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Erminia Fardone

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UNIVERSITÉ DE GRENOBLE

THÈSE

Pour obtenir le grade de

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Spécialité : **Neurosciences - Neurobiologie**

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Présentée par

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préparée au sein de l' **Installation européenne de rayonnement synchrotron** (ESRF)
dans **l'École Doctorale Chimie et Sciences du Vivant**

A new application of microbeam radiation therapy (MRT) on the treatment of epilepsy and brain disorders

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Serendipity

There's a big
a big hard sun
beaten on the big people
in the big hard world

Eddie Vedder – Hard Sun –

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Abstract

A new application of microbeam radiation therapy (MRT) on the treatment of epilepsy and brain disorders

Synchrotron-generated X-ray microplanar beams (microbeams, MBs) are characterized by the ability to avoid widespread tissue damage following delivery of doses ranging from hundreds to over a thousand of Grays. The resistance of normal tissues to high doses of MBs is likely related to the fast repair of the microvessels and to the wide interface between normal and irradiated tissue, allowing fast recolonization of the microbeam ablated columns of tissue. The preservation of the cortical architecture following high-dose microbeam irradiation and the ability to induce non-invasively the equivalent of a surgical cut over the cortex is of great interest for the development of novel experimental models in neurobiology and new treatment avenues for a variety of brain disorders. In this Thesis microbeams transections were delivered to the rat cortex and hippocampus. MBs cortical transections were delivered to the sensory motor cortex (size 100 µm/600 µm, center-to-center distance of 400 µm/1200 µm, peak-valley doses of 360-240 Gy/150-100 Gy) of Wistar male normal rats and rats developing seizures following cortical injections of Kainic Acid (KA). The motor performances following sensorimotor cortex transections were assessed by Rotarod tests. The effect of microbeam transections on cortical architecture was assessed by immunohistology with NeuN and GFAP, markers of mature neurons and astrocytes. No neurological deficit was observed in normal animals undergoing sensorimotor cortex microbeam transections. Convulsive seizure duration was markedly reduced following transections in KA rats. MBs hippocampal transections (75 µm thickness, 400 c-t-c distance and 600 Gy as peak entrance dose) were performed in adult normal Wistar rats. MBs transections were compared with a uniformly delivered X-ray dose (broad beam, BB) at 10 Gy. Our aims were to quantify (i) the impact of microbeams versus conventional irradiation on neurogenesis (using proliferative cells markers such as BrdU and Ki-67), and (ii) to investigate on a long term (1 year) the correlation between neurogenesis and impairments in learning and memory. Preservation of proliferative cells in the microbeam treated group was observed. Microbeam transections delivered to the hippocampus did not induce significant behavioural impairment histological and immunohistochemical findings showed a preserved hippocampal structure with evidence of highly precise transections parcellizing the hippocampus in columns. This work confirms the safe delivery of high doses of radiation to specific and radiosensitive parts of the brain, if performed using microbeams. The development of clinical devices delivering submillimetric beams able to generate cortical or hippocampal transections might become a new powerful new tool for the clinical treatment of epilepsy, and other functional brain disorders.

Key words: Microbeam radiation therapy, radiosurgery, epilepsy, neurogenesis, brain

****

Résumé

Nouvelle application de la radiothérapie par microfaisceaux (MRT) pour le traitement de l'épilepsie et des troubles cérébraux

Les microfaisceaux de rayons X générés par un synchrotron permettent de délivrer des doses de radiation très élevées, jusqu'à plusieurs centaines de gray (Gy), sans pour autant induire de

dommages tissulaires irréversibles dans les zones avoisinant la lésion. La réactivité du réseau vasculaire à se régénérer grâce aux cellules endothéliales, est probablement un mécanisme clef dans la radio-tolérance des tissus sains, puisqu'il permet une recolonisation rapide des zones tissulaires lésées. Cette méthode permet ainsi de reproduire de façon non invasive une incision chirurgicale précise du cortex tout en préservant son architecture. Cette caractéristique présente un intérêt certain et permet d'envisager le développement de nouveaux modèles neurobiologiques expérimentaux ouvrant la voie à des traitements innovants pour de nombreux troubles cérébraux. Durant cette thèse, les microfaisceaux ont été utilisés sur la structure corticale et l'hippocampe de rats. Concernant le cortex, les microfaisceaux ont été appliqués au niveau du cortex sensori-moteur (taille 100 µm/600 µm, 400 µm/1200 µm crête à crête et dose pics - vallée 360-240 Gy/150-100 Gy) chez des rats males Wistar normaux ainsi que chez des rats développant des attaques cérébrales consécutivement à l'injection corticale d'Acide Kaïnique (KA). Suite aux traitements par microfaisceaux, les performances motrices étaient évaluées par le test du Rotarod et les structures corticales étudiées par immunohistochimie grâce au marquage par NeuN et GFAP des neurones et astrocytes matures. Aucun déficit neurologique n'a été observé chez les rats normaux soumis au protocole de traitement par microfaisceaux et une diminution importent de la durée des crises de convulsions a pu être observée chez les rats KA. L'incidence des microfaisceaux (9 microfaisceaux de 75 µm de largeur séparés de 400 µm crête à crête, dose d'entrée: 1000 Gy) délivrés au niveau de l'hippocampe de rats Wistar normaux a été comparée avec un traitement par rayons X de 10 Gy délivré uniformément. Le but de cette expérience était d'évaluer l'impact d'un traitement par microfaisceaux par rapport aux techniques d'irradiation conventionnelles. Pour cela, une évaluation quantitative (i) comparative a été faite sur la neurogénèse grâce à l'utilisation de marqueurs cellulaires (BrdU et Ki-67). Une étude (ii) a aussi été menée sur le long terme (1 an) pour mettre en évidence la corrélation entre la neurogénèse, les troubles de l'apprentissage et de la mémoire. Une préservation des cellules proliférative a été observée au sein du groupe traité par microfaisceaux. Les transsections de l'hippocampe de rats par microfaisceaux n'induisent pas de troubles significatifs (là aussi, il faut avoir des stat ou alors utiliser troubles marquants) du comportement de plus, l'observation histologique et immunohistochimique des tissus a permis de mettre en évidence la conservation de sa structure. Le travail effectué au cours de cette thèse confirme que le traitement spécifique de zones radiosensibles du cerveau par les microfaisceaux est sûr et permet d'améliorer le pronostique des animaux traités par rapport aux techniques conventionnelles. Ainsi, le développement d'appareils permettant de délivrer des faisceaux de rayons submillimétriques capables de générer une destruction précise et limitée au niveau du cortex ou de l'hippocampe pourra permettre une prise en charge innovante plus sûre de nombreuses pathologies fonctionnelles cérébrales comme l'épilepsie.

Mots clés: Radiothérapie par microfaisceaux, radiochirurgie, épilepsie, neurogénèse, cerveaux

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List of abbreviations

AED = Anti-epileptic drugs

Al = Aluminium

AP = Antero-posterior

ASF = Area sampling frequency

AVM = Arteriovenous malformations

BB = Broad beam

Be = Beryllium

BM = Bending magnet

BNL = Brookhaven National Laboratory

BrdU = 5-bromo-2-deoxyuridine

C = Carbon

CA = *Cornus ammonis*

CNS = Central nervous system

CT = Computer tomography

C-t-C = center to center distance

Cu = Copper

DAB = 3,3'-diaminobenzidine tetrahydrochloride

DCX = Doublecortin

DG = Dentate gyrus

DGmo = molecular layer of the dentate gyrus

DGpo = gyrus *polymorph* layer of the dentate gyrus

DGsg = granule cell layer of the dentate gyrus

DMAZ = Dorsal midbrain anticonvulsant zone

DNA = Deoxyribonucleic acid

DSB = Double strand break

DV = Dorso-ventral

EC = Endothelial cells

EEG = Electroencephalogram

EMSC = ESRF multislit collimator

ER = Endoplasmatic reticulum

ESRF = European synchrotron radiation facility

FE = First error

FWHM = Full width at half maximum

GAERS=Genetic Absence Epilepsy in Rats from Strasbour*g*

GCL = Granular cell layer

GFAP = Glial fibrillary acidic protein

Gy = Gray

GUI = Graphic user interface

HA = Horizontal activity

HD = High dose

HHS = Hypothalamic hamar tomas

ID = Insertion device

IntMRT = Interlaced microbeam radiation therapy

J = Joule

KA = Acid kainic

LD = Low dose

LET = Linear energy transfer

MBs = Microbeams

MBRT = Minibeam radiation therapy

MC = Monte Carlo

ML = medio-lateral

MRI = Magnetic resonance imaging

MRT = Microbeam radiation therapy

MST = Multiple subpial transection

MTS = Mesial temporal sclerosis

NDS = Normal donkey serum

NeuN = Neuronal nuclei

NSLS = National synchrotron light source

MTLE = Mesial temporal lobe epilepsy

NTCP = Normal tissue complication probability

OD = Optical density

PAT = Photon activation therapy

PD = Parkinson disease

PVDR = peak-to-valley dose ratio

QF = Quality factor

RAM = Radial eight arm maze

RBE = Relative biological effectiveness

RME = Reference memory errors

RT = Radiation therapy

SE = *Status Epilepticus*

SGZ = Subgranular zone

SNc = *Substantia nigra pars compacta*

SNT = *Subtalamic nucleus*

SPECT = Single-photon emission computed tomography

SRS = Stereotactic radiosurgery

SSB = Single strand break

SSRT = Synchrotron stereotactic radiation therapy

STN = Subthalamic nucleus

SRT= Stereotactic radiotherapy

SVZ = Subventricular zone

T = Tesla

TBS = Phosphate-buffered saline

TCP = Tumour control probability

TSF = Thickness sampling frequency

VA = Vertical activity

VEEG = Video-Elettroencephalogram

VNS = *Vagus nerve* stimulation

WM = Water maze

WME = Working memory errors

Introduction

Les faisceaux microplanaires de rayons-X générés par le synchrotron (microfaisceaux) sont caractérisés par leur capacité à éviter les dommages courants aux tissus induits par des irradiations à des doses allant de quelques centaines à plus d'un millier de Grays (Romanelli *et al* 2011). La grande résistance des tissus sains à des doses élevées par microfaisceaux a été attribuée à la grande tolérance de la vascularisation normale. En plus de cette forte résistance, il a été démontré qu'il y a une régénération rapide des micro-vaisseaux normaux endommagés sur la trajectoire des microfaisceaux (Serduc *et al* 2006, Slatkin *et al* 1995, Blattmann *et al* 2005,). Cet effet a été attribué à l'hyperplasie et à la migration des cellules endothéliales et des cellules gliales irradiées à minima entre les sections irradiées (Slatkin *et al* 1995). L'initiation de la migration, de la prolifération et de la différenciation des cellules gliales progénitrices pourrait être assistée par des effets « bystander[1] » des cellules mourantes (Fernandez-Palomo *et al* 2013, Kashino et al 2009, Dilmanian *et al* 2007). La résistance exceptionnelle du tissu sain à des doses élevées d'irradiation est un phénomène radiobiologique appelé effet dose-volume, c'est un effet protecteur des tissus sains (Romanelli and Bravin 2011, Dilmanian *et al* 2007, Dilmanian *et al* 2006, 2005, Blattmann *et al* 2005, Dilmanian *et al* 2005, Zhong *et al* 2003), indiquant la préservation de l'architecture des tissus traversés par les microfaisceaux transportant des doses dix à cent fois plus élevées que celles associées aux radionécroses observées après une radiothérapie conventionnelle.

Cette grande tolérance du tissu a favorisé la vérification expérimentale de l'utilisation potentielle des microfaisceaux pour une application thérapeutique. L'utilisation thérapeutique des microfaisceaux est déterminée par son faible impact sur les tissus non-ciblés ce qui peut permettre d'employer des doses plus élevées potentiellement curatives dans les cas cliniques pour lesquels la guérison n'est pas possible à ce jour. De cette façon cela pourrait permettre de traiter à nouveau le système nerveux central (SNC) des mois ou des années après le traitement initial, ainsi que de traiter des tumeurs pédiatriques du SNC. La méthode pourrait fournir un dépôt de dose plus localisée et pour cet raison que les applications peuvent inclure le traitement stéréotaxique *i)* des tumeurs en particulier celles positionnées près des organes très sensibles telles que les mélanomes oculaires, les adénomes hypophysaires, et les tumeurs de la moelle épinière, *ii)* des maladies non-cancéreuses telles que l'épilepsie, les malformations artério-veineuses (MAV), les troubles du mouvement (par exemple, les tremblements de Parkinson).

[1] Le bystander est une réponse à l'irradiation des cellules saines qui sont exprimés quand ils se trouvent dans le voisinage des cellules irradiées ou quand ils sont exposés à un milieu de cellules irradié.

À ce jour, beaucoup de recherches sur l'utilisation des microfaisceaux ont été axées sur les tumeurs (Bouchet *et al* 2013, Uyama *et al* 2011, Regnard *et al* 2008, Serduc *et al* 2008, Verant *et al* 2008, Laissue *et al* 2001) et sur quelques applications pour des maladies non-cancéreuses (Romanelli *et al* 2013, Pouyatos *et al* 2013, Dilmanian *et al* 2006).

Mon travail de thèse marque le début d'une nouvelle application de la thérapie par microfaisceaux (MRT, *microbeam radiation therapy*) pour le traitement des maladies du cerveau telles que l'épilepsie et pour l'étude de la préservation des cellules souches de hippocampe et leur implication dans les déficits cognitifs. Ceux-ci sont une conséquence redoutée de l'irradiation crânienne thérapeutique conventionnelle chez les enfants ainsi que chez les adultes.

Transsections corticales par microfaisceaux

L'épilepsie focale réfractaire aux médicaments est un trouble neurologique courant induit par une variété de facteurs conduisant à la genèse d'une région dysfonctionnelle de l'hippocampe ou du néocortex. L'objectif principal de la chirurgie de l'épilepsie est la résection de la zone épileptogène sans produire de nouveaux déficits neurologiques. La limite de cette approche est la nécessité de régénérer une quantité importante du tissu cortical ou de l'hippocampe. Le risque de morbidité est particulièrement élevé lorsque la zone épileptogène implique le cortex dit éloquent comme l'aire de la parole ou l'aire motrice primaire. En outre, la suppression complète des régions du cerveau réalisée au moyen d'une intervention chirurgicale à crâne ouvert est un prix trop difficile à accepter pour de nombreux épileptologues et patients. Les transsections sous-piales multiples (MST) ont été développées pour traiter les patients réfractaire à le médicament et donc, bon candidats pour la chirurgie de l'épilepsie impliquant des régions hautement fonctionnelles du cerveau (Mulligan *et al* 2001, Orbach *et al* 2001, Mountcastle 1997, Morrell *et al* 1995, Morrell 1989, Morrell et Hanbery 1969, Mountcastle 1957), mais pourrait également s'avérer être un bon outil pour traiter les foyers épileptogènes dans le cortex non éloquent.
Les MST sont généralement réalisées par une intervention chirurgicale ouverte et composés par des découpes souvent espacées par des intervalles de 5 mm avec une profondeur de 1,5 à 2 cm. Les études neuroanatomiques montrent que l'unité corticale fonctionnelle de base est disposée verticalement, et que l'activité épileptique se propage horizontalement. Les incisions sont donc orientées verticalement dans le cortex et pourraient interrompre les connexions synaptiques transversales, empêchant la propagation des crises en préservant la colonne verticale et alors la fonction neuronale. Cela donne une preuve supplémentaire que les MST induisent une coupure

sélective laissant derrière elles un cortex fonctionnel intact (Morrell *et al* 1999, Telfeian et Connors 1998, Morrell 1989, Chervin *et al* 1988).

La chirurgie par MST ne peut pas être considérée comme une méthode permanente pour le contrôle des crises car, après cette procédure, l'activité épileptique peut récidiver après une période de 2 à 20 mois. En outre, les risques associés à ce type de chirurgie sont rares et comprennent *i)* des saignements dans le cerveau, où les coupes sont faites, *ii)* des difficultés d'élocution ou de la fonction du langage, *iii)* l'amélioration temporaire, ce qui signifie le retour des crises à un moment donné et *iv)* des changements temporaires dans la fonction neurologique.

Les microfaisceaux de rayons X générés par le synchrotron peuvent induire une équivalence de cette incision chirurgicale dans le tissu cérébral en délivrant des doses très élevées de radiations (des centaines de milliers de Grays) pour des tranches de tissu d'épaisseur microscopique (Laissue *et al* 2007 Bräuer- Krisch *et al* 2010). En utilisant les microfaisceaux, les incisions peuvent être réduites à 60 µm par rapport à la chirurgie conventionnelle par laquelle les incisions sont dans enivrement de 0,2 mm. La résection corticale par microfaisceaux pourrait aussi avoir un rôle important dans le traitement d'autres maladies caractérisées par un dysfonctionnement cortical, y compris les troubles du comportement.

Comme indiqué précédemment la grande tolérance des tissus est une caractéristique particulière de ces microfaisceaux. La radiochirurgie par microfaiseaux a été étudiée au cours de plusieurs expérimentations sur des animaux au synchrotron du Laboratoire National de Brookhaven (National Synchrotron Light Source (NSLS), USA), et au synchrotron européen de Grenoble (ESRF, France). Ils ont utilisés une seule fraction d'irradiation avec des faisceaux de 25 à 90 µm de largeur séparés de 75 à 300 µm crête à crête pour des doses allant jusqu'à des centaines de Gy (Curtis 1967, Zeman *et al* 1961).

La première preuve de faisabilité de l'utilisation des microfaisceaux pour produire une microlésion focale a été accomplie en 2005 grâce à des microfaisceaux parallèles de 950 µm de largeur et séparés de 1200 µm délivrés en tirs orthogonaux se croisant au niveau de la cible. L'évolution de la microlésion a été suivie par IRM 9,4 T à haute résolution (Anschel *et al* 2005). La lésion est apparue 30 jours après le traitement et à l'IRM finale, réalisée 216 jours après, aucune anomalie n'était apparente dans l'hémisphère controlatéral qui n'a reçu que des microfaisceaux non-entrelacés à 115 Gy. Cette technique a ensuite été validée en utilisant des faisceaux de 680 µm avec des doses incidentes allant de 110 à 170 Gy. De plus, l'évaluation préliminaire de l'irradiation de la moelle épinière a été réalisée par Dilmanian *et al* (Dilmanian *et al* 2006), ce qui suggère que la moelle épinière saine est relativement résistante à une irradiation de 400 Gy pour des microfaisceaux allant jusqu'à 680 µm d'épaisseur. En utilisant la dosimétrie par gels BANG et des fantômes, y compris un modèle réaliste de tête humaine

comprenant un crâne, Dilmanian *et al* (Dilmanian *et al* 2008) ont simulé la production d'une lésion 8 cm^3 générée par un entrelacement de faisceaux de 680 µm. En plus des paramètres tels que la largeur du faisceau, l'espace entre les microfaisceaux, la géométrie, la dose peut varier par fractionnement temporel. Siegbahn *et al.* (Siegbahn *et al* 2009) ont montré le bénéfice de délivrer une dose de microfaisceaux en 3 fractions plutôt qu'en une seule.

La radiochirurgie par microfaisceaux, comme mentionné plus haut, pourrait avoir un rôle important dans le traitement des maladies du cerveau telles que l'épilepsie. Dans ce cadre, deux groupes ont récemment travaillé en parallèle sur l'utilisation des rayons X du synchrotron à l'ESRF sur des modèles animaux épilepiques. Notre groupe (Romanelli *et al* 2013) a montré que des transsections par microfaisceaux parallèles délivrant des doses très élevées au cortex sensori-moteur, permettent la réduction de la durée des crises convulsives chez le modèle animal d'épilepsie par injection d'acide kaïnique. De plus par la suite aucun déficit neurologique n'a pu être associé au traitement. En parallèle, un autre groupe (Pouyatos *et al* 2013) a utilisé les microfaisceaux entrelacés (IntMRT, interlaced microbeam radiation therapy) pour cibler les structures cérébrales impliquées dans la production de crises chez les rats GAERS (Genetic absence epilepsy in rats from Strasbourg) , un modèle de rat avec des absences épileptiques. Leurs résultats ont indiqué une réduction des activités synaptiques spontanées.

Les deux expériences ont montré que la radiochirurgie par microfaisceaux pourrait s'avérer être un excellent outil complémentaire aux techniques actuelles de radiochirurgie utilisées pour contrôler les convulsions. À ce stade, d'autres expérimentations pourraient explorer le rôle de la radiochirurgie par microfaisceaux pour les maladies non malignes du cerveau, l'épilepsie, les troubles du mouvement, et les troubles cognitifs liés à la neurogenèse hippocampique.

Mon travail de thèse décrit les transsections corticales au niveau du cortex sensori-moteur sur des rats normaux et sur un modèle animal d'épilepsie, le modèle par injection d'acide kaïnique. Les irradiations ont été effectuées par des microfaisceaux de caractéristiques différentes (25-600 µm de largeur séparés de 200-1200 µm crête-à-crête, doses pic de 150-360 Gy) et la performance motrice des animaux a été évaluée par le test du Rotarod®.

Transections de l'hippocampe par microfaisceaux

La radiothérapie crânienne est une modalité de traitement commun pour les tumeurs cérébrales. Malheureusement si ce schéma thérapeutique est efficace pour éliminer des tumeurs, des dommages aux tissus du cerveau normal sont inévitables. Plusieurs études ont montré que des déficits cognitifs, apparaissant des mois ou des années après le traitement, se produisent pour un

grand nombre de survivants de tumeurs cérébrales (Khuntia *et al* 2006, Zhang *et al* 2005, Welzel *et al* 2008).

L'hippocampe joue un rôle crucial dans l'apprentissage et la mémoire (Izquierdo et Medina, 1997) et un nombre de données considérable montre que l'irradiation entraîne des troubles de ces fonctions (Raber 2010, Yazlovitskaya *et al* 2006). Cette structure du cerveau est constituée de sous-domaines, anatomiquement distincts mais fonctionnellement interdépendants consistant en différents types de cellules, de taille cellulaire, de connectivité neuronale, de propriétés électrophysiologiques et de sensibilité à le dommage (Taupin 2005). Le gyrus denté (DG) est l'une des deux régions du cerveau où la neurogenèse a lieu toute la vie durant (Zhao et Brinton 2005) et il a été montré comme particulièrement sensible aux rayonnements (Monje et Dietrich 2012, Mizumatsu *et al* 2003). En revanche, la dégénérescence neuronale et la perte associée à la maladie d'Alzheimer, l'épilepsie ou aux épisodes d'ischémie/anoxie sont observées plus spécifiquement dans la région CA1 que dans toute autre région du cerveau (Schultz et Rolls 1999). Il y a également des rapports suggérant des différences de réponses entre les cellules pyramidales du CA1 et les cellules granulaires du DG après un stimulus négatif (Shimizu *et al* 1996), mais il manque des informations concernant la spécificité subrégionale des effets de l'irradiation sur l'hippocampe.

Des études sur des modèles animaux suggèrent que ces déficits de l'apprentissage et la mémoire sont associés à l'augmentation des cytokines inflammatoires, à la diminution de la neurogenèse dans l'hippocampe (Lee *et al* 2010, Rola *et al* 2004, Snyder *et al* 2001, Tada *et al* 2000, Parent *et al* 1998), à la dégradation de la matrice extracellulaire (Lee *et al* 2012) et/ou aux modifications de la morphologie synaptique (Shi *et al* 2006). De plus, ils peuvent contribuer à nuire à la fonction du système nerveux central. Les effets des rayonnements sur la plupart de ces processus sont très dépendants de la dose administrée, mais il a été démontré que les cellules endothéliales et le système vasculaire étaient particulièrement sensibles aux effets des radiations. L'apoptose endothéliale (Li *et al* 2003), la rupture de la barrière hémato-encéphalique (Li *et al* 2004), la vacuolisation et l'épaississement de la membrane basale vasculaire (Kamiryo *et al* 2001), ainsi que la raréfaction vasculaire (Brown *et al* 2005) ont été signalés.

L'inhibition de la neurogénèse entraîne des changements comportementaux. La logique voudrait que toute fonction comportementale des nouveaux neurones reflète un sous-ensemble des rôles comportementaux spécifiques des régions où ils résident, dans ce cas, l'hippocampe. Bien qu'il reste encore beaucoup à apprendre sur la fonction du comportement de l'hippocampe, il est clair qu'il joue un rôle important dans la mémoire spatiale et épisodique (Dickerson et Eichenbaum 2010) et que l'hippocampe ventral est important pour la médiation des réponses aux stress (Segal *et al* 2010).

