H]-choline incorporation at a concentration of 5 μM. Thus, the [^{3}H]-choline uptake and washing procedures were performed under 5 μM hemicholinium-3. At the end of the washing periods, the net weight of each slice was determined in a vial, and then the slices were dissolved in 0.5 ml NaOH (1 M) for tritium determination. In each experiment, the total set of materials to be counted consisted of six sets of six vials; each set was prepared independently. Ten millilitres of scintillation cocktail (Ultima-Gold, Packard, Groningen, The Netherlands) was added, the vials were shaken, and radioactivity was determined by liquid scintillation spectrometry (1217 Rackbeta, LKB-Wallac, Germany).

Statistics

The results (tritium contents of the uptake experiments) were expressed as mean ± SEM (d.p.m. per mg − wet weight of the slice). The existence of significant differences between the means of the irradiated and control groups was tested using two-way ANOVA. Time of irradiation was the first independent variable (factor A), and irradiation (groups) was the second independent variable (factor B), with regard to the [^{3}H]-choline uptake in the hippocampal slices. The data obtained from the uptake values of [^{3}H]-choline in the control groups were also analyzed by one-way analysis of variance (ANOVA, Scheffe F-test) to determine the statistical significance of the time-dependent changes. p-Values of less than 0.05 were considered significant.

RESULTS

From 0 to 48 h of pre-condition *in vitro*, there was a significant time-dependent increase in the accumulation of [^{3}H]-choline in the control groups ($p < 0.01$). After 48 h in culture, a significant decrease in the [^{3}H]-choline uptake was observed until the 72nd hour (ANOVA: $p < 0.01$; *Table 1, Figure 1*). Thus, a bell-shaped curve which showed an increase from 0 to 48 h and a decrease thereafter was observed in tritium accumulation.

The changes in choline uptake of the hippocampal slices, which were continuously irradiated with ^{125}I seeds for 72 h, are presented in *Figure 1*. A general depression of [^{3}H]-choline accumulation in the hippocampal slice cultures as an effect of continuous ^{125}I irradiation was evident. The two-way analysis of variance (ANOVA) showed a very significant difference between the control and irradiated groups (factor B, $p < 0.01$), and between the specified time points (hours, factor A, $p < 0.001$). Although depressed significantly, the time-course curve of tritium accumulation was similar to the control groups, showing an increase until the 48th hour and a decrease thereafter. No significant interactions between factor A and B were evident.

Two-way ANOVA revealed no significant changes in [^{3}H]-choline uptake in the cultured hippocampal slices upon exposure to single high-dose irradiation with a ^{60}Co source (factor B, $p < 0.05$), whereas significant differences were found between the specified time points (factor A, $p < 0.001$) (*Table 1, Figure 2*).

Hemicholinium significantly depressed the tritium accumulation in both groups as compared to that

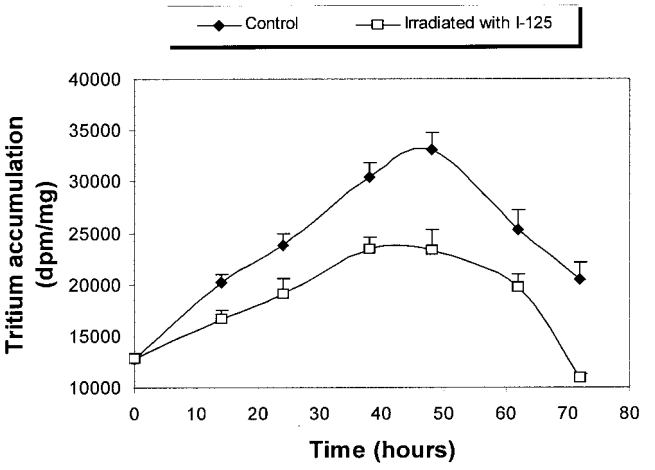

Figure 1: The time course and effect of continuous ^{125}I irradiation on the accumulation of tritium. The average levels of tritium accumulation in hippocampal slices were significantly depressed compared to those in the control group (two-way ANOVA; factor A: $p < 0.001$; factor B: $p < 0.001$). In both groups, from 0 to 48 h in culture there was a significant increase in accumulation values of tritium and decrease thereafter ($p < 0.01$). Each point represents the mean ± SEM of tritium accumulation (dpm mg^{-1} tissue). *Table 1* shows the *n* values