À partir de la littérature il apparaît évident que les dommages induit par l'irradiation sur le processus de neurogenèse de l'hippocampe provoquent un dysfonctionnement cognitif. Connaissant l'effet protecteur des microfaisceaux, cela nous a conduits à nous intéresser à l'impact de ce mode d'irradiation sur la neurogenèse de l'hippocampe et sur les troubles de la mémoire et de l'apprentissage après l'irradiation.

Mon travail décrit l'impact des transsections hippocampique par microfaisceaux chez des rats sains d'un point de vue biologique et comportemental. Nous avons comparé les effets de l'irradiation par microfaisceaux (600 Gy) avec l'irradiation par faisceau homogène (10 Gy). Nous avons choisi 10 Gy comme dose pour le faisceau homogène parce que dans la littérature il a été rapporté que cette dose pouvait provoquer une réduction de la prolifération dans la zone sous-granulaire (SGZ) 2 mois après l'irradiation (Snyder *et al* 2005, Wang *et al* 2005). Les résultats principaux ont été obtenus par imagerie par résonance magnétique (IRM), par des techniques d'immunohistologie et des tests du comportement. Le test comportemental a été fait avec un labyrinthe radial à huit bras (RAM). Le test du RAM a été effectué à la suite du paradigme d'Olton (Olton and Papas 1979, Dubreuil *et al* 2003).

Mon travail de thèse confirme la tolérance du cerveau, même des zones radiosensibles, à l'irradiation par microfaisceaux à des doses élevées. En outre, le développement de dispositifs cliniques délivrant des faisceaux submillimétriques capables de générer des transsections corticales ou de l'hippocampe pourrait devenir un nouvel outil puissant pour le traitement clinique de l'épilepsie et d'autres troubles fonctionnels du cerveau.

En résumé, la thèse est organisée comme suit.

Chapitre I

Ce chapitre décrit brièvement l'utilisation de la radiochirurgie et de la radiothérapie pour le traitement des troubles du cerveau et des tumeurs, ainsi que les caractéristiques et les propriétés du rayonnement synchrotron, avec une attention particulière sur l'instrumentation disponible sur la ligne lumière biomédicale ID17 à l'ESRF. Enfin, les techniques de radiochirurgie et de radiothérapie dans le contexte de la tolérance du tissu cérébral et des effets sur le système nerveux centrale sont décrites.

Chapitre II

La conservation de l'architecture corticale après une irradiation par microfaisceaux est ici décrite. Ainsi la capacité à induire de façon non invasive l'équivalent d'une incision chirurgicale

sur le cortex sensori-moteur d'un modèle animal épileptique par injection d'acide-kaïnique et de rats normaux est détaillée.

Chapitre III

Une description de l'impact des transsections par microfaisceaux sur la neurogenèse de l'hippocampe chez des rats adultes normaux et l'implication sur la mémoire et l'apprentissage est montrée. La possibilité de conserver des cellules souches de l'hippocampe après une dose élevée de rayonnement synchrotron est ainsi mise en évidence.

Chapitre IV

Une discussion plus profonde est menée sur les résultats obtenus à partir des transsections corticales et de l'hippocampe et sur les perspectives futures de l'utilisation de ce nouvel outil comme technique de radiochirurgie pour le traitement clinique de l'épilepsie et d'autres troubles cérébraux.

Introduction

Synchrotron-generated X-ray microplanar beams (microbeams, MBs) are characterized by the ability to avoid widespread tissue damage following delivery of doses ranging from hundreds to over a thousand of Grays (Romanelli et al. 2011). The high resistance of normal tissues to high doses of MBs has been attributed to the high tolerance shown by the normal vasculature. In addition to the high resistance, it has been shown that there is a rapid regeneration of the normal microvessels damaged by the MB tracks (Serduc et al. 2006a; Blattmann et al. 2005; Slatkin et al. 1995). This effect was attributed to hyperplasia and migration of minimally irradiated endothelial and glial cells between the irradiated slices (Slatkin et al. 1995). The initiation of migration, proliferation and differentiation of the progenitor glial cells could be assisted by distant bystander effects from the dying cells (Fernandez-Palomo et al. 2013; Kashino et al. 2009; Dilmanian et al. 2007). The exceptional resistance of the normal-tissue to high dose of microbeam irradiation is a radiobiological phenomenon called "tissue-sparing effect" (Romanelli & Bravin 2011; Dilmanian et al. 2007; Dilmanian et al. 2006; Dilmanian et al. 2002; Dilmanian et al. 2005; Blattmann et al. 2005; Zhong et al. 2003), indicating the preservation of the architecture of the tissues traversed by microbeams carrying doses tens to hundreds times higher than those associated with radionecrosis after conventional radiotherapy.

This high tolerance of tissue stimulated the experimental verification of the potential therapeutical use of microbeams: it might allow the use of higher, potentially curative doses in those clinical cases that cannot be managed by present treatments. In this way could be possible allow retreatment of the CNS (Central Nervous System) months or years after the initial treatment(s), as well as the treatment of paediatric CNS tumours. The sharp dose falloff of the method could provide a more highly localized deposition of the dose, especially for small- and medium-sized targets. Such applications might include stereotactic treatment of *i)* tumours in particularly those positioned near very sensitive organs such as ocular melanoma, pituitary adenoma, and tumours of the spinal cord; *ii)* non-cancerous disorders such as epilepsy, arterio-venous malformations (AVM), movement disorders (*i.e*., Parkinson's disease, tremor).

To date, much research on the use of microbeams has been focused on tumours (Bouchet et al. 2013; Uyama et al. 2011; Serduc, Christen, et al. 2008; Vérant et al. 2008; J.-A. Laissue et al. 2001) and few applications on non-cancerous disorders (Romanelli et al. 2013; Pouyatos et al. 2013; Dilmanian et al. 2006).

In fact, my Thesis work marks the beginning of a new application of microbeam radiation therapy (MRT) for the treatment of brain diseases such as epilepsy and for the study of hippocampal stem cells preservation and their involvement in cognitive deficits. Cognitive deficits are a feared consequence of conventional therapeutic cranial irradiation in children as well as adults.

MBs cortical transections

Medically-refractory focal epilepsy is a common neurological disorder induced by a variety of factors leading to the genesis of a dysfunctional neocortical or hippocampal region. The primary goal of epilepsy surgery is the resection of the epileptogenic zone without producing new neurological deficits. The limit of this approach is the need to reset a vast amount of cortical or hippocampal tissue. The risk of significant morbidity is especially high when the epileptogenic zone involves eloquent cortex, such as speech or primary motor areas. Moreover, complete removal of brain regions accomplished by means of an open surgical procedure is a price too hard to accept by many epileptologists and patients. Multiple subpial transections (MSTs) have been developed to treat patients who are candidates for epilepsy surgery involving highly functional regions of the brain (Orbach et al. 2001a; Mulligan et al. 2001; Mountcastle 1997; Morrell et al. 1995; Mountcastle 1957; Morrell et al. 1989; Morrell & Hanbery 1969) but could also prove to be a good tool to treat epileptogenic foci in non-eloquent cortex.

The MSTs are usually performed by an open surgical procedure and consisted by cuts usually spaced by intervals of 5 mm with a depth of 1.5 to 2 cm. Neuroanatomic studies show that the basic functional cortical unit is arranged vertically, and epileptic activity spreads horizontally. So the incisions are vertical orientated in the cortex and they could interrupt transverse synaptic connections, preventing seizure propagation while preserving the vertical column subserving neuronal function. This gives further evidence that MSTs induce a selective disconnection leaving behind an intact functional cortex (Morrell et al. 1999; Telfeian & Connors 1998; Chervin et al. 1988).

MST surgery cannot be considered as a permanent method for seizure control because after this procedure, the epileptic activity may recur after a period of 2 to 20 months. Furthermore, the risks associated to this kind of surgery are rare and include i) bleeding in the brain where the cuts are made, ii) impaired speech or language function, iii) temporary improvement, meaning the seizures return at some point and iv) temporary changes in neurological function.

Synchrotron-generated X-ray beams (microbeams) can induce the equivalent of this surgical cut through the brain tissue by delivering very high doses of radiation (hundreds to thousands of Grays) to tissue slices of microscopic thickness (Bräuer-Krisch et al. 2010; Slatkin, Blattmann, Wagner, Glotzer & Laissue 2007). Using microbeams the transections can be reduced down, respect to the conventional surgery, to 0.2 mm while producing a transection width sized 60 μm. Microbeam cortical transection could have an important role also in the treatment of other disorders characterized by focal cortical dysfunction, including behavioural disorders.

As previously reported the high tolerance of tissues is a peculiar characteristic of these microbeams. Microbeams radiosurgery has been studied in several animal experiments at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory, and at the European Synchrotron Radiation Facility (ESRF), Grenoble, France using single-fraction in-beam doses of up to hundreds of Grays using 25–90 µm thick microbeams, spaced 75–300 µm on-center (Zeman et al. 1961; Curtis 1967b).

The first demonstration of the feasibility of using microbeams to produce a focal microlesion was accomplished in 2005 by orthogonally fired 950 µm planar beams spaced 1200 µm on-center so that they would interlace, producing a solid (broad beam effect) at the target. The evolution of the microlesion was followed by serial 9.4 T high resolution MRI (Anschel et al. 2005). The lesion first appeared 30 days post treatment, and at the final MRI at 216 days, there was no apparent abnormality in the contralateral hemisphere which received only non-interlaced microbeams at 115 Gy. This technique was then further validated using 680 µm beams with in beam incident doses ranging from 110 to 170 Gy. Additionally, preliminary evaluation of irradiation of the spinal cord was performed by Dilmanian *et al.* (Dilmanian et al. 2006), suggesting that the normal spinal cord is relatively resistant to 400 Gy beams up to 680 µm thick. Using BANG-gel dosimetry and phantoms, including a realistic head model encompassing an actual human skull, Dilmanian *et al.* (Dilmanian et al. 2008) simulated the production of an 8 cm^3 lesion by interlacing of 680 µm beams. This study also confirmed a 43 mm half-value layer as well as a submillimetric 80-to-20% dose fall-off using 120-keV median energy synchrotron beams. Attempts to augment the effectiveness of MRT using gene-mediated immunoprophylaxis have also been attempted with some promising results (Smilowitz et al. 2002). In addition to the spatial parameters of beam width, spacing, and geometry, the dose may varied by temporal fractionation. Siegbahn et al. (Siegbahn et al. 2009) showed the advantage of delivering a microbeam dose as 3 fractions rather than as a single dose.

Microbeam radiosurgery, as formerly mentioned, could have an important role in the treatment of brain disorders such as epilepsy. In this contest, recently two groups worked in parallel on the use of synchrotron X-ray at the ESRF on epileptic animal models. Our group (Romanelli et al. 2013) showed that parallel microbeam (size 25 µm-600 µm, center-to-center distance of 200 µm-1200 µm, peak entrance doses of 100-360 Gy) transections delivered at very high doses on the sensorimotor cortex dramatically reduced convulsive seizure duration in kainic-acid animal model and no subsequent neurological deficit was associated with the treatment. In parallel, another group (Pouyatos et al. 2013) focalized radiation doses via interlaced arrays of microbeams (IntMRT) to target brain structures involved in seizure generation in a rat model of absence epilepsy (GAERS). Their results showed a reduction of spontaneous synaptic activities.

Both experiments showed that microbeam radiosurgery, could prove to be an excellent tool to be added to the current radiosurgical techniques used to control seizures. At this point, more experimentation could explore the role of microbeam radiosurgery in non-malignant brain disease, epilepsy, movement disorders, and cognitive impairments related to hippocampal neurogenesis.

My Thesis work described the cortical transections towards the sensori-motor cortex of normal rats and epileptic animal model, such as kainic-acid-injected. Irradiations were performed delivering different beams array (beam' size from 25 to 600 µm, center-to-center distance from 200 to 1200 µm, peak entrance doses from 600 to 150 Gy), and motor performance was assessed through Rotarod® test.

MBs hippocampal transections

Cranial radiation therapy is a common treatment modality for brain tumours, but unfortunately this treatment regimen is effective in eliminating tumours, damage to normal brain tissue is inevitable. Several studies have shown that cognitive deficits occur in a relatively large percentage of brain tumour survivors, becoming evident months to years after treatment (Welzel et al. 2008; Khuntia et al. 2006; Zhang et al. 2005).

The hippocampus plays a crucial role in learning and memory (Izquierdo & Medina 1997) and considerable data exist showing that irradiation leads to impairment of those functions (Raber 2010; Yazlovitskaya et al. 2006). This structure is composed of anatomically distinct but functionally interrelated subfields consisting of different cell types, cell sizes, neural connectivity, electrophysiological properties and susceptibility to insult (Taupin 2005). The dentate gyrus (DG) is one of the two brain regions where neurogenesis takes place throughout life (Zhao & Brinton 2005) and has been shown to be particularly susceptible to radiation (Monje & Dietrich 2012; Mizumatsu et al. 2003). In contrast, neural degeneration and loss associated with Alzheimer's disease, epilepsy or ischemic/anoxic episodes are seen more distinctively in the CA1 region than in any other brain area (Schultz & Rolls 1999). There have also been reports suggesting differences in responses between the CA1 pyramidal cells and DG granule cells after given injurious stimulus (Shimizu et al. 1996), but there is a paucity of information regarding sub region specificity in the effects of irradiation on the hippocampus.

Studies in animal models suggest that these deficits in learning and memory have been associated to the decreased in hippocampal neurogenesis (Rola et al. 2004; Snyder et al. 2001; Tada et al. 2000; Parent et al. 1998), increases in inflammatory cytokines (Lee et al. 2010), degradation of extracellular matrix (Lee et al. 2012) and/or alterations in synaptic morphology (Shi et al. 2006), and they may contribute to impaired CNS function. The effects of radiation on

many of these processes are highly dependent on dose and radiation treatment, but endothelial cells and vasculature have been shown to be particularly sensitive to the effects of radiation. Endothelial apoptosis (Li et al. 2003) and disruption of the blood-brain barrier (Li et al. 2004), thickening and vacuolation of the vascular basement membrane (Kamiryo et al. 2001), and vascular rarefaction (Brown et al. 2005) have been reported.

The inhibition of neurogenesis demonstrated behavioural changes. Logic suggests that any behavioural function of new neurons will reflect some subset of the behavioural roles for the specific regions in which they reside, in this case the hippocampus. Although there is still much to learn about the behavioural function of the hippocampus, it is clear that it plays an important role in spatial and episodic memory (Dickerson & Eichenbaum 2010) and that the ventral hippocampus is important for mediating stress responses (Segal et al. 2010).

From literature is evident that the irradiation damages the hippocampal neurogenesis process causing cognitive dysfunction. Knowing the sparing effect of MBs generated by synchrotron, this pushed up our interest to study, the impact on hippocampal neurogenesis and memory/learning impairments after irradiation.

My work describes the MBs hippocampal transections impact on the dorsal hippocampus of normal rats from a biological and behavioural point of view. We compared the effects of MBs (600 Gy) irradiation with the broad beam (BB, 10 Gy/homogenous) irradiation, because in literature is reported that a 10 Gy BB irradiation can cause a reduction in proliferation of subgranular zone (SGZ) precursor cells at 2 months later (Snyder et al. 2005; Wang et al. 2005). Main results came out from magnetic resonance imaging (MRI) scan, immuno/histology techniques and behavioural test such as radial eight arm maze (RAM). The RAM test was performed following the Olton paradigm (Olton & Papas 1979; Dubreuil et al. 2003).

My Thesis work confirms the safe delivery of high doses of radiation to specific and radiosensitive parts of the brain, if performed using microbeams. Additionally, the development of clinical devices delivering submillimetric beams able to generate cortical or hippocampal transections might become a new powerful new tool for the clinical treatment of epilepsy and other functional brain disorders.

In summary the Thesis is organized as follows.

Chapter I

The chapter describes briefly the use of radiosurgery and radiation therapy in brain disorders and in the tumour treatment, as well as the characteristics and the properties of synchrotron

radiation, with a particular focus to the instrumentation available at the ID17 biomedical beamline at the ESRF. Finally, the milestones of radiosurgery and radiation therapy in the context of tissue sparing and central nervous system disease curing effect are described.

Chapter II

The preservation of cortical architecture after microbeams irradiation is here described, as well as the ability to induce non-invasively the equivalent of a surgical cut over the sensori-motor cortex in an epileptic Kainic-acid-induced animal model and in normal rats.

Chapter III

A description of MBs transection's impact on hippocampal neurogenesis in adult normal rats and its involvement on memory and learning is showed. The possibility of hippocampal stem cell preservation after high radiation doses delivered in microbeams is as well described.

Chapter IV

A final discussion of results obtained from cortical and hippocampal transections and future perspectives using this new tool as radiosurgery technique to clinical treatment of epilepsy and other brain disorders are given.

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CHAPTER I

General introduction

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Summary

In this first chapter several concepts used along the Thesis will be introduced. First, the use of the radiosurgery and radiation therapy in brain disorders and in the tumour treatment; some basic radiobiology concepts will be also given. Then the characteristics and the properties of synchrotron radiation will be presented, with a particular focus to the instrumentation available at the ID17 biomedical beamline at the ESRF. Finally, the attention will be given to the microbeam radiation therapy technique and to the milestones of this research in the context of tissue sparing and central nervous system curing effect.

Résumé

Dans ce premier chapitre plusieurs concepts seront introduits. Tout d'abord l'utilisation de la radiochirurgie et de la radiothérapie dans les troubles du cerveau traitement des tumeurs, y compris les concepts de base de la radiobiologie seront décrits. Ensuite, les caractéristiques et les propriétés du rayonnement synchrotron seront présentées, avec une attention particulière sur l'instrumentation disponible sur la ligne lumière biomédicale ID17 à l'ESRF. Enfin, la technique de radiothérapie par microfaisceaux et les étapes de cette recherche dans de contexte de la tolérance du tissu cérébral et des effets sur le système nerveux central sont décrites.

I.1 Radiosurgery and Radiotherapy of brain disorders and tumours

Radiosurgery and radiotherapy refer to the precise delivery of a single, high dose of radiation (by x-rays or γ-rays) on a specific area of the brain to treat abnormalities, tumors or functional disorders. Through the use of three-dimensional computer-aided planning and the high degree of immobilization, the treatment can minimize the amount of radiation that passes through healthy brain tissue. Both methods can be performed in either a single treatment or in several applications (fractionated) (Larson et al. 1994; Goetsch 2008) and the highly precise irradiation of targets is planned using information from medical images that are obtained via computer tomography (CT), magnetic resonance imaging (MRI), and angiography of the brain and body.

The difference between stereotactic radiosurgery and stereotactic radiotherapy has to do with the intensity and duration of the radiation treatments. Concerning stereotactic radiosurgery (SRS), it delivers radiation at a very high intensity, all at once, to a small area. It is a way of treating brain disorders with a precise delivery of a single high dose of radiation in a one-day session; whereas stereotactic radiotherapy (SRT) delivers radiation at different times, at lower intensities to larger areas.

The irradiation could be performed in two different ways:

- **External-beam radiation** therapy, which delivers, in the form of photon beams (x-rays or γ-rays), a beam pointed in the direction of the patient's tumor (Lawrence TS, Ten Haken RK 2008). Usually, this type of therapy includes computer and image guided methods to accurately treat the target and minimize the dose to the surrounding tissues. Among the various techniques, one of the most advanced is the stereotactic radiotherapy which will be discussed in details in the second chapter.
- **Internal-beam radiation** includes radioactive material, such as radioactive iodine, inserted in the brain by a catheter or a seed. The radioactive element must be left in place from several hours to several days to effectively kill the tumor cells (Patel & Arthur 2006).

For the external-beam radiation, the devices are Linear accelerator, Gamma Knife and Cyberknife. The machines have many similarities, but also important differences. Gamma Knife was the original machine and uses radioactive cobalt to generate the radiation. All newer

generation machines use a linear accelerator (LINAC) where X-rays are produced instead of gamma rays. **LINAC** machines use a single radiation beam that is delivered in multiple arcs around the target. They can perform radiosurgery on small and large tumors and can fractionate these treatments over several weeks. In contrast, **Gamma Knife** does not move around the target, the ability to treat large tumors is limited, and it does not allow fractionated treatments. All systems need to secure the head in order to achieve the highest targeting accuracy.
For instance, **Cyber Knife** uses active imaging of the tumor during treatment and reacts to any changes in position instantly.

The fundamental limitation of this kind of therapy is the risk of permanent damage of healthy tissues in critical organs. The radiotherapy (RT) treatment can induce cognitive decline that is linked to the impairments of hippocampal neurogenesis. Hence, it has been shown that microbeam radiosurgery employing arrays of parallel and microscopically thin planar synchrotron generated microbeams (MBs) is exceptionally well tolerated by the normal tissues even when delivered in a single fraction.

MBs generated by synchrotron should be a good radiosurgery treatment to modulate cortical functions and to treat focal epilepsy. Microbeam transections, either placed over neocortical seizure foci or through the hippocampus, could prove to be an excellent tool to be added to the current radiosurgical techniques used to control seizures.

I.1.1 Brief overview of radiosurgery

The concept of SRS was developed by Lars Leksell at the Karolinska Institute in Stockolm immediately following World War II (Leksell 1968). Radiosurgery is based on the concept of cross-firing beams of radiation through the target of interest in order to achieve a dose distribution delivering much higher doses within the selected volume in comparison with the nearby tissues. The aim was originally to induce discrete lesions in the brain (similar to those produced by stereotactic surgical procedures) and provide symptomatic improvement to patients affected by a variety of functional brain disorders (e.g., trigeminal neuralgia). In Leksell's vision, this effect would be achieved by delivering a high dose of radiation energy to the intended target in a single session, while steep fall-off dose gradients protect the adjacent brain (Leksell 1951). Leksell developed a rigid frame to be fixed to the patient's head in order to provide the required precision. Extensive research was undertaken to identify the optimal source of high energy beams useful in a clinical setting. Leksell and colleagues tested the possibility to

use KV photons first, then decided to use gamma rays originating from ^{60}Co sources. The so called **Gamma Knife** (Elekta, Stockholm, Sweden) is the device which developed from these efforts: the gamma rays originate from approximately 200 ^{60}Co sources placed in a hemispherical array around the head which are collimated and directed to the target using a helmet (Goodman 1990). The beam collimation is circular, so the high dose–volume at the point of intersection is a sphere. With this system, a relatively spherical dose focused around the target volume, with minimal dose to the surrounding normal cells, could be delivered.

An alternative to the Gamma Knife was developed in the mid-1980s using the conventional linear accelerators (**LINAC**) available in most large hospitals (Rahman et al. 2009). Unlike the radioactive cobalt-based Gamma Knife, these LINAC-based systems use generated X-ray beams. Target localization and treatment delivery, require immobilization of the patient's head with a stereotactic frame. Three-dimensional coordinates of the tumor were precisely determined by the stereotactic frame, and the radiation was delivered accurately to the target volume. Small circular collimators fitted at the end of the treatment head as tresiary collimators were used for narrowing the beam of x-rays from a conventional linear accelerator. Subsequently mini-multileaf collimator (MMLC) replacing the circular collimators enhanced the stereotactic procedures by way of treating irregular lesions in the brain. All these systems require a stereotactic frame to be fixed onto the patient's head, right from imaging to treatment, and hence are termed as invasive or minimally invasive procedures. These methods of conformal dose delivery to the target reduce radiation dose to the surrounding normal tissues substantially.

The **CyberKnife**, invented by Dr. John Adler, a neurosurgeon in Stanford, USA, came into practice by 1990s (Adler 1993; Adler et al. 2006). A lightweight linear accelerator fitted onto an industrial robot makes treatment possible precisely in a desired way. The robotic arm has 6 degrees of freedom of movement; unlike the conventional linear accelerator, which has only rotational movement in one plane. CyberKnife treatments are non-isocentric, where beams can be directed from any desired angle. This system does not require a rigid frame to be fixed onto the skull of the patient for stereotactic setup and verification. Initially the CyberKnife was put into use for treatment of only intracranial lesions, like other stereotactic systems. Subsequent developments made it possible to extend the facility to extracranial lesions also, thereby making it a whole-body stereotactic radiotherapy system. Now, the CyberKnife, with its advanced technologies and complexities, gives maximum flexibility in its use to treat any lesion in any part of the body noninvasively. The CyberKnife also has limitations. One of the major limitations is the prolonged treatment time: approximately 30 to 60 minutes. This does not alter the accuracy, because of repeated verifications before each beam delivery. Large volumes are

not suitable with CyberKnife as the principle of delivery is 'dose painting' from one edge of the tumor to the other. This is most suitable for recurrent and residual tumors after prior radiotherapy treatments. Other than brain lesions, small tumors in lung, liver and spine can be effectively treated using CyberKnife.