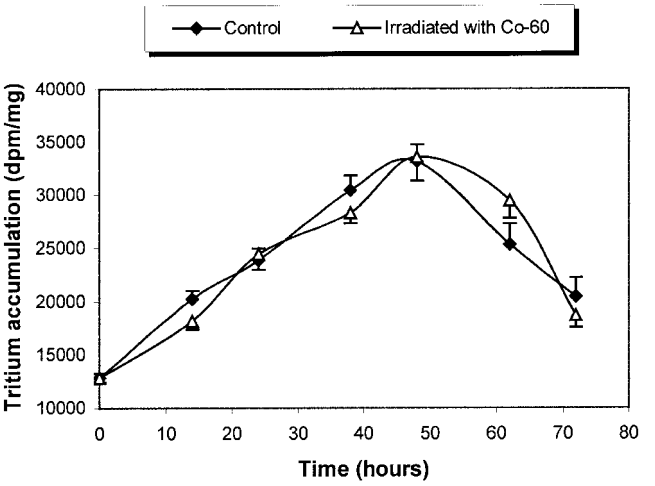

Figure 2: Time course for the effect of single high-dose rate irradiation with ^{60}Co. There were no significant changes in tritium accumulation in the cultured hippocampal slices compared to the control groups, using two-way ANOVA (factor A: $p < 0.001$; factor B: $p > 0.05$). Each point represents the mean ± SEM of tritium accumulation (dpm mg^{-1} slice)). *Table 1* shows the *n* values

observed in the groups treated without hemicholinium (ANOVA; $p < 0.001$). There was no longer a significant difference between the control and the ^{125}I-irradiated groups when both groups were treated with hemicholinium (*Table 1, Figure 3*).

DISCUSSION

An understanding of the neurochemical effects of high-dose radiation in mammalian nervous tissue is essential if radiosurgery is to be used in functional neurosurgery for the ablation of non-neoplastic tissue or the modulation of neuronal function. There is a remarkable potential to use radiosurgery for a variety of brain diseases, as a relatively new treatment modality. Despite widespread clinical experience in radiosurgery of arteriovenous malformations and tumors, the paucity of investigations in experimental models has left many questions unanswered pertaining to the effects of radiosurgery on neurons[1].

The principal advantage of a nervous tissue culture model is that it permits controlled studies regarding continuous observation and direct accessibility of the cells to the pharmacological agents and labelled neurotransmitter precursors. Under *in vitro* conditions, slice cultures of hippocampus provide a model for the study of radiation effects on the various identified types of hippocampal neurons in physiological, pharmacological and morphological experiments[9,10,11]. Moreover, hippocampal slice cultures allow to selectively assess radiation effects on neuronal function, as no vasculature is involved which is supposed to interfere significantly with purely cellular effects due to the radiosensitivity of the endothelial cells[12,13].

Several studies have shown that in mammalian brain, cholinergic synaptosomes possess two saturable uptake systems for choline, one with relatively low affinity and the other with higher affinity[14,15]. The low-affinity uptake system can be regarded as providing choline for more general metabolic pathways; it has been found in various tissues and cell types and is thought to be involved in the synthesis of phospholipids (e.g., glycerophosphorylcholine, phosphorylcholine, and phosphotidylcholine) as part of the biological membranes[16]. The high-affinity choline uptake system appears to be restricted only to cholinergic neurons[17]. This system is energy- and Na^+-dependent, and is especially sensitive

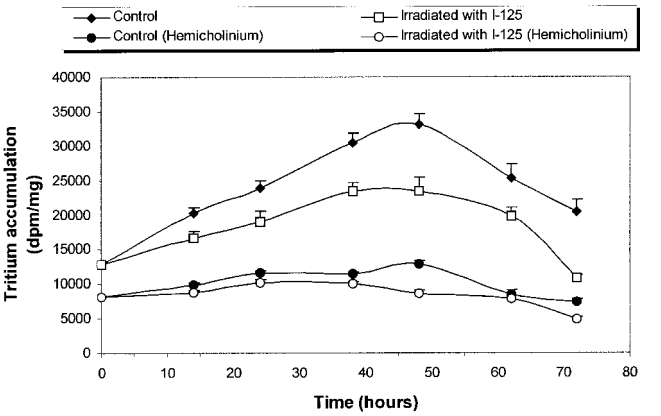

Figure 3: Effect of 5 μM hemicholinium on [^{3}H]-choline (0.1 μM) uptake in cultured hippocampal slices. Hemicholinium significantly depressed tritium accumulation in both the control and the irradiated groups ($p < 0.001$). The differences among the groups treated with hemicholinium (between control and ^{125}I-irradiated) were not statistically significant (ANOVA; $p > 0.05$). The values are mean ± SEM of tritium accumulation (dpm mg^{-1} tissue). *Table 1* shows the *n* values. The values of the control and ^{125}I-irradiated groups treated without hemicholinium are the same as in *Figure 1*

to inhibition by micromolar concentrations of hemicholinium-3[3], and may be rate-limiting in the synthesis of acetylcholine[18]. Slices of the hippocampus and cortex exclusively contain the cholinergic nerve terminals of the cell bodies of neurons that reside in the septum and basal forebrain, respectively[19]. Some authors have noted that the concentration range at which choline acetyltransferase catalyzes the synthesis of acetylcholine is most sensitive to variations in choline concentration[17]. Changes in high-affinity choline uptake in the hippocampus are regarded as an indication of altered cholinergic activity in these regions, and this system is a useful functional marker for cholinergic neurons[2,3].

Our data reveal that the decrease in the uptake after the 48th hour may reflect terminal changes regarding biochemical dysfunction due to prolonged incubation. Although some studies have documented a decrease in high-affinity choline uptake in nerve terminals during incubation of the tissue cultures[20–22], these were very short-term incubations (~80 min) in a medium of normal Krebs buffer; a comparison with our data is therefore difficult. In this study, hemicholinium-3 (5 μM) very significantly inhibited choline uptake in cultivated hippocampal slices and diminished the time-dependent increases and decreases throughout the cultivation. Since the high-affinity choline uptake system is restricted to cholinergic neurons and is very sensitive to inhibition by micromolar concentrations of hemicholinium, this effect has indicated that the observed time course of [^{3}H]-choline uptake in the groups treated without hemicholinium was strongly related to the cholinergic neurochemical mechanisms.

Clinically, the radiation dose-rate effect is a very important factor in radiosurgery, and the effects of the same total dose may differ if delivered continuously, in a fractionated manner, or in one session. Interstitial radiosurgery, by means of stereotactically placed removable radioisotopes, is an alternative way of delivering the dose locally into the brain. Interstitial implantation of radioactive isotopes permits the delivery of high doses of radiation to tumors. This technique has been widely used to treat brain tumors since the 1950s[23]. In recent decades, particularly in Europe, several centers have gained wide experience with stereotactic implantation of radioisotopes[24–26]. The continuous delivery of a relatively low dose rate of gamma-irradiation over a short time may be biologically more efficacious than a course of fractionated or single dose external beam radiosurgery, possibly due to both cell kinetic interactions and oxygen dependency[27,28].