The next step of radiosurgery is likely to involve the development of novel irradiation techniques maximizing the radiobiological potential of stereotactic irradiation. In fact, experimental work performed during the last decade at the European Synchrotron Radiation Facility (ESRF), Grenoble, France and the Brookhaven National Laboratory (BNL), Upton, NY has investigated the clinical potential of submillimetric X-ray beams. **Synchrotron-generated microbeams** exploit the radiobiological principle of spatial fractionation, while conventional radiation therapy is primarily based on relative sparing of healthy tissue by temporal fractionation.

For instance, in the treatment of epilepsy, MBs cortical transections can induce the equivalent of surgical cut (*i.e.* MST) through the tissue, by delivering very high doses of radiation (hundreds to thousands of Gray) to tissue slices of microscopic thickness. Respect to the conventional radiosuergery, MBs can produce very thick transections in the range of 60 µm or less. This kind of radiosurgery could be better because there is a rapid regeneration of vascular network in the lesioned zones.

In general, radiosurgy encourage the treatment of brain disorders and tumours, but it can induce different biological effects due to the interaction between the ionizing radiation and matter, causing damage at DNA level.

I.2 Interaction of ionizing radiation with matter

Living organisms are continuously exposed to ionizing radiation, including high-energy photons, electrons, protons, neutrons, or alpha particles. Those are issued from nuclear disintegration of radioactive materials present in rocks, sands and water, or coming on earth as cosmic particles. Photon absorption in human tissue is determined by the energy of the radiation, as well as the atomic structure of the tissue in question.

The interaction between X-rays and matter is categorized into four main types: photoelectric effect, Compton scattering, pair production and Rayleigh scattering.

Photoelectric effect

In this process, an incoming photon undergoes a collision with a tightly bound electron. The photon transfers practically all of its energy to the electron and ceases to exist. The electron departs with most of the energy from the photon and begins to ionize the surrounding molecules. This interaction depends on the energy of the incoming photon, as well as the atomic number of the tissue. An example of this interaction in practice can be seen on a diagnostic X-ray film. Since the atomic number of bone is 60% higher than that of soft tissue, bone is seen with much more contrast and detail than is soft tissue. The energy range in which the photoelectric effect dominates in tissue is up to ~25 keV.

Compton effect

The Compton effect is the most important photon-tissue interaction for the treatment of cancer using conventional linacs, which are run at 6-20 MeV. Unlike the photoelectric effect, in the Compton interaction both the photon and electron are scattered. The electron begins to ionize with the energy provided by the photon and therefore loose energy and deposit dose in the tissue.

Pair production

In this process, a photon interacts with the nucleus of an atom, not an orbital electron. The pair production has an energy threshold of 1.022 MeV, the rest mass of the produced particles. The photon energy exceeding the threshold is transformed into kinetic energy of the produced pair. The energy range in which pair production dominates is ≥ 25 MeV. This interaction does occur to some extent in routine radiation treatment with high-energy photon beams.

Rayleigh scattering

When a photon interacts with atom, it may or may not impart some energy to it. The photon may be deflected with no energy transfer. This process is called Rayleigh scattering and is most probable for very low-energy photons impinging on matter.

Therefore, the impact of ionizing radiation to the organic matter is measured as exposure, as absorbed dose, as equivalent dose and dose rate:

Exposure

Exposure is a measure of the strength of a radiation field at some point. It is a measure of the ionization of molecules in a mass of air. It is usually defined as the amount of charge (*i.e.* the sum of all ions of the same sign) produced in a unit mass of air when the interacting photons are completely absorbed in that mass. The most commonly used unit of exposure is the Roentgen (R). Specifically, a Roentgen is the amount of photon energy required to produce 1.610×10^{12} ion pairs in 1 cm^3 of dry air at 0 °C. A radiation field of one Roentgen will deposit 2.58×10^{-4} Coulombs of charge in one kilogram of dry air. The main advantage of this unit is that it is easy to directly measure with a survey meter. The main limitation is that it is only valid for deposition in air.

Absorbed dose

Whereas exposure is defined for air, the absorbed dose is the amount of energy that ionizing radiation imparts to a given mass of matter. The amount of energy absorbed per mass is known as radiation dose. In other words, energy (Joules) absorbed per unit mass of tissue and has the (S.I.) units of gray (1 Gy = 1 J/kg). In the past the rad (radiation absorbed dose) was used, where 100 rad = 1 Gy (1 rad = 1 cGy). The size of the absorbed dose is dependent upon the intensity (or activity) of the radiation source, the distance from the source to the irradiated material, and the time over which the material is irradiated. The activity of the source will determine the dose rate which can be expressed in rad/h, mGy/s, etc.

Equivalent dose

When considering radiation interacting with living tissue, it is important to also consider the type of radiation. Although the biological effects of radiation are dependent upon the absorbed dose, some types of radiation produce greater effects than others for the same amount of energy imparted. For example, for equal absorbed doses, alpha particles may be 20 times as damaging as beta particles. In order to account for these variations when describing human health risks from radiation exposure, the quantity called “dose equivalent” is used. This is the absorbed dose multiplied by certain “quality” or “adjustment” factors indicative of the relative biological-damage potential of the particular type of radiation. The quality factor (Q) is a factor used in radiation protection to weigh the absorbed dose with regard to its presumed biological effectiveness. Radiation with higher Q factors will cause greater damage to tissue.

Dose rate

The dose rate is a measure of how fast a radiation dose is being received. Knowing the dose rate, allows the dose to be calculated for a period of time.

I.2.1 Radiobiology efficacy

The rationale of radiotherapy lies in the fact that, in general, healthy tissue presents a higher recovery capability than tumour tissue linked to the response of vascular network (more fast in the healthy tissue). In addition, the tumour control probability (TCP) follows a sigmoid curve in function of dose. As the radiation dose is increased, the TCP rapidly escalates until a plateau is reached. The same is true for the probability of inducing deleterious effects in normal tissue, which is represented by the normal tissue complication probability (NTCP) curve. The balance between the TCP and NTCP is a measure of the therapeutic index of the treatment (Joiner & van der Kogel 2009). As the radiation dose is increased, the TCP rapidly escalates until a plateau is reached. The same is true for the probability of inducing deleterious effects in normal tissue, which is represented by the normal tissue complication probability (NTCP) curve (Figure I-1)

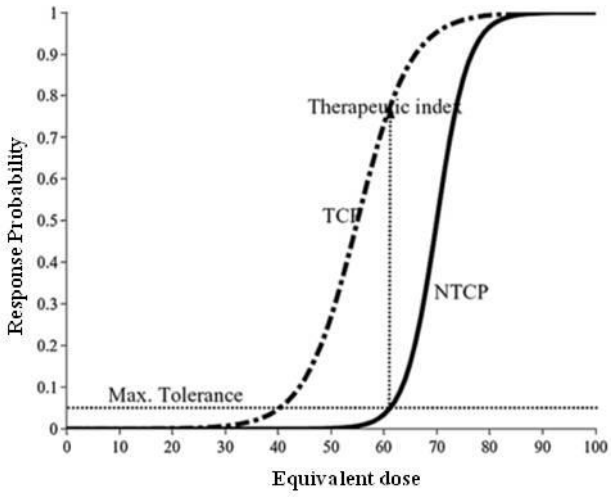

Figure I-1. Sigmoidally shaped response curves (for TCP and NTCP) are constructed as a function of a linear weighting of various factors, for a given dose distribution, which may include multiple dose–volume metrics as well as clinical factors. The units of the *x*-axis may be thought of as 'equivalent dose' units. The probability of tumor control without normal issue complications receives its maximum in the so-called “therapeutic window”. Extracted from El Naqa *et al* 2012.

I.2.2 Basic concepts of biological effects of radiation

Since biological systems consist largely of water (~80% of water in cells), the bulk of ionizations produced by irradiation occur in water molecules. Hence, the knowledge of water radiolysis is therefore critical for understanding radiobiological effects.

As reported in **Figure I-2**, negatively charged free electrons (e^-) that are produced by ionization will rapidly become associated with polar water molecules, greatly reducing their mobility. The water molecule that has lost an electron is a highly reactive positively charged ion. It quickly breaks down to produce a hydrogen ion (H^+) and an uncharged OH radical. The reactive species of oxygen like O_2 and H_2O_2 represent the products of reactions.

The damage effects of water radiolysis are due, in the most of cases, to the geometry of the physical energy deposition of the impacting radiation, referred to as the track structure or LET (Linear Energy Transfer) effects (Roth et al. 1976). LET is closely related to the power of braking radiation, which is the loss of energy of a charged particle per unit path length.

LET essentially indicates the quality of different types of radiation and is important because the biological effect of a radiation (its relative biological effectiveness, RBE) depends on its average LET. LET is measured by the ionization density (*i.e.* ion pairs/cm of tissue) along the path of the radiation. Higher LET causes greater biological impact and is assigned a higher Quality Factor (QF). Example QF values: X, gamma, and beta have QF = 1; alpha QF=20; thermal neutrons QF=3; "fast" neutrons (>10 KeV) QF = 10; fission fragments QF>20.

High value of LET (>10 keV/µm) indicates the presence of ionizations taking place in high density. Radiations having LET<10 keV/µm are considered as low LETs. Hence, radiations with high LET value cause double strand break (DSB)of the DNA molecule. Unfortunaately, the DSB is very difficult to be repaired causing the total destruction of the cell.

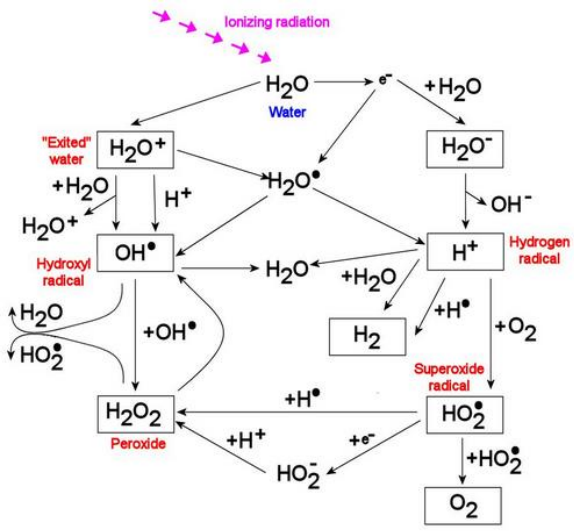

Figure I-2. Water Radiolysis. Exposure of cells to ionizing radiation induces high-energy radiolysis of H_20 water molecules into H^+ and OH radicals. These radical are themselves chemically reactive, and in turn recombine to produce a series of highly reactive combinations such as superoxide ($H0_2$) and peroxide (H_20_2), which produce oxidative damage within the cell.

I.2.2.1 Ionizing radiation induced DNA damage

DNA is the most important cellular component that can be stricken from radiation because it carries the genetic code. The most sensitive part of the DNA molecule is considered to be the pyrimidine bases (timine (T) and cystine (C)). Other important molecules that can be damaged from radiation are the enzymes and the proteins of the cellular membranes. What is sensitive in proteins is the peptide bond.

The most common lesions produced are base damages, single-strand breaks (SSB) and DSB (Ward 1985). DSB are often considered critical lesions and thought to be probable key precursors of most early and late effects of stemming from ionizing radiation exposure (Frankenberg-Schwager et al. 1994; Bryant 1985; Campa et al. 2005).

The number of DNA lesions generated by irradiation is large, but the number giving rise to cell kill is extremely small. The numbers of lesions induced in the DNA of a cell by a dose of 1-2 Gy are approximately as follows: base damages >1000; SSB ~1000; DSB ~40.

An additional level of damage complexity is added when two or more damage sites (strand breaks, abasic sites or oxidized bases) are closely spaced on the same or opposing DNA strands. These clustered lesions are tough to be particularly difficult to repair and have even been postulated to be the damage type mainly responsible for both the lethal and mutagenic effects of ionizing radiation (Figure I-3) (Belli et al. 1996; Pastwa et al. 2003; Shikazono et al. 2006).

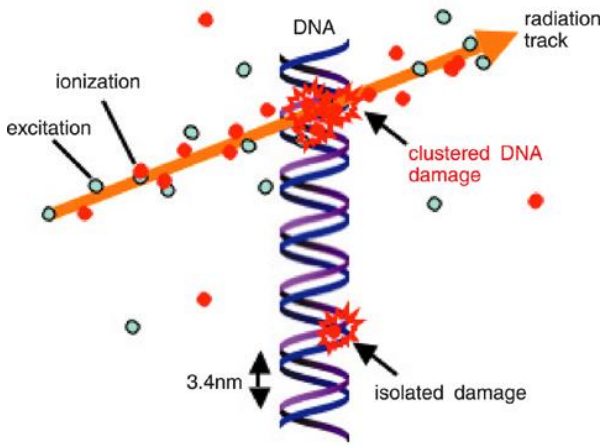

Figure I-3. DNA damage by ionizing radiation. DNA is damaged due to ionization or excitation caused by radiation. Clustered DNA damage would be produced where the density of ionization/excitation is high, whereas the isolated damage would be generated where it is low. Taken from Shikazono *et al* 2006.

DNA damage can be either direct or indirect from the irradiation (**Figure I-4**). The direct damage is when parts of DNA are ionized directly from the radiation. In other words, the damage occurs as a result of ionization of atoms on key molecules in the biologic system. This causes inactivation or functional alteration of the molecule. Instead, the indirect action involves the production of reactive free radicals (H_2O_2, H° and H_2) whose toxic damage on the key molecule results in a biologic effect. These are effects mediated by free radicals and reactions involved are discussed previously (Figure I-2).

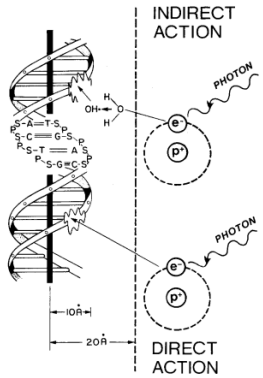

Figure I-4. Direct or indirect irradiation induced DNA damage (10Å=2nm; 20Å=4nm). Taken from (Hall & Giaccia 2006) .

Radiation induces many kinds of lesions to the DNA molecule a lot of which the cell succeeds to repair; some others are transmitted to posterities and a small portion of these lesions lead to cell death.

Damage recognition during the cell cycle

A radiation induced DNA damage could be recognized during the cell cycle through check points, arranged by kinase activity which phosphorylates and activates other kinases (Figure I-5). The radiosensitivity of cells varies considerably as they pass through the cell cycle. Briefly, the S and M portions of the cell cycle are separated by two periods (gaps) G1 and G2 (stages of interphase, is the time when the cell is growing and coping its chromosomes in preparation for cell division). The sages G2, G1 and G0 are described as followed:

- G1 occurs just after the cell has split. During this phase, the cell begins to manufacture more proteins in preparation for division. It also experiences other growth: metabolism increases, RNA synthesis is elevated and organelles duplicate. During the S phase, the DNA is copied, so that when the cell divides, both cells will have a copy of this genetic information. More precisely, at the beginning of the S phase, each chromosome is composed of one coiled DNA double helix molecule, which is called a chromatid. At

the end of this phase each chromosome has two identical DNA double helix molecules, and therefore is composed of two sister chromatids. The end result is the existence of duplicated genetic material in the cell, which will eventually be divided into two. The S phase is followed by the G2 phase.

- G2 phase occurs just before the cell begins to divide into two cells, and is a preparation stage for its chromosome duplication. The cell can then enter the M phase where the cell division occurs and two new cells are formed. After cell division occurs, the cell may enter a resting phase, referred to as the G_0 phase.
- G_0 phase is very important when modeling radiation treatment of cancer because the cell is less sensitive to radiation when in this phase. The time between successive divisions (mitoses) is called cell cycle time.

For mammalian cells growing in culture the S phase is usually in the range of 6-8 hours, M less than an hour, G2 in the range of 2-4 hours, and G1 from 1-8 hours, making the total cell cycle in the order of 10-20 hours. In contrast, the cell cycle for stem cells in certain tissues is up to about 10 days (Figure I-15).

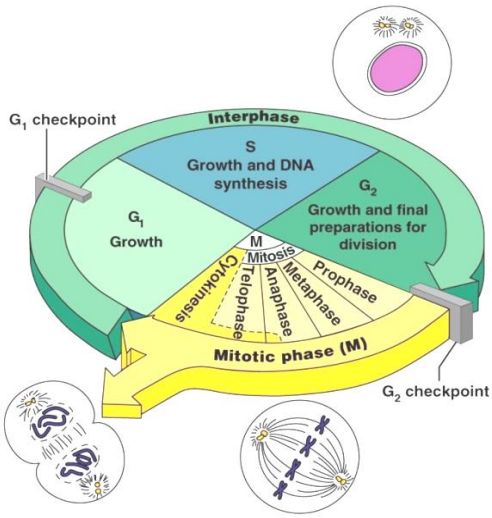

Figure I-5. Scheme of the eukaryotic cell cycle. The cell cycle can be divided in two periods: interphase, during which the cell grows, accumulating nutrients needed for mitosis and duplicating its DNA; the mitotic (M) phase, during which the cell splits itself into two distinct cells, often called "daughter cells" and the final phase, cytokinesis, where the new cell is completely divided. Taken from http://csir-lifescience.blogspot.fr

Consequences of unrepaired DNA damage: chromosome damage

Ionizing radiation can influence base pairing, coding, transcription and gene expression. For instance, genetic mutations are alterations in the genetic code that can cause either cell death or the altered genetic code can be transmitted to the posterities; therefore, quantitative alterations

of the cellular DNA, creation of multipeptide gigantic cells; and morphological chromosomal alterations; then, chromosome aberrations occur when a cell is irradiated in the early stages of interphase.

As explained before (I.2.2.1, page 42), G1 and G2 are stages of the interphase and at these stages radiation can cause several segments of the chromosomes to brake. These segments tend to adhere to other parts of the defective chromosomes, not to unimpaired ones resulting in chromosomes with uncommon morphology. Sometimes, these segments reunify to the correct vacancies of the stricken chromosomes, causing the damage to be repaired. It is also possible to have no segment to chromosomes reunion at all and so a chromosome with an eliminating part to occur.

Repair of ionizing radiation-induced DNA damage

Several distinct enzymatic mechanisms are involved in deletion of damaged bases occur in living cells. The *excision repair of base* damage can be initiated by DNA repair enzymes called DNA glycosylases, which catalyze the hydrolysis of N-glycosylic bonds linked to chemically altered bases. DNA glycosylases recognize either specific base modifications or a limited spectrum of similarly modified nucleotides. Excision repair that is initiated by DNA glycosylases is called base excision repair because the chemically modified moieties are excised as free bases. Many of the known forms of base damage, particularly those caused by the interaction of DNA with environmental agents, are not recognized by DNA glycosylases. There is yet another class of repair specific enzymes that generate incisions in DNA near sites of base damage (*nucleotide excision repair*).

Inadequate DNA strand breaks-repair

Inadequate repair of DNA strand breaks, especially double-strand breaks, can have catastrophic consequences for the cell. A variety of lesions can result from a mis-joined or unrepaired double-strand break, including small deletions that can inactivate a single gene, translocations resulting from non-homologous rearrangements, and loss of large segments of the genome caused by the formation of acentric chromosomes. Chromosomal rearrangements and genetic loss can occur even when the primary lesion resides in a nonessential part of the genome. If genes inactivated by such events encode essential cellular components, then cell death will occur.

I.2.2.2 Radiobiological definition of cell death

The radiation-induced cell death is an important effect for controlling cancer. The organized cell death is crucially important for normal tissue turnover, and that derangements of the pathways that control it can lead to disease. Cell death has a complex relationship to the immune system. Under appropriate circumstances, it can prompt a vigorous immune response to eradicate infectious agents in the process of damaging tissues. At other times, however, it can be immunologically silent or even suppress the immune system. There are several types of cell death caused by radiation, the more important are:

- mitotic catastrophe: it is caused by altered mitoses and/or irreparable chromosome damage and is accompanied by micronucleation and multinucleation. Mitotic catastrophe causes a delayed mitosis-linked cell death and finally leads to apoptosis or necrosis (Kroemer et al. 2009; Vakifahmetoglu et al. 2008). The cell morphology is characterized by micronuclei, *i.e.* pieces of DNA outside the nuclei.
- Apoptosis: it is defined as a programmed cell death mechanism and was first described by Kerr *et al.*(Kerr et al. 1972) It is a genetically regulated process that is a normal physiological method for removing cells that are not needed or are damaged; it does not involve inflammation response. The mechanistic pathways controlling apoptosis have become an important target for cancer treatments, although in many tumor cells apoptosis can be switched off, especially if the main signaling molecule, P53, is mutated (Tamm et al. 2001; Hu & Kavanagh 2003).
- necrosis: cells have undergone an apoptotic program for a certain time before losing their membrane integrity (Gaipl et al. 2001). The disruption of the tumor cell membrane leads to the release of danger signals and consecutively to immune activation (Matzinger 2002).

Proliferating cells suffer DNA damage through ionization or release of free radicals when irradiated, subsequently leading to mitotic catastrophe, growth arrest, and apoptosis (Monje et al. 2002). Brain irradiation early in life targets proliferating cells, resulting in severe cognitive impairment in humans (Oi et al. 1990; Abayomi 1996; Mulhern et al. 2004). The neurogenic regions of the adult brain are highly sensitive to irradiation, due to high proliferative activity in these regions (Mizumatsu et al. 2003). Even low-to-moderate doses can result in persistent negative effects on hippocampal neurogenesis (Tada et al. 2000; Fukuda et al. 2005; Mizumatsu et al. 2003). Because of the extreme sensitivity of the neurogenic regions to irradiation, compared with surrounding tissue, irradiation can advantageously be used as a tool to study adult neurogenesis and its functional importance in the brain (Winocur et al. 2006).

I.3 Synchrotron X-ray generated microbeams

As previously mentioned, microbeam radiosurgery could be done using Synchrotron X-ray source. At the ID17 Biomedical Beamline of the ESRF (Grenoble) three types of RT are applied: contrast-enhanced synchrotron stereotactic radiation therapy (SSRT), microbeam radiation therapy (MRT, where we performed the microbeam radiosurgery) and minibeam radiation therapy (MBRT). Contrast-enhanced SSRT consists of loading the brain tumour with a high-atomic-number element, such as iodinated contrast media, and irradiating it with monochromatic X-rays from a synchrotron source (tuned at an optimal energy) in stereotactic conditions. The highly conformal irradiation geometry and the increase in the photoelectric cross section produce localised dose enhancement in the tumour. This leads to improved dose distributions when compared to conventional high energy RT treatments (Mesa et al. 1999; Boudou et al. 2005; Adam et al. 2008). The other two techniques, *i.e.*, MRT and MBRT, combine submillimetric field sizes (25–100 µm and 500–700 µm-wide beams in MRT and MBRT, respectively) with the spatial fractionation of the dose (interbeam separation of 200–400 µm in MRT and 1000-2000 µm in MBRT).

For the aim of this Thesis the following paragraphs are focusing on MRT techniques but before the Synchrotron radiation, the ESRF and the biomedical bemaline ID17 will be briefly introduced.

I.3.1 Synchrotron radiation sources

Synchrotron radiation (SR) is generated when a charge is moving at a relativistic speed following a curved trajectory. The emission of electromagnetic radiation is along the tangent of the trajectory of the particles; the emitted radiation is highly collimated (Jackson 1998).
Thanks to its usefulness in several research fields, like in material science, chemistry, biology, etc dedicated facilities have been built all round the world. In these facilities electrons are accelerated and confined them in a circular orbit by means of electromagnetic fields. Figure I-7 shows the main different parts constituting a synchrotron (left) and a storage ring (right) where electrons are generated by heating a cathode (typically made of tungsten) and then they are pulled out by a strong electric field through a hole at the end of a gun. These electrons are first accelerated in a linear accelerator (LINAC) and subsequently, bunches of electrons are transmitted into a circular accelerator (BOOSTER), where they are speeded up to operational

energies. At this point, electrons are injected into a large storage ring, where they circulate in ultra high vacuum (<< 10^{-8} mbar) for many hours. The storage ring is not circular, it is actually a polygon that comprise both straight and curved sections under. During one full turn, the electrons are deflected from their path by 64 bending magnets each inducing beam deviation of 5.625°.

Dipole magnets (bending magnets) are located in the curved sections to bend the electrons trajectory in order to maintain them in a closed orbit and/or produce SR with a continuous spectrum. The electron beam is steered by quadrupoles and sextupoles in order to keep the beam within a defined orbit.

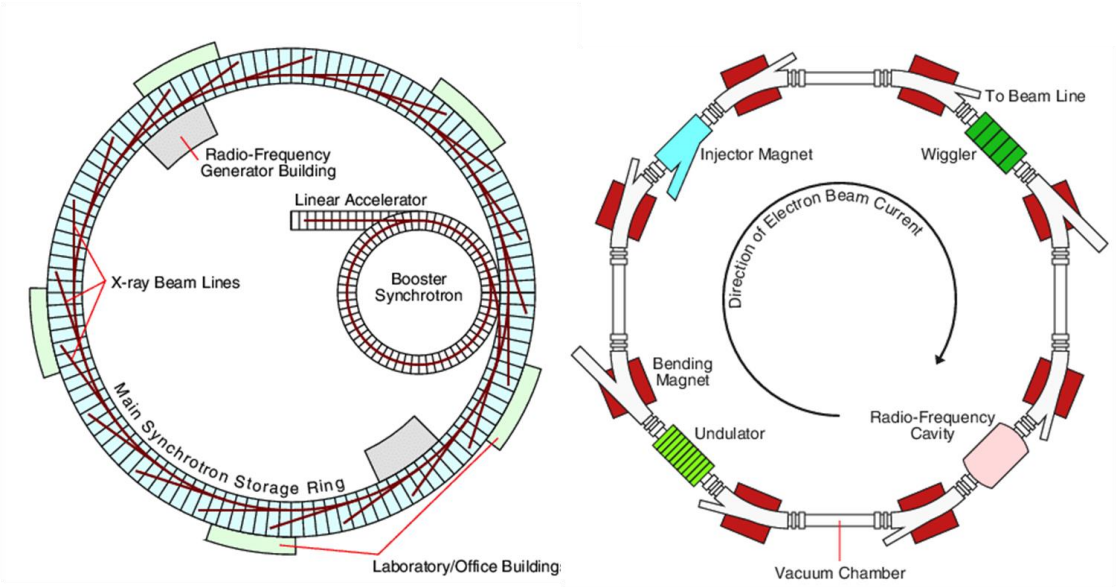

Figure I-6. Scheme of the different parts constituting a synchrotron (left) and a storage ring (right)

On straight sections between two bending magnets are the IDs, responsible for the high brightness of third generation synchrotron devices. There are two types of IDs at synchrotron facilities: wigglers and ondulators (Figure I-7). They both produce significantly higher brightness SR than bending magnets. They consist of a succession of small magnets having an alternate polarity. The electrons thus have a swinging while maintaining overall rectilinear path. The cones of radiation will be superimposed and thus increase the brightness.

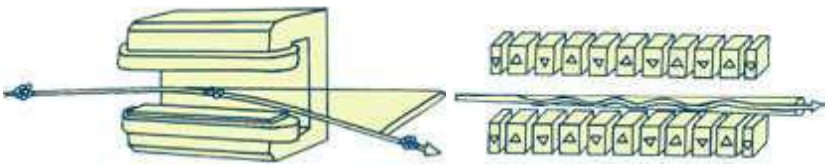

Figure I-7. Electron beam crossing (on the left) a bending magnet (BM) and (on the right) an Insertion device (ID). Both systems are used to produce extremely bright SR. IDs (ondulators and wigglers) are present in third-generation synchrotron sources and they consist of periodic magnetic structures. Extracted from www.esrf.eu

I.3.2 The European Synchrotron Radiation Facility (ESRF)

The ESRF is a third-generation synchrotron able to produce high-brillance X-rays, called “hard” X-rays, which have wavelengths of 0.10 to 0.01 nm or energy in the range 10 to 120 keV (Figure I-8). The brilliance is the most important advantage of synchrotron radiation. Then, a synchrotron source like the ESRF has a brilliance that is more than a billion times higher than a laboratory source. Brilliance is a term that describes both the brightness (a visual perception in which a source appears to be radiating or reflecting light) and the angular spread of the beam.

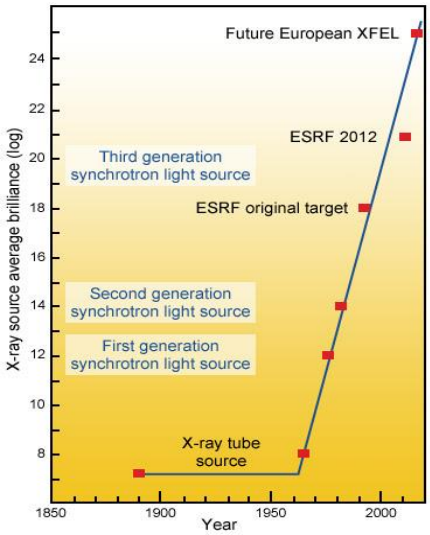

Figure I-8. Comparison between the brilliances of different X-ray sources. Taken from www.esrf.eu

ESRF is one of three most powerful synchrotrons in the world with the Advanced Photon Source in the United States and the Spring 8 in Japan (**Figure I-9**). ESRF was founded in 1988 in Grenoble (France) and, nowadays, the ESRF is the result of an international consortium of nineteen countries devoted to cutting-edge science with photons.

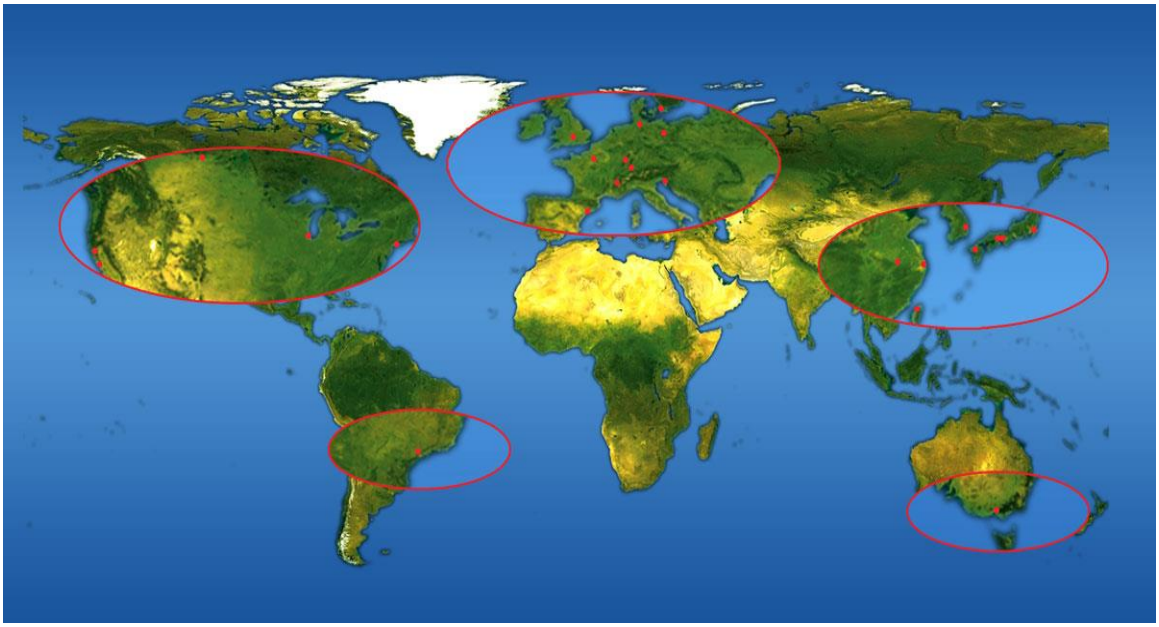

Figure I-9. Map of the twenty-five synchrotron light sources around the world which entered operation in the past 15 years. Extracted from www.esrf.eu

At the ESRF, the synchrotron radiation X-ray beam is produced by a LINAC that accelerates electrons up to 200 MeV; a booster with a circumference of 300 m and a repetition rate of 10 Hz; and a 6.04 GeV electron storage ring (844.4 m of perimeter). The beam is guided by 64 bending magnets and focused by 320 quadrupoles and 224 sextupoles (www.esrf.eu).
The 42 beamlines available at the ESRF are used for a wide variety of scientific topics as mentioned at the beginning of this paragraph. In particular, ID17 is one of the few synchrotron beamlines in the world dedicated to biomedical research.

I.3.3 Beamlines

The radiation emitted by the bending magnets (BM) or by the insertion device (ID) is transferred to the experimental stations inside in-vacuum beamlines. Each beamline is specialized in a particular research area, and is usually formed by hutches (**Figure I-10**). In general, they are defined as follow:

- optical hutch(es), closest to the ring, where all the optical instruments necessary to model the beam according to the requirements of different experiments are located (monochromators, slits, attenuators);
- experimental hutch(es), comprising different types of detectors for measuring and recording the information generated by the radiation-matter interaction;
- control room(s), used by scientists to control the experiment remotely and acquire data.

Key features useful in radiation therapy of SR are:

- Wide energy range, ranging from infrared to hard x-rays (>100 keV). Experiments can be conducted with white radiation or with monochromatic beams.
- Small horizontal (a few mrad) and vertical (fractions of mrad) divergence.
- High intensity and brightness. This enables measurements to be conducted at a high dose rate.

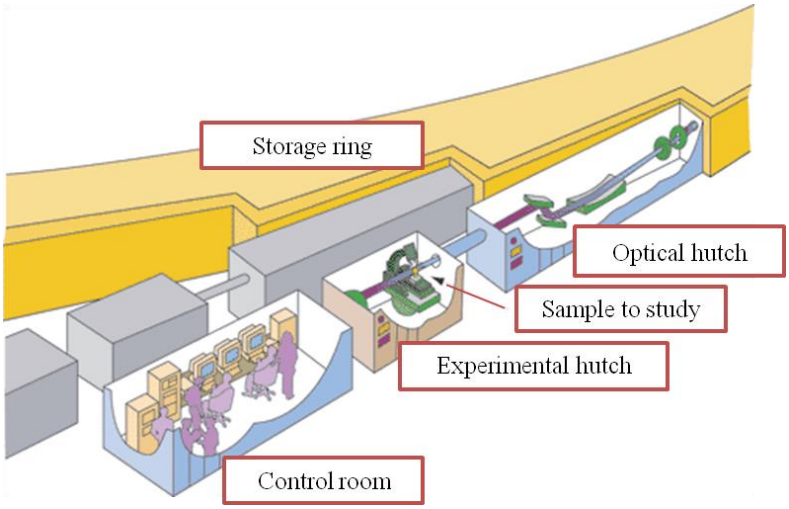

Figure I-10. Schematic representation of a beamline at the ESRF. Extracted from www.esrf.eu

I.3.3.1 ID17 the biomedical beamline

The photon source at ID17 is composed of two wigglers with periods of 15 cm (named w150) and 12.5 cm (w125). In spatially fractionated synchrotron RT techniques, only the first wiggler (w150) is used. The second wiggler (w125) was installed to further increase the photon beam flux in other applications such as SSRT.

Exacting researchers at the ID17 beamline carry out preclinical and clinical studies in radiation therapy and imaging. Different kinds of programs are performed, including preclinical MRT, photon Activation Therapy (PAT), phase contrast imaging techniques, bronchography, brain perfusion studies, and radiobiology investigations (Bravin and Suortti 2011, Thomlinson *et al* 2005, Baruchel *et al* 2008, Suortti and Thomlinson 2003). ID17 possesses two experimental hutches and two optical hutches. The first one, named MRT hutch, is located at around 40 m from the wiggler source and is used for spatially fractionated RT techniques (MRT and MBRT).

MRT is also referred in this Thesis work to Microbeam radiosurgery. In the RT technique the irradiations are performed with the white-beam coming from the wiggler after some filtering.
The second station is located at about 150 m from the source and it uses monochromatic x-rays for synchrotron stereotactic radiation therapy (SSRT) (Adam et al. 2011) or for medical imaging (absorption, time-resolved, K-edge subtraction, phase contrast, analyzer-based and diffraction-enhanced modes) (Baruchel et al. 2008).
The MRT instruments are bypassed by a vacuum pipe, which allows the beam to flow through the entire length of the long pipe tunnel. The reason for this larger-than-normal length of the beamline is to profit of the natural horizontal divergence to make the beam large enough for the requirements of the imaging programs. The geometric definition of the beam is obtained by means of diaphragms, slits, and filters, while a monochromator system located in the monochromator hutch) and serving the satellite building, is used to select the desired energies from the white beam (Figure I-11).

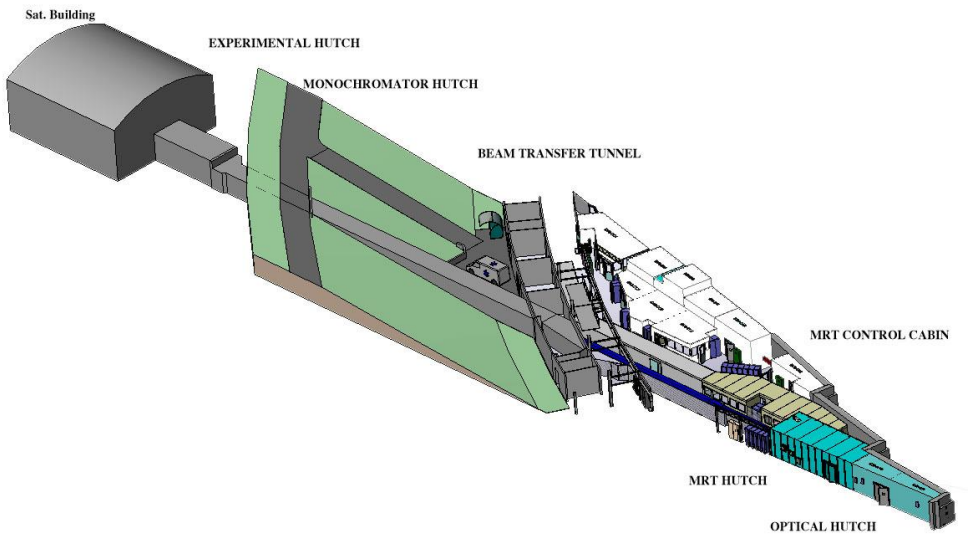

Figure I-11. ID17 Biomedical Beamline layout. Extracted from www.esrf.eu

The beamline starts at the front-end, where it is connected to the storage ring, which is also under ultrahigh vacuum. The front-end contains a shutter, used to enable or disable the beam's passage through the beamline, and various other elements, such as safety devices for vacuum and radioprotection. The beam exiting the wiggler travels in vacuum inside a long stainless steel pipe. Several valves and Be windows (300–500 μm-thick) allow isolation between different vacuum sections. Along the way, different detectors such as diodes or ionization chambers (IC) are used to monitor the beam.

In the MRT hutch, the white beam is directed towards in the first experimental hutch, which is located after the first optical hutch, where all optical elements directly hit by the white beam, are water cooled to dissipate the heat load induced by the radiation. In its path, the beam encounters several beam modifiers. First of all, the low energy part of the spectrum is eliminated progressively through a series of filters: 0.5 mm beryllium (Be), 0.5 mm carbon (C), 1.25 mm aluminum (Al), and 1 mm copper (Cu) (Figure I-12).

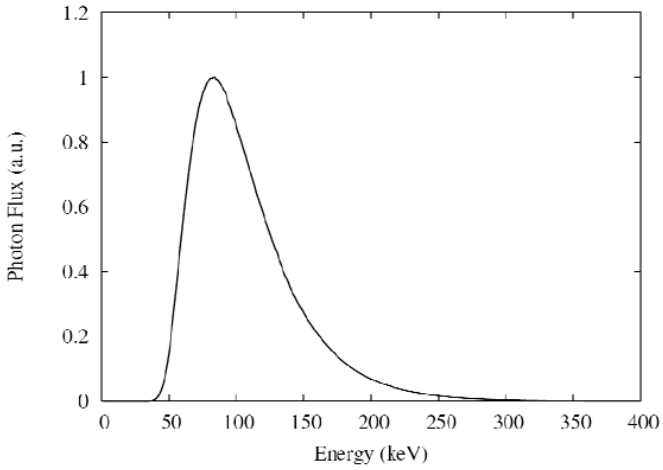

Figure I-12. The ESRF measured photon spectrum used in MRT (1 mm Cu, 1.25 mm Al, 0.5 mm C and 0.5 mm Be). Extracted from Bräuer-Krisch *et al*., 2003 (Bräuer-Krisch, A. Bravin, et al. 2003)

The resulting spectrum ranges approximately from 50 to 350 keV, with a maximum at 83 keV.
As shown in Figure I-13, primary slits allow a first spatial selection of the beam after filtration. These primary slits are in vacuum and located before the filters, and are constituted by four independent blades, a couple for defining the beam height, and a second one for determining the overall horizontal beam width. The 'fast shutter' and the photon absorber are key elements since they are responsible for stopping the radiation in less than 5 ms, after the irradiation process has been completed. The fast shutter consists of two 15-mm-thick tungsten carbide blades coupled to two actuator magnets. During the closing time of the 'fast shutter', the beam is absorbed by the photon absorber (cooled 40-mm-thick Cu block), which has a longer reaction time (about 1 s). More information on this system can be found in the work by Renier *et al*. (Renier *et al 2002)*. Therefore, the SR beam passes through a system composed by slits (vertical and horizontal) that defined the total width of the synchrotron beam used downstream.
In the experimental hutch horizontal tungsten slits limit the beam width to 38 mm and three adjustable positions offer a choice of different beam heights (50, 100, and 500 µm). Vertical

tungsten slits, mounted on a translation stage and positioned upstream, just before the collimator, are aligned with the center of the beam to allow horizontal scanning of the individual microbeams with 1 µm steps. The beam passes from ultrahigh vacuum into air through a beryllium window, which separates the vacuum beamline from the external atmosphere; beryllium has the advantage of being very transparent to X-rays for energies above 10 keV. After the window, the beam goes through an ionization chamber (IC) before impinging on the multislit collimator, called EMSC (**E**SRF-made **m**ulti**s**lit **c**ollimator). The collimator is used to fractionate the synchrotron radiation into microbeams. The used spectrum contains photons in the interval from 30 up to 600 keV with a mean energy of 107 keV.

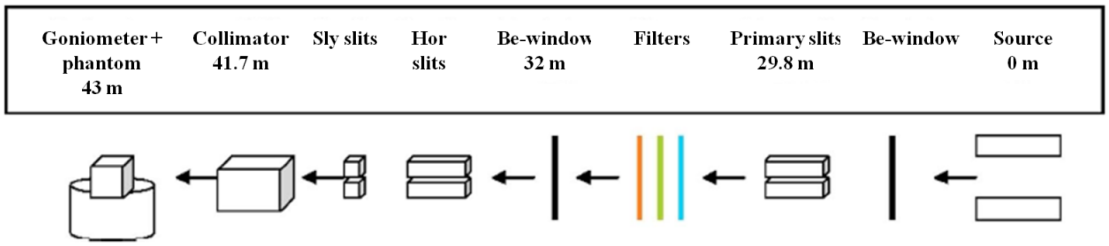

Figure I-13. Sketch of the MRT experimental setup with the distance of the elements from the X-ray source. Extracted from Brauer-Krisch *et al*. 2009(Bräuer-Krisch et al. 2009). Distances are not to scale.

The EMSC is assembled with two identical ~8 mm thick blocks made of tungsten carbide (86.8%, weight percentage) and Cu (12.0%) movable one in front of the other to selectionate the desired microbeam width; each block is composed by 125, three mm high, and 100 µm wide equidistant slits to fractionate the oncoming seamless X-ray beam, regularly repeated with a uniform pitch of 400 µm center-to-center (c-t-c) (Figure I-14) (Bräuer-Krisch et al. 2009).

Before irradiation, and with the animal patient already sitting on the 3-axis Kappa-type high-precision goniometer (Huber, Germany), the target is identified by acquiring low dose radiographs following an imaging protocol (Serduc et al. 2010). In this phase, the collimator is temporarily removed.

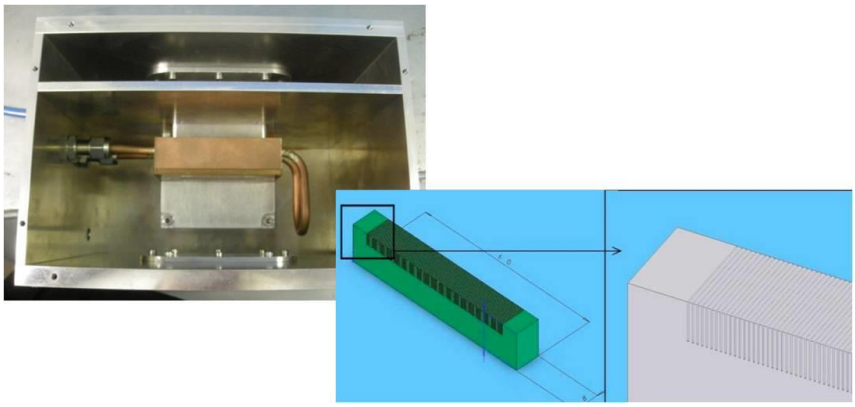

Figure I-14. On the left, top view of the multislit collimator whole block used for MRT. On the right, a graphical representation of the EMSC (ESRF **m**ulti**s**lit **c**ollimator) with 100 µm slit width and a pitch of 400 µm. Extracted from Bräuer-Krisch *et al.*, 2009 and Martìnez-Rovira 2012.

Images area acquired by vertically scanning the goniometer and simultaneously acquiring the X-ray images with a Frelon camera (Bravin et al. 2003). Thanks to a graphical user interface system (GUI) the target area of the treatment can be identified according with the skull structures (Figure I-15).

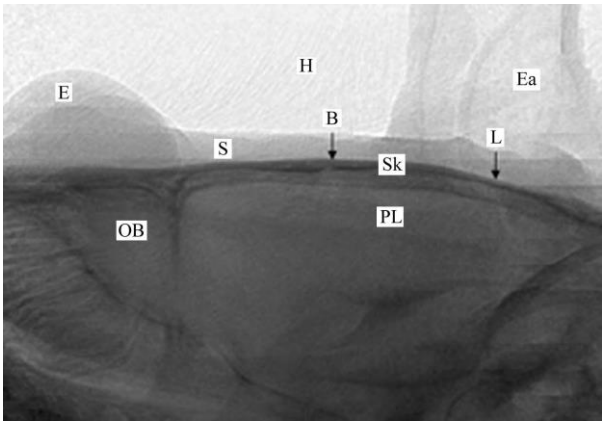

Figure I-15. A typical in vivo beam-eye-view X-ray radiograph of a rat head acquired in the MRT hutch of ID17 . The principal anatomical characteristics are clearly visible: eye (E), skin (S), hair (H), ears (Ea), olfactory bulb (OB) and parietal lobe (PL). Furthermore, the skull surface (Sk), the bregma (B) and lambda (L), reference points used in the rat head atlas for stereotactic surgery, are easily located (arrows). Extracted from Serduc *et al*. (Serduc et al. 2010)

I.3.4 Dosimetry concepts applied to microbeam radiosurgery

The dosimetry is an important aspect that allows us to correlate the dose effectively deposited into the tissues with the radio-biologically induced effects. Today several approaches exist to measure or simulate the dose deposition in samples. The dose distribution depends on several parameters such as the sample composition, the incident photon spectrum, the number, the shape, and the dimension of microbeams as well as their separation. This crucial task, essential

to prepare treatment plans, and to correlate the deposited dose with the biological findings, can be carried out using two alternative methods.

The first method consists in experimentally measuring the dose distributions using dosimeters with an adequate resolution (down to 1 μm), which have recently become available. The X-ray energies involved are relatively low (~50-600 keV) compared with photon energies used in hospital, linear-accelerator based radiotherapy (~4-20 MeV), which makes the choice of detector material extremely important. Furthermore, the very intense beam used in microbeam radiosurgery can cause saturation in the detected signal. This fact limits the available instruments and techniques which can be used for measurements. At the ESRF, the absolute dose determination in homogeneous fields (1x1cm^2) is evaluated using ionization chambers and TLD dosimeters, while radiochromic films and metaloxide-semiconductor field-effect transistor (MOSFET) dosimeters are used for relative dosimetry.

The second method estimates the dose deposition using Monte Carlo (MC) simulations, which are a relatively easier to be performed than experimental dosimetry. Furthermore, MC methods allow determining the 3D distribution of the dose within the sample. To date, different MC codes, such as EGS4, GEANT, and PENELOPE that use different physical interaction models and transport algorithms, have been used to validate results and increase accuracy (Sempau et al. 2003; Siegbahn et al. 2006; Martìnez-Rovira 2012; Felici et al. 2008; Spiga 2008; Spiga et al. 2007).

PENELOPE is a general purpose MC simulation package developed at the University of Barcelona (Sempau et al. 2003). The developers of this code have put special emphasis on the implementation of accurate low-energy electron cross sections. A mixed simulation scheme is implemented in the code, according to well-established terminology, where hard interactions are simulated collision by collision and small angular deflections and energy losses are treated in a grouped manner. Compton scattering is the dominant photon interaction in low atomic number materials of interest in radiation therapy, for the X-ray spectrum generated by the ESRF wiggler (Siegbahn et al. 2006). The elastic scattering of low-energy electrons generated in the Compton interactions is of particular importance since it determines how far into the valley region the electrons are transported. The beam X-ray energies are typically sampled from the experimental ESRF spectrum (Bräuer-Krisch et al. 2003). The most probable energy is 83 keV and the mean energy is 107 keV. All photons are assumed to impinge unidirectionally perpendicular to the phantom surface. Siegbahn and coworkers (Siegbahn et al. 2005) studied the different results between Monte Carlo simulations and the Gafchromic® films (International Specialty Products, New Jersey, USA) (Figure I-16).