Continuous low-dose irradiation using point sources offers some considerable therapeutic advantages: the dose distribution allows for a higher central dose within the target volume, and in addition, the steep dose fall-off results in lower doses to the adjacent normal brain tissue increasing the therapeutic ratio. Other factors, such as the decreased oxygen enhancement ratio with this kind of radiation[29] and possible reoxygenation during protracted irradiation, may also be considered advantages of interstitial radiosurgery[30]. The efficacy of continuous irradiation does not depend on the proliferative rate of the cells as in fractionated therapy. A number of investigators have studied interstitial radiation in the brains of experimental animals[25,31–36]. These reports comprised a qualitative appraisal of morphological changes induced by chronic irradiation with implanted sources. Little quantitative data exist concerning the effects of different dose rates on specific cell functions[30] or on the tolerance of the normal brain[36–39]. While current radiobiological data support the view that interstitial irradiation with a low dose rate has a therapeutic advantage, there is a need for further experimental studies to define more clearly the possible advantages of interstitial radiosurgery at intermediate or high dose rates and to evaluate possible enhancement of effects by chemical modifiers.

Our results have shown that continuous exposure to radiation decreased the choline uptake in hippocampal tissue slices, and that single-dose irradiation – although the same total doses were delivered by the end of irradiation – had no significant effect on choline uptake. Hemicholinium depressed very significantly this effect, i.e., the effect is rather specific to the cholinergic terminal. Continuous irradiation might cause cellular dysfunction regarding reduced activity of high-affinity choline uptake, possibly by peroxidation of cell membrane components[40,41]. The selective effect of continuous interstitial irradiation may be directly related to its physical properties: relativley low dose rate, sustained continuous irradiation, and possible high 'relative biological efficiency' of low-energy gamma irradiation from the iodine seeds.

The amygdala–hippocampus complex plays a role in both the origin and the spread of complex partial seizures; these epileptic phenomena also involve the orbital surface of the frontal lobe. The study of the regulation of neuronal excitability may lead to a clearer understanding of some of the clinical phenomena observed in seizure disorders. An alteration in excitatory and inhibitory influences may underlie epileptic phenomena. GABA (gamma aminobutyric acid) serves as the major inhibitory transmitter in the hippocampus. The activation of epileptogenic neurons may be due to the excitatory amino acids – e.g., glutamate release before the seizure – and glutamate may act as a paracrine factor to produce synchronization[42]. On the other hand, the net effect of muscarinic cholinergic activation is an increase in the responsiveness of neurons to excitatory inputs[43,44].

The radiosurgical effects on hippocampal slices may have some bearings on epilepsy surgery of this region. A considerable interest in the radiosurgical treatment of epilepsy has become evident in recent years[45,46]. Medically intractable seizures originating from the mesiobasal limbic structures can be treated by amygdalohippocampectomy[47]. High dose-rate focused irradiation procedures coupled with stereotactic techniques may be an alternative to more invasive surgical procedures by providing greater accuracy to resect small portions of the epileptogenic circuitry in the treatment of the mesiobasal limbic type of temporal epilepsy[46,48–52]. Most data regarding the beneficial

effect of radiosurgery on epilepsy have been collected from cases with arteriovenous malformations and cerebral neoplasms after treatment with external beam–single dose radiosurgery[52–56]. On the other hand, several studies have shown that stereotactic insertion of radioactive sources within epileptogenic cerebral tumors is quite effective in suppressing epilepsy[12,57,58]. Continuous irradiation may be more efficacious in suppressing epileptogenic activity in the mesial–temporal circuits and may be a rational alternative to other radiosurgical techniques.

CONCLUSION

Mature hippocampal tissue culture is an appropriate model for the study of the effects of focused irradiation on the normal brain. Our data demonstrated that continuous radiation is more efficacious than single-dose irradiation on high-affinity choline uptake activity in cultured hippocampal slices in rabbits. The effect must be investigated further with serial assessments of metabolism, and the knowledge gained from such experiments will be the basis for further studies on the use of stereotactic radiosurgery for the treatment of functional disorders of the central nervous system.

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