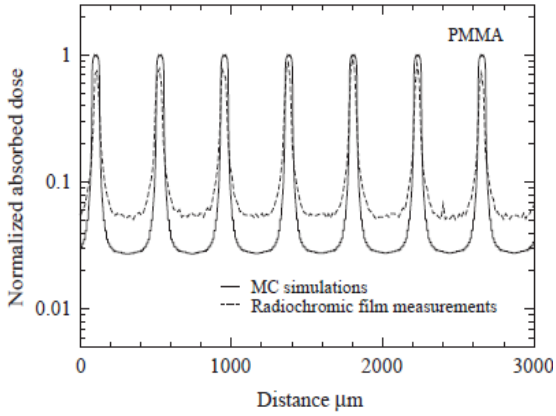

Figure I-16. Comparison between an MC calculation and a film measurement when using a multislit collimator with 50 micron wide rectangular microbeams and 400 micron microbeam separation. PMMA=polymethylmethacrylate phantom, MC=Monte Carlo. Extracted from Siegbahn *et al.* 2005.

Gafchromic® films were used for several years at the ESRF not only to measure the dose but also for checking the positioning of the animal, size of the microbeam and distance between beams.

There are two dosimetry protocols, one used for microbeams and the other one used for homogenous beam (or broad beam).

The dosimetry protocol for microbeam radiation consists in placing these films at different depths in a polymethylmethacrylate phantom (~water equivalent) simulating the head of an animal that should be irradiated. This sensitive film polymerizes when irradiated and change their color (become blue) in proportion to the dose (Figure I-17). The readout of all films is done with a microdensitometer (3CS Microdensitometer, J. L. Automation). This system has a wide dynamic range (it is sensitive to optical densities (OD) from 0.2 to 6) and a very high spatial resolution (around 5 µm). Before irradiation, the films are kept in their light tight black envelope and at room temperature. They are cut and prepared in a relatively dark room and kept in aluminum foil except during exposures.

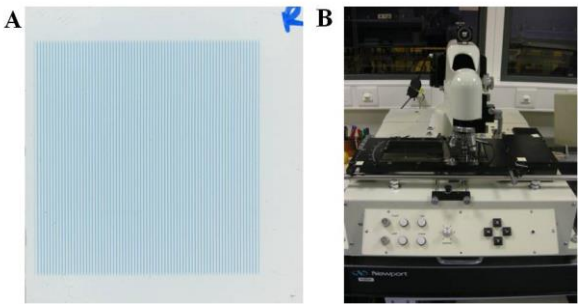

Figure I-17. A) A Gafchromic® HD-810 film after an irradiation with microbeams (3×3 cm^2 field size). B) Microdensitometer (3CS microdensitometer, J. L. Automation) used to read the radiochromic films exposed to radiation. All pictures are extracted from Martinez-Rovira (Martìnez-Rovira 2012).

The absolute dosimetry calculated for homogenous beam is performed by following the recommendations compiled in the International Atomic Energy Agency (IAEA) Technical Reports Series (TRS) 398 (IAEA-TRS-430, 2004). The microbeam fits within the medium-energy x-ray range since its aluminum half-value layers (HVL) was found to be 16 mm (Siegbahn 2007). In this case, the dose measurement in reference conditions (broad beam or homogenous beam) is performed at 2 g/cm^2. The reference field size was chosen to be 2×2 cm^2 instead of 10×10 cm^2 since it is closer to the field sizes that are going to be used for microbeam treatments. Cylindrical ionization chambers (IC) with a cavity volume in the range of 0.1–1.0 cm^3 are recommended for reference dosimetry in medium-energy x-ray beams. The IC was calibrated in kilovoltage X-rays. During measurement, the reference point of the chamber (on the chamber axis at the centre of the cavity volume) was placed at the reference depth. Water is the recommended medium for measurements of absorbed dose. There should be a margin of at least 5 g/cm^2 beyond all four sides of the largest field size and 10 g/cm^2 beyond the maximum depth of measurement. Additionally, the window of the phantom should be made of plastic with a thickness of 0.2–0.5 cm. Dose measurements for horizontal beams performed with a water tank containing distilled water. Standard dosimetry and reference calibration protocols assume exposure of the IC to a uniform beam. However, as has been mentioned, the vertical dimensions of the beam at the patient position are defined by the vertical slit size (50–800 µm). The beam is then scanned in the vertical direction at constant speed (Prezado et al. 2011).

I.4 Development of microbeam irradiations

The use of unidirectionally delivered parallel planar microbeams or multidirectionally from different entry points was indicated with the term of MRT.

During the past decade, potential applications of MRT have been studied experimentally at the National Synchrotron Light Source (NSLS) at Upton, New York, USA (Slatkin et al. n.d.; Slatkin et al. 1995; Dilmanian et al. 2001) and at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France (Laissue et al. 1998; Dilmanian et al. 2001; Romanelli et al. 2013). Numerous experiments have been carried out at the NSLS and, subsequently, at the ESRF, with the aim of assessing the therapeutic effectiveness of microbeam radiosurgery. Nowadays, the use of microbeam radiation has spread to other synchrotrons around the world (Spring8 in Japan, Canadian Light Source, Australian Synchrotron).

I.4.1 Historical Overview

Microbeam radiation concepts seems to be related to the ability of normal tissues to tolerate a very high radiation dose in small volumes; this is call dose-volume effect (Withers et al. 1988), resulting in the preservation of the tissues' architecture.

This was clearly illustrated in 1961 by Zeman *et al*. (Zeman et al. 1961) that simulated in laboratory the effects of spatially fractionated radiation on living animals, aiming at mimicking and studying in a controlled environment the effects of cosmic radiation on future astronauts. Their study showed that 280 Gy administered with a beam (22 MeV deuterium) of 1 mm of diameter induced tissue necrosis extended further on the beam's path. Instead, tissue irradiated with 25 µm-wide and an entrance dose of 4000 Gy remain intact (Figure I-18).

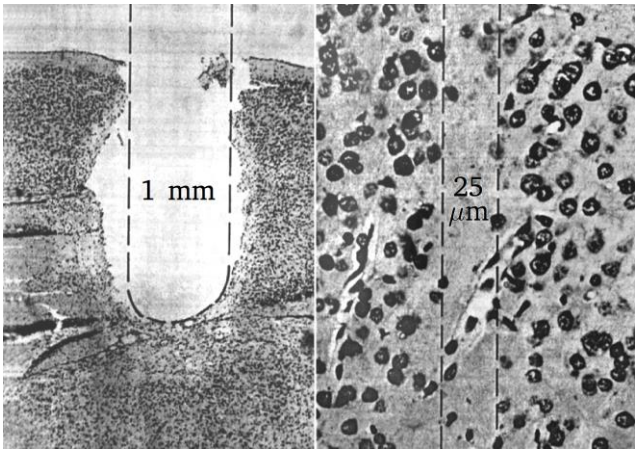

Figure I-18. Histological example of mice brains illustrating the dose-volume effect. On the left, the tissue was irradiated with deuteron beam of 1 mm and an entrance dose of 280 Gy. On the right, tissue irradiation with 25 µm-wide beam and an entrance dose of 4000 Gy. Extracted from Zeman *et al*. (Zeman et al. 1961).

The study on biological effect of ionizing microbeam radiation continued at Brookhaven National Laboratory, where the work went in the same direction producing 25 µm-wide beam at 22 MeV, to specifically mimic the ionization track of a single cosmic-ray particle. The initial experiments established that a dose of 500 rad (5 Gy) delivered to a hair follicle was sufficient to cause graying of the hair. Tissue volume irradiated was not a factor, so long as the follicle was hit. Next, the mouse brain was irradiated with a beam between 0.025 and 1 mm wide and at various doses. The exact details of these results are unclear, as they are published in several reports, some of which appear in conflict. What is apparent is that the dose required to produce a lesion within 24 days by a 0.025 mm wide beam is vastly greater than that required by a 1 mm wide beam. The hypothesis, already advanced at the time, was the rapid regeneration of blood

vessels in the passage of the microbeam by endothelial cells located either side of the microbeam. Preservation of vascular network ensured the constant supply of oxygen and nutrients, thereby reducing the appearance of necrotic areas of tissue irradiated.

The principle behind the microbeam radiosurgery seems to be related to the ability of normal tissues to tolerate a very high radiation dose in small volumes (dose-volume effect), resulting in the preservation of the tissues' architecture.

The majority of recent microbeam studies have used arrays of parallel beams. MRT used can vary between 25 and 100 micron width and the distance between two microbeams is typically 100–400 mm. The dose rate in the air reaches ~16000 Gy s^{-1}. The dose profile consist of a pattern of peaks and valleys, *i.e.*, with high doses along the microbeam path and low doses in the spaces between them. Most studies describe beam spacing as "on-center". The valley dose must not exceed a certain threshold dose to assure efficient tissue repair within intensely irradiated microslices. These valley doses depend on several parameters including photon energy, number of microbeams, microbeam width and height, center-to-center distance, as well as the depth, shape, and composition of tissues proximal to, within, and distal to the target (Figure I-19).

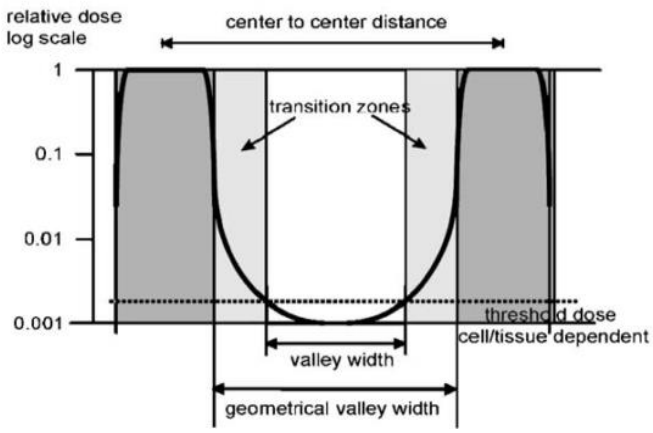

Figure I-19. Schematic dose profile between two microbeams (gray dark, peak areas), the valley zone is defined as the area between the two microbeams and the transition zones (area adjacent to the peaks). Extracted from Blatmann *et al* (Blattmann *et al* 2005).

The ratio between the peak dose and the valley dose (peak-to-valley dose ratio, PVDR) is an important dosimetric parameter in spatially fractioned techniques (Dilmanian et al. 2002). PVDR lies in the simple fact that radio-biologically the cells in the path of the peak will be essentially destroyed, while the valley dose must remain low enough for the normal tissue to provide sufficient repair.

The work in MRT has investigated the sparing and tumoricidal effects of microbeams and the developing of methodology which would be effective at treating malignant brain tumors.

I.4.2 The sparing effect of microbeams

The first publication on biological experiment was described in 1995 by Slatkin and collaborators (Slatkin et al. 1995) and performed at the BNL where they delivered in a single exposure 20 and 37 µm-wide microbeams (spaced by 75 and 200 µm, respectively) on rat brains. Histology analysis showed cerebral necrosis in rats irradiated at 10000 Gy after 15 days. Whereas, brain tissues in and between all the 312- and 625-Gy-entrance slices appeared normal. Only cells located in the passage of microbeam die between 15 days and one month after irradiation. This unusual resistance to necrosis was a central to the rationale of microplanar beam radiation therapy.

Laissue and collaborators (J. A. Laissue et al. 2001; Slatkin, Blattmann, Wagner, Glotzer & Laissue 2007) used the larger brain of the piglet as a surrogate for human pediatric MRT. The cerebellum received between 66 and 263 Gy of MRT with valley doses at approximately 5% of peak dose. The irradiated area was 1.5×1.5 cm^2 with 20–30 µm width beams spaced at 210 µm intervals. The animals were studied out to 465 days with no behavioral or MRI evidence of pathology. Histology showed evidence of cell destruction along the beam path only ("stripes") for all groups (Figure I-20).

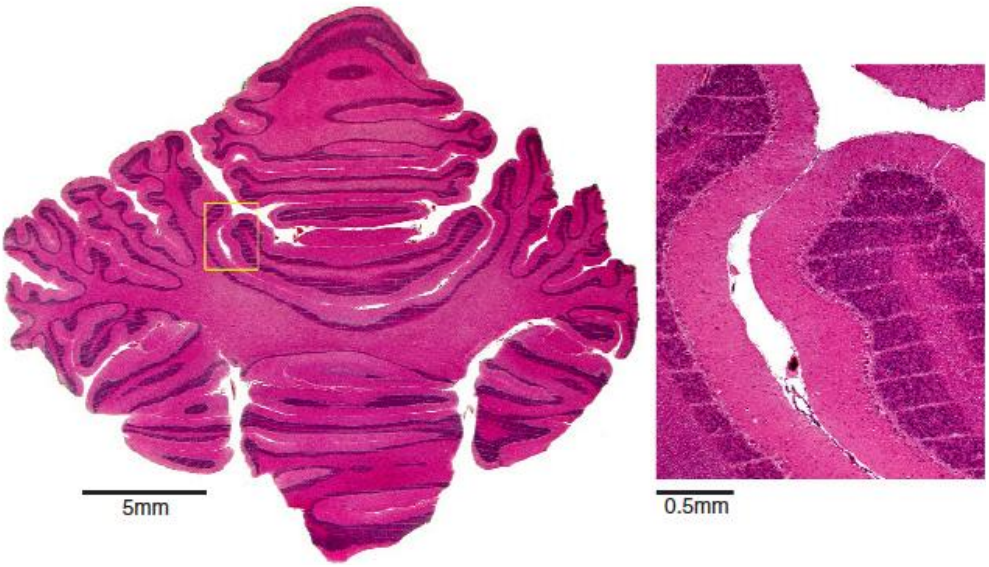

Figure I-20. Hematoxylin and eosin stain of a horizontal section of the cerebellum of a piglet 15 months after microbeam irradiation with an entrance dose of 300 Gy (20–30 µm-wide microbeams spaced by 200 µm). The beam paths are clearly visible in the histology images, but no tissue destruction is observed. Extracted from Laissue *et al* 2001.

Later on, Dilmanian and co-workers (Dilmanian et al. 2002) performed a microbeam irradiation on duck embryos using at 40, 80, 160, and 450 Gy compared with broad beam irradiation at 6, 12, and 18 Gy. Results showed that 450-Gy treatment caused vascular lesions outside of the microbeam paths. The sacrifice and subsequent histopathology of one of these ducks was performed at day 23 from irradiation. Histopathology was reported as normal for the lower dose microbeam groups as well as broad beam at 6 or 12 Gy. This is due to the fast regeneration of healthy vasculature. This repair is depicted by neovascular anastomoses (communication between blood vessels by means of collateral channels) between intact capillary networks, bridging parallel columns of tissue previously containing identical intact capillary networks that had been ablated by the microbeams (Figure I-21).

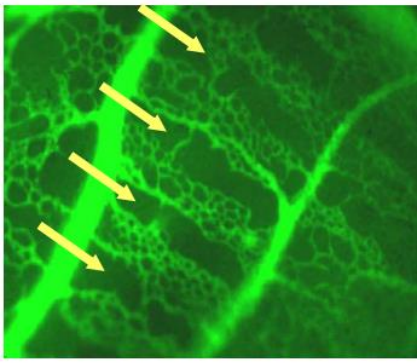

Figure I-21. Microscope picture of anastomotic repair (yellow arrows) of microbeam damage observed in vivo in a chick-embryo chorioallantoic membrane. Extracted from Blattmann *et al* 2005.

Serduc and co-workers (Serduc et al. 2006b) confirmed the lack of changes in the blood volume and vascular density in normal vasculature at very high entrance doses on healthy mice. In addition, no disruption of the blood brain barrier or significant oedema was observed and only a transient increase in the blood-barrier permeability was detected. This experiment was conducted by the same research group (Serduc, van de Looij, et al. 2008) some year later using diffusion weighted MRI and specific micro-gravimetry. They evaluated early (1–28 days post-irradiation) edema in the brains of Swiss nude mice exposed to microbeams. Results suggested mild cellular edema (apparent diffusion coefficient increase) 1 day following 312 Gy (entrance dose) MRT, which resolved by day 7, while 1000 Gy resulted in both cellular (apparent diffusion coefficient increase) and vasogenic edema (cerebral water content increase) which determined by day 7. In both cases, the amount of edema was quite modest despite the high doses employed. Another explanation for less edema is that repair of microbeam damage may be more efficient. Indeed, the absence of important edema may represent an important advantage of MRT with respect to other RT treatments.

I.4.3 The tumoricidal effect

In addition to the exploration of MRT in healthy animals, experiments involving animal models of cancer, including primary brain tumors. The first model was proposed by Laissue and co-workers (Laissue et al. 1998) they applied the same technique described by Dilmanian and collaborators (Dilmanian et al. 2002) on intracranial rat 9L gliosarcomas tumor model. They choose this tumor model because is immunologically compatible with rats and extremely radioresistent compared to other models (Barth & Kaur 2009; Bencokova et al. 2008). They used microbeams of 25 µm wide and spaced 100 µm unidirectional and bidirectional arrays of microbeams using 312.5 and 625 Gy as skin entrance doses. The irradiation was performed on caudate nucleus of Fischer rats 344 implanted 14 days before irradiation. The results showed that microbeam radiation protocols significantly increased the survival of the animals, which was accompanied by a reduction in tumor growth after irradiation. The longest survival was observed for the 625 Gy bidirectional group, with 50% of the animals alive at day 115 (control rats died within 31 days). Furthermore, the complete ablation of the tumor was also observed in more than half of the rats. This suggested the possibility of a differential effect between healthy and tumor tissue after MRT irradiations (Figure I-22).

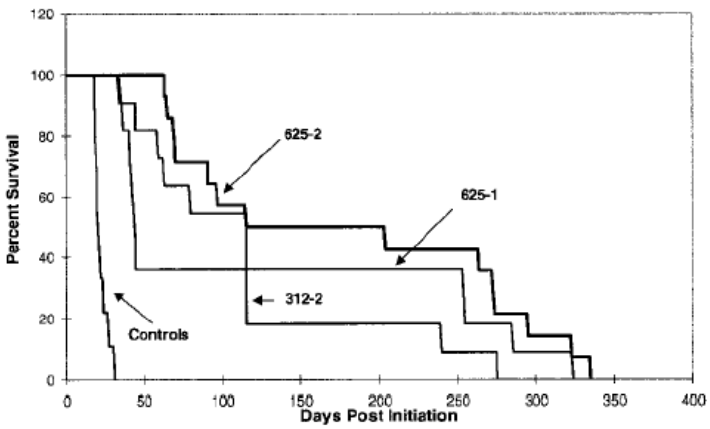

Figure I-22. Survival curves of 9L gliosarcoma rats implanted 14 days before irradiation using microbeams of 25 µm-wide and spaced 100 µm. The two types of irradiation used were: bidirectional irradiation at 312.5 (named 312-2) and 625 Gy (named 625-2), and unidirectional irradiation with an entrance dose of 625 Gy (625-1). At 115 days after implantation the percentage of survival animals was for the bidirectional irradiation: 50% at 625 Gy and 18% at 312 Gy; for the unidirectional irradiation was 36% at 625 and 0% of non-irradiated controls. Extracted from (Laissue et al. 1998).

In 2008 Regnard *et al*. (Regnard et al. 2008) showed the possible achievement of a good balance between sparing normal tissues and controlling the tumor by tuning a single parameter comparing, 25-µm microbeam arrays spaced either 100 or 200 µm (center-to-center) at 625 Gy

to broad beam irradiation (20 Gy). The median survival time (post-implantation) was 40 and 67 days at 200 and 100 µm spacing, respectively. However, 72% of rats irradiated at 100 µm spacing showed abnormal clinical signs and weight patterns, whereas only 12% of rats were affected at 200 µm spacing. Hence, the 100 µm series can be considered as the upper limit achievable, while the 200 µm series could be the lower limit.

Therefore, the high therapeutic index of synchrotron X-ray generated microbeams has been confirmed later on by Miura and co-workers (Miura et al. 2006) by the significant increase in survival time of mice implanted subcutaneously aggressive human squamous-cell carcinoma cells (SCCVII) model. These animals were irradiated in two orthogonal exposures of 200 µm spaced microbeams, with peak skin entrance dose (for each exposure) ranging from 442 to 884 Gy for 35 µm-wide beams and with 442 Gy for 70 µm-wide beams. The best median survival times (37 days post-irradiation) was obtained with the combination 884/35 exceeding the 20 days from 35-Gy irradiation. This high survive of mice is due to dose-dependent effect.

Schültke *et al*. (Schültke et al. 2008) studied in two animal models of malignant brain tumor (C6 cells implanted in Wistar rats and F98 cells in Fischer rats) the survival and memory development after MRT. Thirteen days after implantation of tumour cells, animals were submitted to MRT using two orthogonal 1 cm wide arrays composed by 50 microplanar microbeams of 25 µm-wide and a center-to-center distance of about 200 µm (skin-entrance dose of 350 Gy for each direction). Object recognition tests were performed at day 13 after tumor cell implantation and in monthly intervals up to 1 year later. In both animal models, MRT with buthionine-SR-sulfoximine (BSO) and without adjuvant therapy significantly increased survival times. The adjuvant therapy with BSO was tested because from literature seems that increased the radiosensitivity of the cells. Irradiation with microbeams seems not affect memory as there was no significant difference between healthy untreated and tumour-free animals that underwent MRT at either 1 month or 1 year after MRT.

As explained formerly, the resistant of normal brain tissue to spatially fractioned irradiation has been attributed to the radiotolerance of normal vascular networks which supplies nutrient to exposed cerebral tissue reducing the hypoxia/ischemia mechanism. It also been hypothesized that the microvascular blood supply to tumors is unable to regenerate in the same way as that of healthy tissue. Following this research pathway Bouchet and co-workers (Bouchet et al. 2010; Bouchet et al. 2013) provided new evidence for the differential effect of MRT on tumour vasculature that leads to tumour hypoxia. They analysed the effects of irradiation on blood vessel, oxygenation and vascular networks, in a 9L gliosarcoma rat model, using intersecting orthogonally microbeam, arrays of 50 µm-wide and separated at the center by 200 µm (2 arrays

x 400 Gy). They showed that the vasculature of the normal brain exposed to irradiation remains sufficiently perfused to prevent any hypoxia.

Several other articles published in the recent years report the result of investigations of the sparing and curing effect of microbeam radiation. In their ensemble, microbeam radiation therapy appears to be very well tolerated by brain tissue, especially by the vascular system, which is very sensitive to ionizing radiation. So, this extraordinary tolerance to synchrotron X-ray generated microbeams gave us the input to use it in radiosurgery for treating non-cancerous central nervous system pathologies.

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CHAPTER II

Parallel and convergent microbeam transections of the sensorimotor cortex in normal adult rats and in an epileptic animal model

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Summary

The preservation of cortical architecture following high-dose microbeam irradiation and the ability to induce non-invasively the equivalent of a surgical cut over the cortex i s explored in this second chapter. Results showed the different biological effects of healthy tissue to microbeams delivered at a range doses between 150 and 600 Gy. The sensorimotor cortex microbeam transections did not cause neurological deficit in normal rats and caused reduction of convulsive seizures in an epileptic Kainic-acid animal model.

Résumé

La préservation de l'architecture corticale suite à l'irradiation par microfaisceau à haute dose et la capacité d'induire de façon non-invasive l'équivalent d'une incision chirurgicale au niveau du cortex est exploré dans ce second chapitre. Les résultats ont montré que des irradiations par microfaiseaux délivrés à des doses comprises entre 150 et 600 Gy induisaient différents effets biologiques sur le tissu sain. Les transections par microfaisaux sur le cortex sensori-moteur n'ont pas généré de déficit neurologique chez les rats normaux et entraînent la réduction des crises convulsives sur les animaux épileptiques (modèle de rat acid-kaïnique).

II.1 Introduction

Epilepsy is a neurological disorder affecting 2% of population when in association with other encefalopathic form and about 0.5% referring with the simple disease. This neurological disorder is characterized by recurrent and sudden excessive electrical discharges, i.e. epileptic seizures, which disrupt the normal activity of neurons and change the patient's behavior/function temporarily (Barry & Hauser 1993; Hauser et al. 1993).

Status epilepticus (SE) is defined as a condition in which epileptic activity (*i.e.* seizure) persists for more than 30 min or recurrent seizures over period of 30 min without full recovery of consciousness between seizures (Beghi et al. 2005; Lowenstein 1999; Berg et al. 2010). Seizure-induced neurodegeneration is caused by excessive stimulation by neurotransmitters (excitotoxity) such as ionotropic glutamate receptors by glutamate (the major excitatory neurotransmitter of the brain), leading to excessive calcium ion (Ca^{2+}) influx inside the neuronal cell that could be damage or killed in this way (Olney 1969).

The first choice to treat epilepsy seizures is the use of anti-epileptic drugs (AEDs). About 70% of people with epilepsy have their seizures controlled with AEDs. Unfortunately, approximately 20–30% of patients with epilepsy continue to have seizures despite efforts to find an effective combination of AEDs and these seizures becomes intractable (Romanelli et al. 2012; Cascino 2004; Kuzniecky & Devinsky 2007). Hence, epilepsy surgery is an effective option for selected patients with intractable epilepsy. Hence, radiosurgery could be considered an emerging therapeutic approach for the treatment of medically intractable epileptogenic foci. A favorable seizure outcome was first reported in studies of the effects of radiosurgery in the treatment of arteriovenous malformations (AMV, a blood vessel abnormality) and tumours. Radiosurgery has since been applied to the treatment of complex partial seizures with mesial-temporal-lobe onset. Nearly simultaneously, experimental evidence supporting the usefulness of radiosurgery to improve or abolish seizures has confirmed that stereotactic irradiation can preferentially affect epileptogenic versus normal cortex.

The goal of a resective surgical treatment depends on how good the epileptogenic zone can be identified and fully removed, without injuries of functional important cortex areas (Awad et al. 1991). Additionally, identification of eloquent cortical areas near the region to be resected is essential to prevent postoperative neurological deficit. A wide range of imaging techniques is valuable for imaging the epileptogenic zone, MRI, MR spectroscopy, diffusion imaging, positron emission tomography (PET), single-photon emission computed tomography (SPECT) and simultaneous EEG-fMRI (Matsuda et al. 1989; Fish & Spencer 1995; Cendes 2013). Eloquent cortex has in the past been mapped using highly invasive techniques; and functional

magnetic resonance imaging (fMRI) of motor and cognitive tasks holds great promise for future non-invasive mapping strategies (Cascino 2004; Go & Snead 2008).

Two important concepts in this context are the eloquent cortex and the epileptogenic zone. Eloquent cortex refers to any cortical area in which injury produces symptomatic cognitive or motor deficit. Examples of such areas are primary sensorimotor cortex, essential speech areas, occipital visual areas and mesial temporal regions crucial for episodic memory. Many other cortical areas can be resected without obvious consequences for the patient, although detailed neuropsychological testing may reveal subtle deficits. The epileptogenic zone refers to the region of cerebral cortex that is both necessary and sufficient to generate epileptic seizures, hence its entire removal is required for a successful outcome (Engel et al. 2007). This area is not necessarily the same as the region of cortex which produces interictal spikes on the scalp EEG (the irritative zone is measured by magnetoencephalography (MEG) and fMRI; nor is it necessarily completely overlapping with any structural lesion (epileptogenic lesion) which may be the underlying cause of the epilepsy and which may be seen on an MRI brain scan. Furthermore, any of these zones and lesions may encroach into eloquent cortex; the brain lesion itself may sustain normal functions (Richardson 1998); and cortex around the epileptogenic zone may show impaired function prior to surgery, but recover post-operatively (Hajek et al. 1994). Furthermore, the normal allocation of functions to particular brain regions may be altered in patients with epilepsy – for example, language representation may be in the right hemisphere rather than the left in the presence of left hemisphere pathology present since early childhood – so cortex may be unexpectedly eloquent in a typically non-eloquent area. It will be immediately clear that planning the cortical resection in surgery for epilepsy is extremely challenging. Besides, when resective surgery is not indicated, neuromodulation could be an emerging surgical option (Anderson et al. 2009). It includes the delivery of electrical stimulation to selected brain regions or to the *vagus nerve* as well as stereotactic radiosurgery, multiple subpial transections (MST), and the more recent generation of neurostimulators.
Nowadays epileptic foci irradiated by conventional clinical devices, such as Gamma-Knife®, Cyberknife® or Linacs (described in deepest in the paragraph II.2.3.62.1II.1.1II.2.3.6), have reported as acceptable outcomes for hypothalamic hamartomas, arteriovenous malformations and cavernomas (Romanelli & Anschel 2006; Quigg & Noachtar 2012; Sims et al. 1999; St George et al. 2002). In such clinical context, the synchrotron-generated X-rays could provide attractive physical properties in terms of precision and conformation of the dose delivery (Romanelli & Bravin 2011).

The main objectives of this Thesis were to study the equivalent of a surgical cut, *i.e.* MST in the clinical surgery, through the brain cortex by delivering very high doses of radiation (hundreds to

thousands of Grays) (Slatkin, Blattmann, Wagner, Glotzer, Laissue, et al. 2007; Bräuer-Krisch et al. 2010) to normal Wistar male rats and we have also used an experimental model of epilepsy induced by local injection of kainic acid (KA) in the rat sensorimotor cortex as a pilot experiment. The irradiation field was involved in the motor locomotion and for this reason we performed a Rotarod® test to verify their mobility on an accelerated rod. Microbeam and minibeam cortical transections might prove to be an innovative approach to cure medically-refractory focal epilepsy. In such case, it will provide a less invasive tool to effectively parcellize and disconnect the seizure focus while sparing adjacent neural tissue (Romanelli et al. 2011).

II.2 Epilepsy and epileptic seizures

Epilepsy is a chronic disorder characterized by recurrent and unprovoked seizures (Dreifuss 1987; Hauser et al. 1993). It is the second most common neurological disorder (after stroke), as it heavily affects the quality of life of afflicted individuals. Basically, epilepsy is characterized by sudden onset of rhythmic and synchronized discharges of several neurons, in restricted brain areas or the whole brain. In according to the definition proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy, an epileptic seizure is "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher et al. 2005). The etiology of epilepsy is various among different age groups and geographical locations. Besides, clinical manifestations are affected also by genetic and environmental elements (Shorvon 2009; Shorvon 1996). Epileptic seizures depend on several factors, such as (*i*) the dimension, (*ii*) the localization and (*iii*) the seizure spread to other brain areas. The seizure frequency is very variable and can range from one in a lifetime to several hundred a day or even evolve into SE, with lack of seizure termination (Noachtar & Rémi 2012). The epileptogenic zone is the region from which the epileptic seizure originates. An epileptic discharge that is limited to the seizure-onset zone does not necessarily lead to clinical symptoms, probably because the epileptogenic zone does not overlap with the symptomatogenic zone (Lüders et al. 1992). The "symptomatogenic zone" refers to the area of the cortex that produces certain clinical symptoms as a result of epileptic activation. For example, seizures that originate in the frontal convexity remain asymptomatic as long as they do not spread into the symptomatogenic zones. If the epileptic activation reaches the primary motor area, focal clonic seizures occur (Noachtar & Borggraefe 2009). In 1981, ILAE classified different types of epileptic seizures:

- ✓ **Partial (focal) seizures:** A seizure is classified as a partial seizure when there is no alteration of cognition and the seizures manifestations are confined to a part of one cerebral hemisphere.
    - ✓ Simple partial seizures (without alteration of cognition, includes attention, memory, producing and understanding language, learning, reasoning, problem solving, and decision making): the patient remains conscious and is able to remember what happens. In this type of seizures, the patient may exhibit motor and somatosensory or special-sensory symptoms (simple hallucinations), or autonomic signs such as epigastric sensations, flushing, or sweating.
    - ✓ Complex partial seizures (with alteration of consciousness, including sentience, awareness, subjectivity, the ability to experience or to feel, wakefulness, having a sense of selfhood, and the executive control system of the mind): It may appear as a simple partial seizure followed by impairment of consciousness, or it is possible that the consciousness is lost at the seizure onset. The *aura* which is used frequently in description of epileptic seizures refers to the portion of the seizure occurring before the consciousness is impaired. Some partial seizures (simple or complex) may evolve to generalized seizures. This type of partial seizures is known as secondarily generalized.
- ✓ **Generalized seizures:** the absence seizures are characterized by an abrupt onset, interruption of the patient's current activity, and a blank stare. The consciousness is impaired during the seizure but regained quickly after the seizure. Generalized seizures are categorized in: absence seizures (typical and atypical absence), monoclinic seizures, clonic seizures, tonic seizures primary tonic-clonic seizures, tonic seizures. The most frequently experienced type of generalized epileptic seizures is tonic-clonic. This type of seizure may commence with a vague warning (*i.e.*, *aura*) in some patients, but most people lose consciousness without any premonitory signs.
- ✓ **Unclassified epileptic seizures:** If a seizure cannot be classified due to some reasons such as inadequate or incomplete data, it is labeled as an unclassified seizure. Epileptic seizures frequently evolve from one seizure type into another. That reflects the seizure spread with involvement of different brain areas.

Seizure activities can be generally divided into two categories based on the way they terminate (Engel 2006): self-limited epileptic seizures and SE. Indeed, when the natural mechanisms responsible for seizure termination fail, the seizure tends to continue indefinitely and status epilepticus occurs (Fisher et al. 2005; Engel 2006).

II.2.1 Animal model of epilepsy

Animal models for seizures and epilepsy have played a fundamental role in our understanding of the physiological and behavioral changes associated with human epilepsy. Today they remain an important goal in the development of new AEDs (Patsalos 2013; Aneja & Sharma 2013). In *vivo* animal models have been categorized into models of seizures and those of epilepsy. Many of the models are acute, and many are, in fact, models of status epilepticus which may have important differences from isolated seizures (Engel et al. 2013; Avanzini 1995). Major categories of models are those for simple partial seizures (Avanzini 1995; Fischer & Tetzlaff 2004): topical convulsants, acute electrical stimulation, cortically implanted metals, cryogenic injury; for complex partial seizures: kainic acid, tetanus toxin, injections into area tempesta, kindling, rodent hippocampal slice, isolated cell preparations, human neurosurgical tissue; for generalized tonic-clonic seizures: genetically seizure-prone strains of mouse, rat, gerbil and baboon, maximal electroshock seizures, systemic chemical convulsants, metabolic derangements; and for generalized absence seizures: thalamic stimulation, bilateral cortical foci, systemic penicillin, gamma-hydroxy-butyrate, intraventricular opiates, genetic rat models. The lithium-pilocarpine, homocysteine and rapid repetitive stimulation models are most useful in studies of status epilepticus. These animal models are important in discovering therapy that may prevent or modify the development of epilepsy after brain insults.

In particularly, for this work we choose the Kainic-acid (KA) animal model, because the injection of the excitotoxic agent (KA) in rats comprises the same three stages as seen in the mesial temporal lobe epilepsy (MTLE) *i.e.* initial insult, a latency period, and spontaneous motor seizures. MTLE is the most interesting epilepsy disease for non-pharamcological therapies due to its high incidence of poorly responsive forms. It specifically consists of atrophy and gliosis within the limbic system and is the most frequent cause of medically intractable epilepsy in adults (Quigg & Noachtar 2012). Hence, KA-injected rats mimic perfectly this SE and this is what we needed to understand the effect of our new tool of radiosurgery using X-ray generated microbeams

II.2.2 Diagnosis of epilepsy

Epilepsy is usually difficult to diagnose quickly because, first of all many other conditions, such as migraines and panic attacks, can cause similar symptoms; and because in most cases, it cannot be confirmed until there is more than one seizure. Diagnosing epilepsy is a multistep process, usually involving: i) neurological exam and supporting blood and other clinical tests that the patient has epileptic seizures and not some other type of episode such as fainting,

breath-holding (in children), transient ischemic attacks, hypoglycemia or non-epileptic seizures; ii) identification of the type of seizure involved; iii) determination of whether the seizure disorder falls within a recognized syndrome; *iv*) a clinical evaluation in search of the cause of the epilepsy; *v*) based on all previous findings, selection of the most appropriate therapy. Definitive diagnosis of epilepsy is often only made after an extended period of follow-up (Hauser & Kurland 1975; Sander et al. 1990). For a correct diagnosis the use of an EEG is important to detect unusual brain activity associated with epilepsy, but also a MRI scan, which can spot any defects in the structure of your brain.

II.2.2.1 Elettroencephalogram (EEG)

An EEG is a simple procedure that generally causes no pain or discomfort. Most of the time, it only takes about an hour, and the room is usually dark, quiet, and relaxing. EEG helps determine seizure type and epilepsy syndrome in patients with epilepsy, and thereby choice of antiepileptic medication and prediction of prognosis. There are several different types of EEG tests that can diagnose epilepsy, such as routine EEG (basic EEG test that is most often used to diagnose epilepsy and other neurological conditions); ambulatory EEG (may be recommended to capture more activity and give a better idea of when seizures occur); video EEG (it uses a regular EEG machine, but adds video monitoring); special electrode EEG (electrodes to be inserted in the skin instead of glued on, it or be inserted directly into the brain in via tiny holes that are drilled into the skull or in a surgical procedure).

II.2.2.2 Magnetic resonance imaging (MRI)

MRI is the diagnostic tool that identifies structural abnormalities in the brain. In epilepsy context, can help to classify both seizure type and syndrome. After the first seizure, MRI can be used to identify any serious disorder that may have provoked the seizure, such as a brain tumor or AMV. If MRI shows a structural lesion that is the likely source of the seizures, then they can be correctly classified as partial in nature. The classification often is crucial for the best treatment. Therefore, information from MRI is used to identify, for instance mesial temporal sclerosis (MTS) or cavernous angioma, which carries a favorable surgical prognosis, and malformations of cortical development, which have a less favorable prognosis for postoperative seizure control. Before surgery, MRI helps to identify the surgical approach and the volume of tissue to be removed.

II.2.3 Treatment of epilepsy

The common epilepsy treatments, medication and surgery, not only may have severe side effects but also fail to satisfactorily control seizures in ~20-40% of epileptic patients (Brodie & Kwan 2012). The treatment of epilepsy depends on several factors, including the frequency and the severity of the seizures and the person's age, overall health, and medical history. As reported in the introduction, when epilepsy patients do not respond to AED or other treatment options, focal cortical resection may provide relief from seizures. Using advanced brain imaging and EEG testing, neurologists can determine where in the brain a patient's seizures are originating (Schulze-Bonhage et al. 2010; Alarcon et al. 1995). The most common area of the brain for epilepsy surgery is the temporal lobe. Sometimes, it is necessary remove a specific brain structures such as hippocampus or amygdala. It is important to remember that the part of the brain causing seizures is already damaged and interfering with normal brain activity. Here, are listed the most common therapies to treat epilepsy disorders:

- resective therapy, means the surgical removal of epileptogenic focus;
- non-resective surgical therapies such as implantation of neuromodualtion devices (*i.e.* Vagus nerve stimulation -VNS- and deep brain stimulation -DBS-), callosotomy, and multisubpial transections (MST).

II.2.3.1 Resective surgical therapy

Resective surgery is a type of epilepsy operation in which the area of the brain responsible for seizures is surgically removed, without injuries their functions (Awad et al. 1991). The basic requirements for a surgical treatment are the exact diagnosis of epilepsy, such as pharmacoresistance, disability of the seizures, resectable focus, motivation of the patient, no progressive etiology (exception Rasmussen encephalitis), high probability that seizure freedom increases the quality of life. The goal of a resective surgical treatment depends on how good the epileptogenic zone can be identified and fully removed, without injuries of functional important cortex areas (Awad et al. 1991). The surgery approach is difficult, when foci are located in close vicinity to eloquent cortex, even when cortical mapping is used (Bauman et al. 2005). For patients resistant to drug treatment or who cannot benefit from resective surgery an emerging surgical option is the neuromodulation (Saillet et al. 2009; Romanelli et al. 2012). Neuromodulatory treatments include the *vagus nerve* stimulation (VNS), the deep brain stimulation (DBS), the callosotomy, the multiple subpial transections (MST) and the stereotactic radiosurgery (SST). The neuromodulation are less invasive surgical treatments; given their

importance in the present clinical practice, these methodologies will be described more in details in the following paragraphs.

II.2.3.2 *Vagus nerve* stimulation (VNS)

The *vagus nerve* is a part of the autonomic nervous system, which controls functions of the body that are not under voluntary control, such as heart rate and digestion. In the neck, the *vagus nerve* travels near the carotid artery and jugular vein. Interestingly, neurologists in the 19th century noted that applying pressure on the carotid artery in the neck, and thus the *vagus nerve*, could stop seizures. After having proven the seizure reduction in animals, today VNS is consider the most common neuromodulation procedure for epilepsy in humans. When the epilepsy is intractable to medications and many others have their seizures controlled at the expense of unacceptable adverse effects from pharmacotherapy.

VNS is a type of treatment for epilepsy that involves a stimulator (called a pulse generator) which is connected, inside the body, to the left *vagus nerve*. The device is programmed to deliver these signals (or "stimulate") at periodic intervals. Further reduction of seizure frequency was reported with long-term treatment, even after 1 year. However, longer follow-up did not confirm the initial impressive effect with less than 2% of patients becoming seizure-free after VNS (Mapstone 2008).

In addition, the profile of adverse effects can be considered minimal with postoperative infection occurring in 3-6% of patients, most of them treated with oral antibiotics and rarely requiring pulse generator or electrode removal. Relatively common side effects include dysphonia (20-30%) and cough (6%), but are usually transient and related to the intensity of stimulation (Ben-Menachem et al. 1999; Uthman et al. 2004). Sleep apnea or other clinically significant cardiopulmonary dysfunction is much rarer. It was proposed to perform VNS prior to callosotomy. After surgery, about 1/3 of patients have had the number of their seizures reduced by half or more; less than 5% of patients become seizure free; about 1/3 have shown benefit but have had their seizure frequency reduced by less than half; about 1/3 have had no worthwhile benefit (Spencer & Katz 1990).

II.2.3.3 Deep brain stimulation (DBS)

DBS for epilepsy is a more recent and evolving treatment approach. DBS has been considered as an alternative therapy for some patients with to date intractable epilepsy, since subcortical networks are assumed to control cortical excitability. To date, current data suggests that DBS in epilepsy can be carried out safely in several brain structures such as anterior or centromedian nucleus of the *thalamus*, subthalamic nucleus (STN), caudate nucleus, hippocampus, hypothalamus, cortex, and cerebellum, resulting in variable effects on seizures (Ellis & Stevens

2008). The STN is considered the principal target for DBS in movement disorders and has become widely adopted for this indication (Tabbal et al. 2007). STN has been proposed as a possible therapeutic target in refractory epilepsy, based on the knowledge that the inhibition of the excitatory effect of the *substantia nigra pars reticulata* (SNr) can reduce the firing of the γ-aminobutyric acid neurons in the deep layer of superior *colliculus*, i.e., the dorsal midbrain anticonvulsant zone (DMAZ) (Iadarola & Gale 1982). The first successful and effective STN-DBS for epilepsy was reported by Benabid and coworkers (Benabid et al. 2001) in a child with cortical dysplasia. Subsequently, other neurosurgeons have performed STN electrode implantation with overall seizure reduction ranging between 60% and 80% in refractory focal epilepsies (Saillet et al. 2009; Dinner et al. 2002; Vonck et al. 2003), and a lower efficacy (50% seizure reduction) in progressive myoclonic epilepsy (Vesper et al. 2007). However, so far only a limited number of patients have received STN-DBS and additional trials are required to assess its therapeutic potential and indications.

II.2.3.4 Callosotomy

The *corpus callosum* is a body of specialized nerve tissue that connects the left and right hemispheres of the brain. Information passes back and forth through the *corpus callosum*, allowing both hemispheres of the brain to "talk" with each other. The *corpus callosum* helps coordinate brain function in both hemispheres, it can also transmit abnormal electrical activity that can lead to seizures involving the entire brain. *Corpus* callosotomy, unlike other types of epilepsy surgery, does not remove the actual origination point of the seizures, it can greatly reduce their frequency and severity through severs cuts. These cuts interrupt the spread of seizures from hemisphere to hemisphere (Morrell 1989). Seizure frequency is reduced by an average of 70% to 80% after partial callosotomy and 80% to 90% after complete callosotomy. Partial seizures are often unchanged, but they may be improved or worsened. In many cases, especially after partial callosotomy, seizures are less frequent but persist (Asadi-Pooya et al. 2013; Park et al. 2013).

II.2.3.5 Multiple subpial transections (MST)

MST is a potential surgical treatment for patients with epileptogenic foci located in cortical areas with higher functions. It was conceived after the discoveries of the columnar organization of neocortex and that expression and spread of seizures utilize the transverse fiber network (Smith 1998). The rationale for MST is based on the concept that the cerebral cortex is functionally organized in vertically oriented columns of neurons and the transmission of electrical signals, both efferent and afferent, is mostly independent from the horizontal spread of seizure activity (Mountcastle 1957; Mountcastle 1997; Chervin et al. 1988). On the other hand,

pathological epileptic electrical activity follows a non uniform horizontal spatial pattern, in which the neuronal cell layer V is always involved, sometimes as an epileptic trigger (Telfeian & Connors 1998). Studies have shown the ability of MSTs to stop the propagation of epileptogenic activity and improve seizure control without inducing devastating neurological deficits (Morrell 1989; Mulligan et al. 2001; Orbach et al. 2001b). Several studies highlight the that independent epileptic regions can become synchronous when they are <5 mm apart, this fact provoked the initial attempt to transect the cortex at 5-mm intervals perpendicular to the long axis of the gyrus with the goal of limiting seizures while sparing functions (Engel et al. 2007; Dichter & Spencer 1969; Lueders et al. 1981; Goldensohn et al. 1977).

Indeed, surgery should avoid the disruption of the *pia matter* and lesioning sulcal vessels and this is achieved through the use of specially designed epilepsy knives (Mountcastle 1957). The number of transections are estimated pre-operatively and then refined based upon electrocorticography recordings during the procedure. MST provide a unique tool for the treatment of Landau-Kleffner syndrome in children, an epileptic disorder characterized by speech deterioration (Spencer et al. 2002). MST can help reduce or eliminate seizures arising from vital functional cortical areas. The side effect is that the epileptic activity may recur after a period of 2 to 20 months. It is uncertain whether this procedure can achieve long-term seizure control.

II.2.3.6 Stereotactic radiosurgery (SRS)

SRS has made the depths of the brain accessible to surgery and the use of radiation has made it possible to operate through the intact skull. It used directs highly focused beams of ionizing radiation to a defined target, within a well-immobilized patient and with a very rapid dose fall-off to surrounding normal tissues. It is an invasive treatment of benign and malignant tumors (Romanelli & Anschel 2006).

There are three basic types of radiosurgery, and each type uses different equipment and radiation sources. Cobalt 60 systems or Gamma-knife (Figure II-23A) use cobalt as a source for gamma rays, Linear accelerator (LINAC) systems (Figure II-23 B) use high-energy x-rays to treat a tumour or other lesions and Cyber-Knife (Figure II-23 C) uses x-rays to ensure the radiation beam is optimally directed to the patient throughout the treatment. The image-guided approach continually tracks, detects and corrects for any movement.

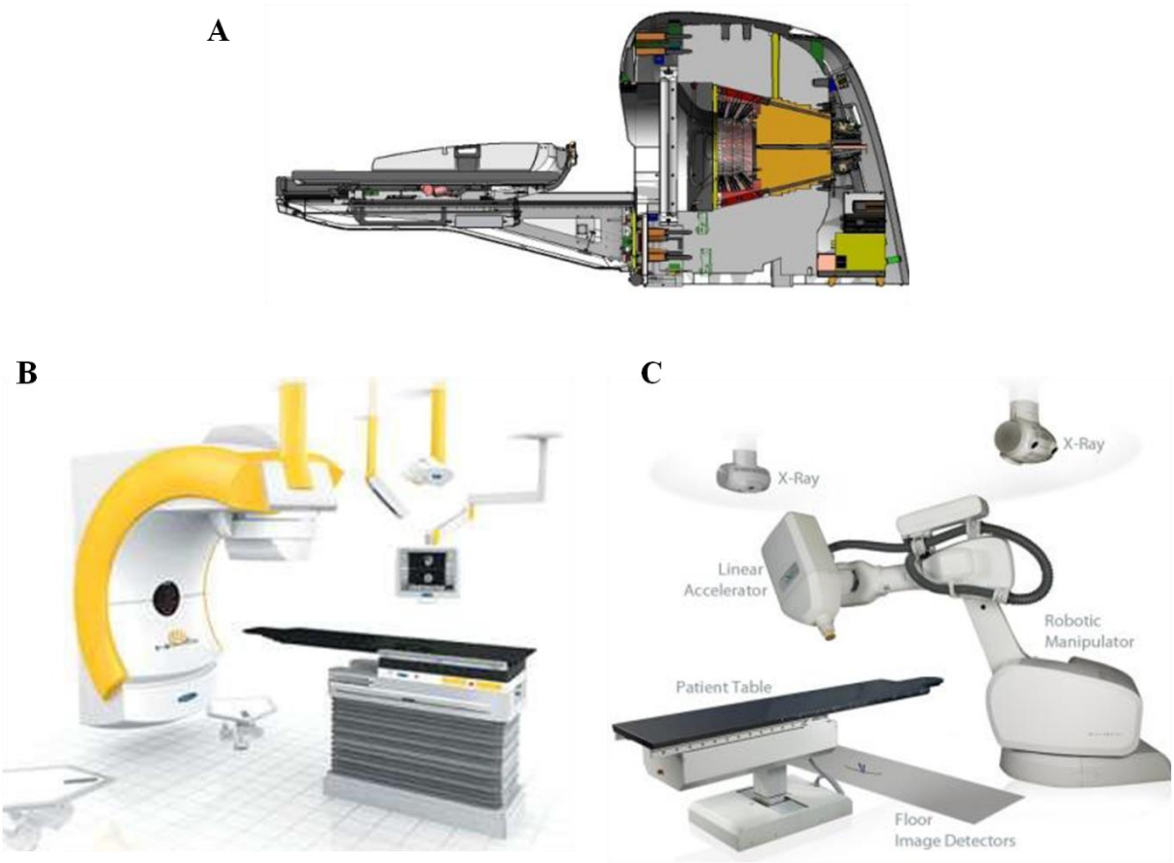


Figure II-23. Common radiosurgical modalities. A) Gamma-Knife (extracted from www.elekta.com), B) Linac based stereotaxy (extracted from www.radonc.ucla.edu) and C) Cyber Knife (extracted from www.accuracy.com).

General indications for radiosurgery include many kinds of brain lesions, such as acoustic neuromas, meningiomas, gliomas, metastases, trigeminal neuralgia, AVM and skull base tumors, among others. However, more recently, results have been published on SRS as the primary treatment of epilepsy secondary to mesial temporal sclerosis or hypothalamic hamartoma (Romanelli et al. 2008). SRS is also increasingly being considered with good safety profile for the treatment of hypothalamic hamar tomas (HHs), typically associated with pharmacologically intractable gelastic seizures and which often develop into complex partial and/or generalized tonic-clonic seizures. The choice of treatment must be individualized depending on the age and clinical circumstances of the patient and the size and anatomic relationships of the hamartoma (Rosenfeld & Feiz-Erfan 2007). Although it does not replace conventional surgery, SRS is a good option in selected cases such as highly functioning teenagers and adults where it is important to minimize memory deficit which has a higher chance of occurring following open surgery (Arita et al. 1998; Régis et al. 2004).

Procedure of administering stereotactic radiosurgery in patients

The patient's head is attached to a head ring called “stereotactic head” frame which ascertains an orientation in the reference framework with a co-ordinate system for target determination and for precise patient positioning. A noninvasive fixation device with a meshed thermo-transformable mask mood for each patient, or head immobilizers or markers used for localization with frameless equipment and aiding as co-ordinate systems placed on the bony skeleton of the patient, detectable by computer image detectors are used in frameless stereotactic technology. Real time online continuous updating of the patient's position during the treatment has made utilization of the stereotaxy based radiation to other parts of the body as well as in the spinal cord. A series of images with a specific reference coordinate attached to each is obtained by a CT, MRI, SPECT, and/or PET. Images are obtained with the head ring in place and are transferred with the underlying coordinate system to the computer workstation. With advancement in the 3D visualization of the images the virtual lesion is outlined by the surgeon using computer software to analyze the cross-sectional anatomy, determine the tumour area to be treated, and to note the critical structures adjacent to the lesion that should be avoided.

To define the treatment session a simulation for the dose delivery must be done. Simulation mimic the geometry of an isoelectric treatment machine, representation of the beam entry angle, beam trajectory, visualization of beam direction and treatment field with an understanding of the area of tissue at risk and tumour recurrence. Quality control and final verification is performed more so with LINAC based systems than with Gamma-knife on a target positioner such as stereotactic hardware with radiographic film, laser-guided systems or newer electronic detector systems and a trial run is then performed on the positioner or the phantom base before treating the patient. When everything is ready, the patient is positioned on the table and the planned radiation treatment is administered.

Advantages and disadvantages of SRS procedure

As explained before, SRS is an external radiation therapy that delivers precise, small and focused beams of radiation to a targeted area. The aim is to destroy the lesioned area or in case of a tumour, to control its growth without harming nearby healthy tissue. SRS involves the use of a stereotactic frame and high resolution imaging such as CT or MRI. The advantages of the SRS are attributed to the fact that is a non- or low-invasive technique. Since it includes shorter surgery times (shorter than an open brain surgery, reducing the probability of errors due to fatigue), shorter patient recovery times (the trauma is confined to the surrounding tissues, allowing a quicker recovery), less invasive respect to conventional surgery (no need for opening the skin or cutting the skull), ability to non-invasively reach deep brain tissues (high penetration of X-rays).

Also, SRS treatment has few disadvantages. Sometimes, the curative effects of SRS appear only months after the treatment; several irradiation sessions are necessary; there is the risk of neurological injury (if radiation is misaimed and damages healthy brain tissue or causes a bleed, there is risk of harm to the patient. These risks are less common in SRS than in open brain surgery); risk of mechanical inaccuracy, potential necessity of multiple visits (some tumors and AVM will take repeated brain surgery that could be causing severe side effects). Another example of the limitation of radiosurgery is given by the sensitivity of particular area such as the optic nerves and optic chiasm to radiosurgical doses. Most centers limit the dose to these structures to 8 Gy (compared with doses of 15 to 20 Gy to treat meningiomas and metastatic lesions, respectively). Because of these limitations, treatment of tumors adjacent to the optic chiasm such as meningiomas can be technically demanding, and treatment of tumors such as optic gliomas can be impossible. Another problem can occur during SRS is the radionecrosis. It refers to a combination of cytotoxic and microvascular tissue injury within the treated field as a consequence of the delivered radiation. The onset of injury may be delayed several months following treatment and usually persists for months. The injury may be asymptomatic or may mimic a severe stroke and require steroids, hyperbaric oxygen, or surgical decompression. The risk of radionecrosis increases with higher doses and with larger treatment volumes, and clinicians typically choose doses to minimize these risks (Flickinger et al. 2002; Shaw et al. 2000; Giller & Berger 2005; Gigante & Goodman 2012).

Looking to these disadvantages, synchrotron X-ray generated microbeams could be a new powerful tool for the radiosurgery. The application of microbeam radiation is limited to the trials, but its properties are linked to the lower impact on the non-targeted tissue, to the no retreatment of the CNS months or years after the initial treatment(s), the sharp dose falloff of the method could provide a more highly localized deposition of the dose, especially for small- and medium-sized targets.

Microbeam radiosurgery

At the ID17 Biomedical Beamline of the European Synchrotron Radiation Facility (ESRF) radiosurgery using Synchrotron X-ray beam could open a new tool to apply at the current radiosurgery technique to avoid the disadvantages occurred after conventional radiosurgery. Typically, the technique uses using high radiation doses delivered through near-parallel thin (25–75 μm) beams separated by a few hundred microns. This unique irradiation modality slows, and sometimes ablates, brain tumors in rodents (Laissue et al. 1998). In addition to its high

precision, the technique allows users to take advantage of the rapid regeneration of normal microvessels damaged in the direct paths of thin microbeams (Slatkin et al. 1995; Smilowitz et al. 2002; Blattmann et al. 2005; Serduc et al. 2006b). The preferential tumoricidal effects, or strong tumor palliation properties, are due, in part, to the lack of recovery of the tumor vasculaturel (Laissue et al. 1998; Dilmanian et al. 2003; Dilmanian et al. 2002), presumably because of structural differences between microvessels of tumor and those of the surrounding normal tissue (Denekamp et al. 1998).

II.3 Experimental outline

To evaluate whether microbeam cortical transections could be performed on eloquent cortex without induction of neurological damage in an animal model and to assess the efficacy of this approach on seizure control, we used normal rats and an animal model of epileptic seizures induced by local injections of kainic acid in the sensorimotor cortex. Microbeam cortical transections were generated in the left motor cortex of normal two month-old Wistar rats. We examined the tolerance of normal rats' motor cortex by histo/immunochemistry and motor performance by Rotarod® test. In KA-injected animal the verification of the duration of seizures after X-ray Synchrotron generated microbeams was assessed by VEEG. VEEG is a more sophisticated method of recording the EEG over long periods, which uses both EEG and video recording. For the cortical transections we used different beams geometries (minibeams and microbeams with a convergent or parallel conformation) delivered at different skin-entrance dose in the 150-600 Gy range. The results obtained provide a less invasive tool to effectively parcellize and disconnect the seizure focus; in fact it allows for very high dose deposition without inducing severe damages to normal brain tissue surrounding the irradiated field.

II.4 Materials and Methods

II.4.1 Animal preparation

The study was performed on forty-five adult Wistar male rats (250–270 g, Charles River Laboratories, L'Asbresle, France) maintained under controlled environmental conditions at a temperature of 22±2ºC, 40% humidity and a 12-hour light/dark cycle with food and water *ad libitum.* The local animal ethics committee had approved all the experiments. The care and use

of animals were in accordance with the 2010/63/UE directive. All efforts were made to minimize the pain, discomfort, and number of experimental animals.

II.4.2 Kainic-acid injected rats

Three male Wistar rats were implanted with intracerebral guide cannulas (Bilaney GmbH, Schirmerstr, Germany), under isoflurane (2%) anesthesia, in a Kopf stereotactic frame. The site of implantation was the left cerebral cortex (coordinates: 1.0 mm anterior to the bregma, 2.0 mm lateral to the midline, 1.0 mm ventral from the outer surface of dura mater (Paxinos & Watson 1986) (Figure II-26A). KA (20 µg/1 µl) was slowly injected by a microcannula into the left sensorimotor cortex. The cannula was left in place for 2 min before being withdrawn. Rats were allowed to recover in a large heated box for 1 hour and then were transferred to their home cages. Two hours after seizure induction, epileptic activity spread maximally, leading to SE.

Video EEG (VEEG) data was recorded immediately following KA injection using Grass Technologies Aura system (Grass Technologies, West Warwick Rhode Island) and a wire-commutator system (Plastics One, Roanoke, VA) allowing the rats to have relatively free movement. Between 2-4 hours after injection, VEEG recording was suspended while the rats underwent irradiation.

Kainic-acid model was chosen because is widely used to create epilepsy models, in which spontaneous recurrent seizures, hippocampal damage, and behavioural alterations develop after SE (*Epileptic status*) (Nørmark et al. 2011).

II.4.3 Irradiation campaign on normal rats

Male Wistar rats were anesthetized in a chamber circulated with a mix of air/isoflurane 5% for 5 min, then maintained with intraperitoneal injection of xylazine/ketamine (64.5/5.4 $mg.kg^{-1}$). Eleven rats were irradiated with parallel beams and other 12 with convergent beams.

II.4.3.1 Parallel cortical transections irradiation protocol

Each animal was placed vertical prone and fixed by ear bars and teeth on a custom-made Plexiglas stereotactic frame and placed on the Kappa type goniometer, by which the rat could be translated and rotated in front to the X-ray beam. The quasi-laminar beam obtained so far is collimated into an array of rectangular microbeams of variable size depending on the chosen collimator slits, which are placed about 42 m from the photon source and 1 m upstream from the head of the experimental animals. Details on the technique and of the related instrumentation are well described in the first chapter (I.3.3.1). In a first group, the sensorimotor cortex of rats

was transected by an array of 7 microbeams having a thickness of 100 µm, center-to-center (c-t-c) distance of 400 µm with a skin entrance dose of 360 Gy along the beam trace (peak dose) (Figure II-24).

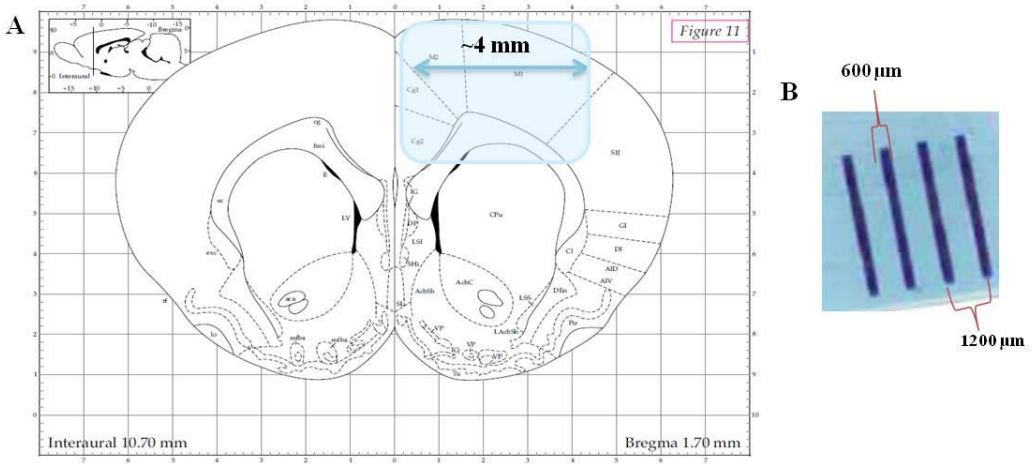


Figure II-24. Representative picture of the cortical irradiation field (4 mm high and 4 mm large) for 4 beams 600 µm-wide and 1200 µm center-to-center distance (A) and the relative Gafchromic® film irradiated (B). The brain sketches are extracted from Paxinos and Watson 1986)

Therefore, two groups underwent to 4 cortical transections made with 600 µm wide beams spaced by 1.2 mm and depositing a skin entrance dose of 150 or 240 Gy .In the two cases, the X-ray doses delivered to the tissue volume in the middle between the beams (valleys) were limited to 4 Gy and 6 Gy, respectively (which are known to be well tolerated) (Bräuer-Krisch et al. 2010). The irradiation coordinates were 4 mm on the antero-posterior direction (1 mm anterior to -3 mm posterior to the bregma) and, respectively, 2.5 mm and 4.2 mm on the lateral direction (starting 1 mm lateral to midline in the first case (Group 1) and from the midline itself in the second case (Group 2 and 3) (Figure II-2) (Paxinos & Watson 1986).

	Sham-irradiated	**Group 1**	**Group 2**	**Group 3**
No of animals	11	8	8	3
Peak dose (Gy)		360*	150*	240*
Beam width (µm)		100	600	600
Beam number		7	4	4
Center to center distance (µm)		400	1200	1200
PVDR		67.92	25	25

Table II-1. Irradiation parameters of the parallel beams geometry. The table shows three different configurations of irradiation; group 1 was irradiated with microbeams of 200 µm-width (in yellow highlighted) wherease groups 2 and 3 with thick-microbeams of 600 µm-width.*Peak dose measured (II.4.3.3).

The alignment of the target area was performed by in-situ X-ray image guidance (Figure II-25). The technical details about instrumentation and methods applied are described in the first chapter (I.2.2.1). Briefly, in our case the bregma was identified on the radiographs allowing positioning the target following the rat atlas (Paxinos & Watson 1986). Briefly, the dose was delivered by vertically moving the Kappa goniometer, at a speed that was inversely proportional to the dose to be delivered. The irradiation itself takes a fraction of a seconds; the overall procedure, including the human positioning, the X-ray imaging, the identification of the irradiation target, the remotely controlled swap of the setup from imaging to irradiation, and finally the irradiation take between 10 and 15 minutes.

Figure II-25. The control panel of the rat irradiation and imaging systems. After an optical prepositioning of the target performed through 3 remotely controlled video cameras (three upper panels), high resolution target determination is performed by X-ray imaging. A computer-guided robotic arm (Kappa goniometer, below the rat and not visible in the Figure) moves the stereotactic headframe to acquire radioscopic image necessary to individuate the bregma (radiographic image, bottom right) from which stereotactic coordinates allow the irradiation.

All movements were remotely controlled and irradiation values were decided by the operator before the treatment. The animal immobility during exposure was checked on 3 control screens located in the control hutch (Figure II-25).

II.4.3.2 Convergent cortical transections irradiation protocol

Each animal was positioned horizontally on the computer-controlled goniometer, by which the rat could be translated and rotated in front to the X-ray beam. Convergent cortical transections were induced using different dose protocols; peak-valley doses of 600 Gy and 400 Gy were delivered to rats irradiated with 25 µm and 60 µm wide microbeams respectively, whereas 330 Gy and 240 Gy were delivered to rats irradiated with 100 µm and 600 µm beams wide respectively (Figure II-26 and Figure II-24). The irradiation was performed in the antero-posterior (AP) direction on the left sensory motor cortex:

- 1st group: 4.0 mm lateral to bregma (starting from 1 mm) and 4.5 mm medial (starting from 1,5 mm lateral to midline);
- 2nd group: 4.0 mm lateral to bregma (starting from 1 mm) and 6.5 mm medial (starting from 1,5 mm lateral to midline);
- 3rd group: 4.0 mm lateral to bregma (starting from 1 mm) and 5.5 mm medial (starting from 1,5 mm lateral to midline);
- 4th group: 4.0 mm lateral to bregma (starting from 1 mm) and 6.0 mm medial (starting from 1.5 mm lateral to midline);

The uniformity and size of microbeams were checked by a radiochromic film, and the incoming spatially non-fractionated dose was measured using ionization chambers.

	Sham-irradiated	Group 1	Group 2	Group 3	Group 4
No of animals	3	3	3	3	3
Peak dose (Gy)		600*	400*	330*	240*
Beams width (µm)		25	60	100	600
Beam number		8	8	8	3
Center to center					
Max (µm)		200	400	100	1200
Min (µm)		400	800	200	2400
Angle bw beams					
I microbeam		0°	0°	0°	0°
II microbeam		6.31°	6.31°	6.31°	--
III microbeam		12.78°	12.78°	12.78°	--
IV microbeam		19.1°	19.1°	19.1°	19.1°
V microbeam		25.46°	25.46°	25.46°	--
VI microbeam		31.83°	31.83°	31.83°	--
VII microbeam		38.2°	38.2°	38.2°	38.2°
VIII microbeam		44.56°	44.56°	44.56°	--

Table II-2. Irradiation parameters of convergent beams geometry. Groups a, 2 and 3 were irradiated with microbeams of 25 to 100 µm-width, wherease group 4 (in pale blue highlighted) was irradiated with rhick-microbeam of 600 µm-width. The table shows 4 different types of irradiation performed on a 4 mm height cortical field *Peak dose measured (II.4.3.3).

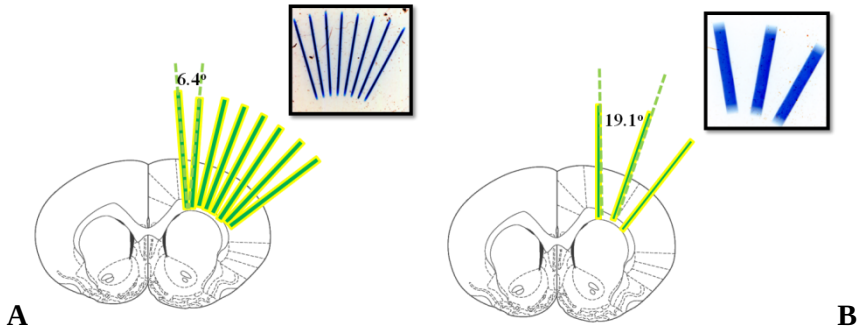


Figure II-26. Irradiation geometry of convergent cortical transections of A) 8 microbeams transecting the cortex belonging to groups 1, 2 and 3 (see Table II-2) and B) 3 minibeams transecting the same zone, irradiation belonging to group 4 (see Table II-2). Small squares on the top of each sketch shows a Gafchromic film.

II.4.3.3 Dosimetry calculation

Microbeam irradiation necessitates of an accurate calculation of the dose distribution function, which depends on several parameters, like the sample composition, the incident photon energy, the number, the shape and the dimension of microbeams as well as their separation.

The absolute dose was calculated starting from measurements performed in solid water phantom using a PTW pinpoint chamber (Freiburg, Germany). The reference dose is measured for an homogeneous field at 2 cm depth for a 2 cm x 2 cm field size. Measurements are performed by vertically scanning the PTW chamber in front of the beam at a known speed. The dose rate at the sample position is calculated then using the formula:

$$D_r\ [Gy * s^{-1} * mA^{-1}] = D\ [Gy] * h^{-1} [mm] * v^{-1}\ [mm * s^{-1}] * I^{-1}\ [mA]$$

where D_r is the instantaneous dose rate normalized on the current (I) circulating in the storage ring, D is the measured dose, h is the beam height, v is the scanning speed. To obtain the dose at 3 mm depth (more significant than the standard 2 cm depth in the case of small animals like rats) a correction factor must be applied (Clavel 2012).

Monte Carlo simulation

The dose distribution in the tissues was calculated using Monte Carlo methods, best accounting for the high variability in the X-ray absorption and scattering of the X-rays in the energy range used in MRT including the heterogeneity of the tissue composition and the spatial fractionation of the radiation. The Monte Carlo code PENELOPE 2006 (Salvat et al. 1999; Sempau et al.

2003) was used for these calculations. PENELOPE simulates the coupled transport of photons, electrons and positrons in the energy interval from 50 eV to 1 GeV, and in arbitrary material systems. PENELOPE has been widely used in MRT dose assessment (Siegbahn et al. 2009; Prezado et al. 2009; Martínez-Rovira et al. 2010). The simulation algorithm is based on a scattering model that combines numerical databases with analytical cross section models for the different interaction mechanisms and is applicable to energies (kinetic energies in the case of electrons and positrons) from a few hundred eV to 1 GeV. It uses a mixed simulation scheme in which hard interactions are simulated collision by collision and small angular deflections and energy losses are treated in a grouped manner.

In order to determine the dose in the peak and in the valley on the cortex, the simulations have been performed using a rat head phantom. The phantom was modelled by three concentric ellipsoids whose volumes were 13.00 cm^3 for the brain, 3.13 cm^3 for the skull, and 7.15 cm^3 for the skin; these values have been extracted from MRI images acquired on the rats of the same strain and weight; the skin thickness was 0.7 mm and the cranial bone thickness of 0.4 mm. Peak and valley dose calculations were performed for the different irradiation geometries used in the in-vivo experiments.

Peak-valley dose ratio (PVDR)

Since the biological processes underlying the microbeam effects depend on the shape of the dose distribution function, including its peak and "valley" – the dose between the microbeams – a realistic simulation of this distribution is necessary for treatment planning and evaluating the experimental results. A one-parameter characterization of this distribution is possible using the peak-to-valley dose ratio (PVDR), namely the ratio between the dose value at the center of the peak and the dose value in the region between two adjacent peaks (valley dose).

As formerly explained in the first Chapter, the valley dose is a very important parameter for the irradiation, it must not exceed a certain threshold dose to assure efficient tissue repair within intensely irradiated microslices. In order to calculate the valley dose, the different parameters like peak dose deposited, distance between the microbeams, thickness of each microbeam, number of microbeams, volume of the target zone, deepest of the tissue irradiated and the energy of the beam, need to be taken into account as well as realistic material composition. The valley dose of each microbeam adds to its "neighbour" and in this way the average of the valley dose increases.

The MC simulation was performed on parallel configurations and for the convergent beam case, the MC simulations were not repeated in the rat head phantom, but results were scaled for the different beam geometries using a simple water phantom. For this case, a maximum and

minimum PVDR was computed and the PVDR is decreasing from the top of the skull towards the motor cortex, thus the valley dose was increasing from the skull towards the deeper structures of the brain due to the tighter spacing between the microbeams.

Table II-3 summarize the different beam geometries used for all studies with the peak entrance dose values, the PVDR and the resulting valley dose calculated by MC simulation.

Geometry	Beam width (µm)	Beam spacing (µm)	# of beams	Peak dose * (Gy)	PVDR	Valley dose** (Gy)
Parallel	100	400	7	360	67.9	5.3
	600	1200	4	150	25.0	6
	600	1200	4	240	25.0	4
Convergent	25	Max: 200 Min: 400	8 8	600	136.3 351.5	1.7 4.4
	60	Max: 400 Min: 800	8 8	330	206.5 242.4	1.6 1.4
	100	Max: 400 Min: 800	8 8	330	90.9 120.7	3.6 2.7
	600	Max: 1200 Min: 2400	3 3	240	44.7 63.8	3.8 5.4

Table II-3. Irradiation parameters of parallel and convergent beams geometries. The table shows *peak dose measured and **valley dose calculated through MC simulation method (see text). The calculation of the valley dose is dependent on a number of factors such as the peak dose deposited, distance between the microbeams, thickness of each microbeam, number of microbeams, the volume of the target zone, the deepest of the tissue irraited and the energy of the beam. The valley dose rises with the size of the radiation field and therefore for the number of microbeam. The errors bar in the calculation of PVDR should be around 15%.

II.4.3.4 Evaluation of neuronal deficit

After irradiation, rats belonging to the groups irradiated with a skin entrance dose of 360 Gy and 150 Gy were placed in large cages hosting two animals and observed daily for the week following the irradiation and then weekly. The evolution of their body weight was noted each

month and neurological observations (in particular to verify the potential presence of signs of contralateral hemiparesis) were performed once a week.

II.4.3.5 Rotarod® test

Motor behavior was assessed by the Rotarod® (Panlab/Harvard Apparatus) in order to evaluate motor coordination after sensorimotor cortex irradiation. The Rotarod® test, in which rodents walk on a rotating cylinder as presented in Figure II-27. Adaptation to the Rotarod® commenced the same day of the test. Animals were first habituated to low rotation (4 rotations per minute -RPM-) for 30s, after which they were allowed 1 min of rest. Sham-irradiated and irradiated rats were submitted to three trials per day, once per month for 6 months after irradiation, of 4 to 40 RPM acceleration during 5 min, with 10 min resting interval between them. The time spent on the rotarod and the number of RPM at which the animal was able to maintain balance in the moving wheel were measured. The average performance was compared with the pre-irradiation values.

Figure II-27. The rotarod test device used in our experiment. It is based on a rotating rod with forced motor activity being applied by the rat. Supplier: Panlab/Harvard, Les Ulis, France.

II.4.3.6 Immunohistochemistry analysis

Tissues collection

Two days after irradiation 1 naïve Wistar male rat for parallel MBs transections and 1 for convergent MBs transections were randomly chosen and sacrificed with Dolethal® overdose. The brain was rapidly excised, frozen in pre-cooled isopentane at −50 ˚C and stored at −80 ˚C.

Fifteeen micron coronal brain sections were obtained at −20 °C using a cryotome (Microm HM560, France). The slices were mounted onto Superfrost® Plus glass slides (Fisher Scientific, Pittsburgh, PA).

All other animals were sacrificed at 3 and 6 months after transections by intraperitoneal injection of Doléthal®. Brains were dissected out and immediately fixed in a solution composed of ethyl alcohol (60%), acetic acid (10%), and chloroform (30%). Twenty hours later, brains were placed in 70% ethanol until they were included in paraffin. Ten micrometers of serial sections were cut through a microtome (RM2245 semiautomatic Leica, Milan, Italy) and used for immunohistochemical analysis.

Histology: NISSL staining

Nissl is a term used by classical cytologists for the endoplasmic reticulum (ER). Since all cells contain ER, cresyl violet will stain both neurons and glia. We distinguished neurons from glial cells in all subregions of hippocampus. Neurons has centrally located nucleolus, distinctive nucleus, visible cytoplasm, presence of dendritic processes and longer cell body size; while glial cells have heterochromatine clumps, sparse cytoplasm and smaller cell body size. Tissue slices were stained for 8 minutes with 0.1% thionin. Thereafter, they were dehydrated in alcohol series, cleared in toluene, and coverslipped with Pertex (Medite, Burgdorf, Germany).

Immunohistology: pH2AX, GFAP and NeuN labeling

Immunohistochemistry is a sensitive method and an important tool for determining cell distribution and morphology. The method is based on the specific binding of the primary antibody to an antigen on the tissue/cell. The outcome and quality of the binding is influenced by factors such as the fixation and the specificity of the antibody for example. Non-specific binding of secondary antibodies can be detected by incubating some sections without adding primary antibodies. The damage caused by irradiation in the brain was identified using immunohistological markers for astrocytes (GFAP) and neurons (NeuN). Additionally, DNA double strand breaks caused by different irradiations was identified by the pH2AX marker.

pH2AX as a marker that stain DNA double strand breaks. Detection of this phosphorylation event has emerged as a highly specific and sensitive molecular marker for monitoring DNA damage produced by ionizing radiation and a variety of genotoxic drugs (Bouquet et al. 2006; Paull et al. 2000; Pilch et al. 2003). Therefore, the rate of loss of foci and the presence of residual foci has been correlated with cellular radiosensitivity (Taneja et al. 2004; MacPhail et al. 2003). The nucleosome, the fundamental unit of chromatin organization in eukaryotes, consists of 200 bp of DNA wrapped around an octamer of four pairs of histones H2A, H2B, H3,

and H4 (Wolff 1998). The histone globular domains comprise the nucleosome core, whereas the histone tails protrude, providing potential sites for protein modification such as acetylation, methylation, and phosphorylation. These histone tail modifications alter DNA accessibility and nucleosome dynamics (Fischle et al. 2003), thereby affecting regulation of gene expression (Eberharter & Becker 2002), DNA repair (Mahaney et al. 2009), and other nuclear events. Histone phosphorylation is rapid and extensive, covering large regions of the chromosome adjacent to each break and producing foci that can be visualized microscopically after antibody labeling (Limoli et al. 2002; Liu et al. 2003). IR exposure induces rapid phosphorylation of aminoacid residue (Ser139) to form foci of H2AX immunoreactivity at DNA double strand break sites (Paull et al. 2000; Pilch et al. 2003). Foci continue to grow for about 1 hour after exposure to X-rays and then disappear slowly over time, consistent with rejoining of DNA breaks but with slower kinetics. Phosphorylation of H2AX (pH2AX) is proposed to concentrate repair factors at sites of DNA damage (Celeste et al. 2003; Bassing et al. 2003). In fact, absence of H2AX is associated with genomic instability and radiation sensitivity (Hudson et al. 2011).

Therefore, we performed immunohistochemistry analysis using glial fibrillary acidic protein (GFAP), which is a marker for astrocytes. A series of studies indicates that astrocytes influence in particular synaptic activity. This due to the fact that they are key cells in the central nervous system (CNS) involved in the maintenance of the extracellular environment and in the stabilization of cell-cell communications under physiological and pathological conditions (Wang & Bordey 2008; Nedergaard et al. 2003; Parpura et al. 2012; Hanisch & Kettenmann 2007). Recent studies have provided compelling evidences that reactive gliosis can exert both beneficial and harmful effects on tissue repair (Sofroniew 2009; Silver & Miller 2004; Faulkner et al. 2004). On one hand, those reactive astrocytes would form physical barrier that isolates the lesion site from normal brain tissue, and help the restoring of blood brain barrier (Faulkner et al. 2004). On the other hand, the physical barrier and the extracellular microenvironment, those reactive astrocytes help to maintain, would inhibit neurite outgrowth and neural regeneration (Goldshmit et al. 2004).

NeuN (Neuronal Nuclei) has been identify as a neuron-specific nuclear protein (Mullen et al. 1992). It was found expressed widely in the adult nervous system of multiple species, including humans(Mullen et al. 1992; Wolf et al. 1996; Sarnat et al. 1998). Intriguingly, these previous studies also documented that expression of NeuN-immunoreactivity is regulated developmentally and is found only in postmitotic neurons. NeuN-antibody does not stain the immature nerve cells until they exit from the cell cycle and achieve a stage of development that at least approaches mature function. This is coincident with the migration of the neuroblasts from their birthplace in the embryonic neural tube to their fifro position, outgrowth of axons and generation of synapses (Sarnat et al. 1998; Molliver & Kristt 1975; Weyer & Schilling 2003; Wolf et al. 1996).

- Fluorescence immunohistochemistry for pH2AX: sections were fixed with PFA 4% for 15 min and blocked with donkey normal serum (DNS, Interchim) diluted in 10 mM phosphate buffered saline (PBS) for one hour (10 mM PBS/DNS 5%). The primary antibody used was pH2AX (1/500, 05636, Upstate) diluted in PBS/NDS 1%. Sections were washed 4 times with 10 mM PBS, then incubated with the secondary antibodies Alexa Fluor-conjugated donkey F(ab9)2 (1:200, Invitrogen, Carlsbad, CA, USA) for 2 hours at room temperature. Finally, the tissue was rinsed three times in 10 mM PBS and the sections were then mounted on glass slides and coverslipped with DAPI mounting medium (Vectar Shield, Laboratories).

- Brightfield immunohistochemestry for GFAP and NeuN: endogenous peroxidase activity was blocked with H_2O_2. Unspecific binding was blocked and the sections were permeabilized. Primary antibodies against glial cells (GFAP, 1/1000, Sigma-Aldricht) and mature neurons (NeuN, 1/100, Chemicon) were used. Thereafter the sections were incubated with biotinylated conjugated secondary antibodies followed by avidin-biotinperoxidase complex. The peroxidase was detected by 3, 3´-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldricht) solution in the presence of H_2O_2. Rinsing in water stopped the reaction and the sections were dehydrated and mounted. For both immunohistochemistry, negative controls were performed by omitting the primary antibodies and applying the secondary antibodies alone.

II.4.3.7 Microscopy

The sections were viewed by bright field microscopy and images were captured with the optical microscope Olympus BX51, equipped for epifluorescence and objectives X10, X20, X40. The slices were photographed with a video camera connected to the microscope. The images were then processed by the software Cell^B (Olympus microscope, Life science instrument).

II.4.3.8 Statistical analysis

All statistical analyses were performed using the GraphPad Prism software program (version 3.0, GraphPad Software, San Diego, CA, USA). Values reported are means ± SEM (number of observations). The level of significance was set at $p<0.05$ or $p<0.001$.

I. The weight gained by each irradiated and not-irradiated group during six months observation period were analyzed using Two-way ANOVA followed by Bonferroni multiple comparison test.

II. Rotarod performances were assessed with one-way analysis of variance (ANOVA). We evaluated the differences between each irradiated-group vs sham-irradiated one

during 6 months observation period, followed by Bonferroni multiple comparison test.

II.4.4 Irradiation campaign on KA-injected rats

After KA injection, rats were observed for signs of seizures. Seizures were classified as non-convulsive limbic seizures (staring, automatisms) and convulsive seizures (unilateral forelimb clonus, bilateral forelimb clonus, generalized tonic-clonic seizures). After KA injection continuous observation was performed by observers in alternating blocks of 8 hours during the first 24 hours. During the second and third day time, continuous observation was done in blocks of 6 hours. Then the rats underwent observation for 1 hour once a day for 4 days and, after the first week, weekly observations of 1 hour. The period between the first and last observed convulsive seizure was annotated. Then, epileptic animal models were irradiated. The preparation of animals and the alignment of the target area for irradiation is the same followed for the first irradiation campaign (II.4.3.1) using parallel transection on Wistar normal rat brain. These rats were divided into two groups: sham-irradiated rats (n= 6) and rats receiving microbeam transections (n= 12). Using the same irradiation geometry previously described for normal rats, parallel transections (II.4.3.1) were induced using either 100 µm wide (n= 6) and 600 µm wide (n= 6) beams (Figure II-28). A high dose (HD) and a low dose (LD) protocol were tested for both microbeam groups: peak valley doses of 360-6 Gy (HD) versus 240-4 (LD) were delivered to rats irradiated with 100 µm wide microbeams, whereas valley doses of 150-6 Gy (HD) and 100-4 Gy (LD) or 360-6 Gy (HD) versus 150-5 Gy (LD) were delivered to rats irradiated with 600 µm wide beams (Table II-4; Figure II-28). Dosimetric calculations were performed by using MC simulations as described for normal rats, in the first campaign.

	Sham-irradiated	**Group 1**	**Group 2**	**Group 3**	**Group 4**
No of animals	6	3	3	3	3
Peak dose (Gy)	--	360*	150 *	240*	100 *
Valley dose (Gy)	--	6**	6**	4**	4**
Beam width (µm)	--	100	600	100	600
Beam number	--	7	4	7	4
C-t-C distance (µm)	--	400	1200	400	1200

Table II-4 Irradiation parameters of parallel beams geometry in KA-animal model. Groups 1 and 3 (in yellow highlighted) were irradiated through microbeams of 100 µm-width; whereas groups 2 and 4 with thick-microbeams of 600 µm-width. *Peak dose measured and **valley dose calculated through Monte Carlo simulation method (the MC method is explained in details in the paragraph II.4.3.3).

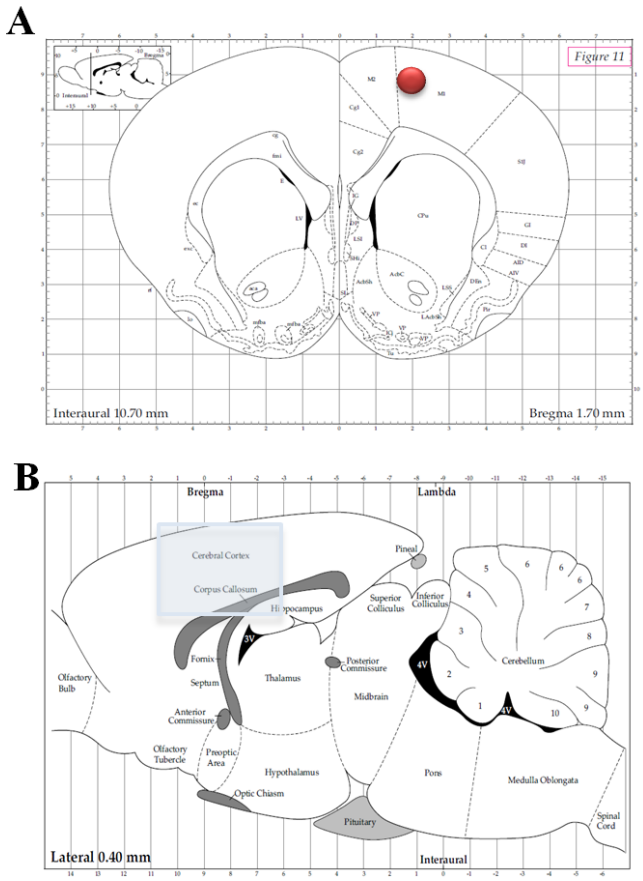


Figure II-28. Schematic representation of microbeam irradiation geometry in rats injected with KA**.** Coronal (A) and sagittal (B) section of the Paxinos and Watson's rat brain atlas as demonstration. In A is indicated the point (red ball) where the kainic acid injection was performed to induce the epileptic status (a seizure that lasts longer than 30 minutes) and in B a sagittal view of the irradiated region. Images modified from *Paxinos and Watson* (Paxinos & Watson 1986).

II.4.4.1 Video recording: VEEG

The presence of convulsive seizures was revealed by electrocorticography; this registration was not performed during irradiation, given the possibility of dosimetric distortions induced by the interaction of X-ray beams with the EEG electrodes in the brain, and the lack of metal-free electrodes at the time of the irradiation.

II.4.4.2 Evaluation of seizure duration

To mimic epileptic symptoms we used in this study a rat model of Kainic Acid (KA)-induced convulsive epilepsy. KA is an exogenous agonist of KA ionotropic glutamate receptors which induces severe convulsive seizures following local injection into the sensorimotor cortex (Hashizume et al., 1998). All rats (n=18) injected with KA developed convulsive seizures

(forelimb controlateral or bilateral jerks) within 26±12 min, followed by generalized tonic-clonic seizures and status epilepticus (within 2 hours). Rats were then divided into two groups: control rats (n= 6) and rats receiving microbeam transections (n= 12). Using the same irradiation geometry previously described for normal rats, transections were induced using either 100 mm wide (n= 6) and 600 mm wide (n= 6) beams. A high dose (HD) and a low dose (LD) protocol were tested for both microbeam groups: peak-valley doses of 360-6 Gy (HD) versus 240-4 Gy (LD) were delivered to rats irradiated with 100 mm wide microbeams, whereas peak-valley doses of 150-6 Gy (HD) and 100-4 Gy (LD) were delivered to rats irradiated with 600 mm wide beams.

II.4.4.3 Post-irradiation behavior in kainic acid-injected rats

Rats were observed for signs of seizures. Seizures were classified as non-convulsive limbic seizures (staring, automatisms) and convulsive seizures (unilateral forelimb clonus, bilateral forelimb clonus, generalized tonic-clonic seizures). After KA injection continuous observation was performed by 3 skilled observers in alternating blocks of 8 hours during the first 24 hours. During the second and third day time, continuous observation was done in blocks of 6 hours. Then the rats underwent observation for 1 hour once a day for 4 days and, after the first week, weekly observations of 1 hour. The period between the first and last observed convulsive seizure was annotated.

II.4.4.4 Statistical analysis

The statistical significance of duration of seizures between KA-injected irradiated and KA-injected not irradiated rats was measured with One-Way ANOVA using Bonferroni's post hoc test.

II.5 Results

II.5.1 Assessment of rats' body weight

Body weight of both, normal rats and KA-injected rats were observed for a period of 6 months and their body weight was recorded as normal. Figure II-29 shows the weight gained by normal Wistar rats before and after parallel or convergent beams irradiation during 6 months observation period. At 60 days after parallel microbeams irradiations, rats irradiated with an entrance dose of 150 Gy showed a transient reduction of body weight. One-hundred-fifty and

180 days after parallel microbeams transections, rats irradiated with 360 Gy showed a reduction in weight gain compared to sham-irradiated group ($p<0.05$).

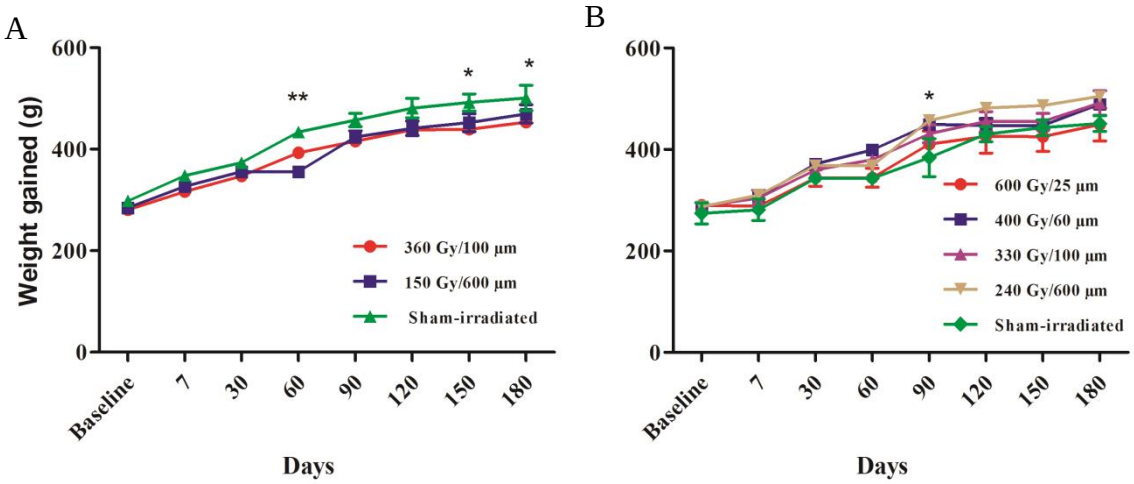


Figure II-29. Weight curves for normal animals before and after irradiation. The plots represent the weight gained during 6 months observation period. A) Rats irradiated with parallel microbeams and B) rats irradiated with convergent microbeams. The irradiation doses in the legend are given as peak dose. Data are means ± S.E.M. (n=4/parallel irradiated group and n=4 sham-irradiated; n=3/convergent group and n=4 sham-irradiated). Two-Way ANOVA plus Bonferroni. The level of significance is *$p<0.05$ and **$p<0.001$.

II.5.2 Parallel and convergent cortical transections and motor behaviour

Rotarod test in normal adult Wistar rats was performed after 7 days and then monthly for 6 months. Parallel or convergent transections of the sensory motor cortex did not cause severe motor impairments, neither paralysis nor minor paralysis, all animals were able to run on the rod. Rats irradiated with parallel cortical transections (Figure II-30A) showed that there are no significative differences between irradiated and sham-irradiated groups and the performance' decrease is due to their age, probably. Convergent cortical transections of microbeams delivered at 400 Gy and and minibeams delivered at 240 Gy affected significantly ($p<0.05$) the motor

performance on the rod compared to sham-irradiated rats (

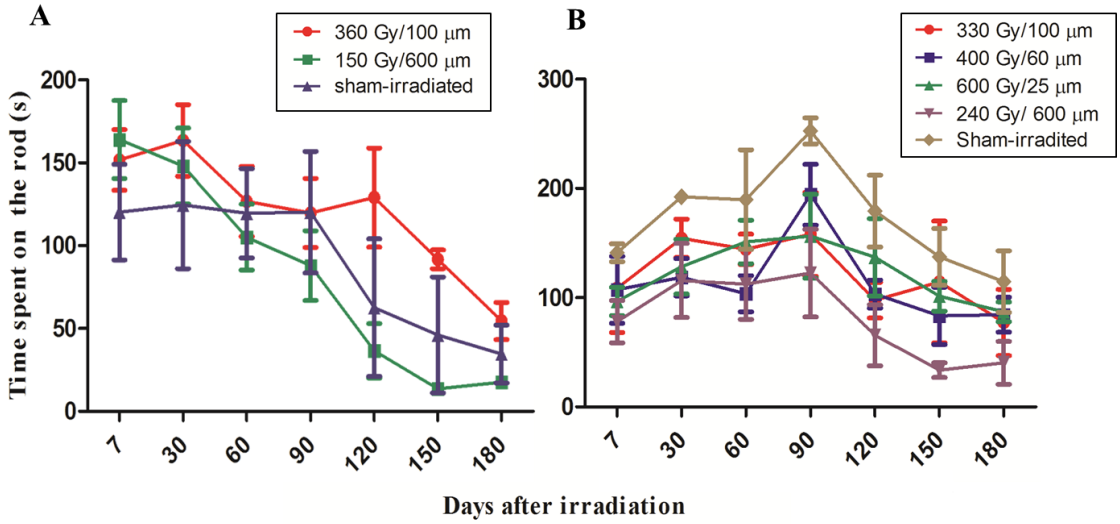


Figure II-30 B).

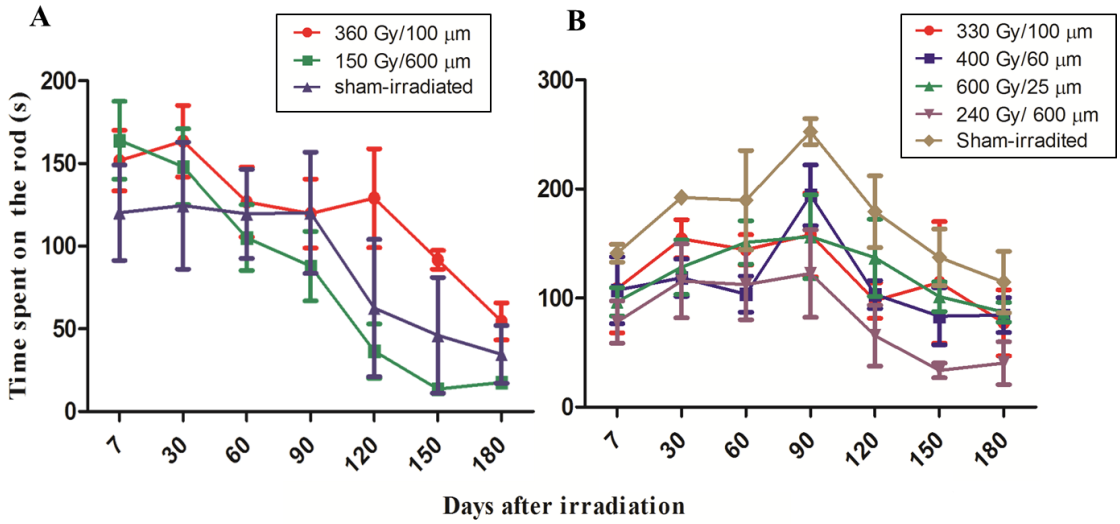


Figure II-30. Duration of performance on the rotarod over the 6 months observation period after parallel (A) and convergent (B) cortical microbeam (≥ 25 µm) or minibeams (≥ 500 µm) transections. One-way analysis followed by Bonferroni multiple comparison tests, irradiated groups vs sham-irradiated group. A) No significative differences between parallel irradiated groups vs sham-irradited; B) signifciative differences ($p<0.005$) between 400 Gy/60 µm or 240 Gy/600 µm *vs* sham-irradiated group

II.5.3 Parallel cortical transections and seizure duration

Seizure duration was significantly reduced in rats undergoing transections as compared to control rats (Figyre II-31) ($p<0.05$). In control non-irradiated rats convulsive seizures disappeared after 40±12 hours. In the group receiving 100 µm transections, convulsive seizures disappeared after an average of 3.0±0.5 hours and 3.3±0.5 hours in the HD and LD protocols,

respectively (n= 3 for each group). A similar result was observed in the group of rats treated with 600 mm beams, with seizures disappearing after an average of 2.2±0.2 hours and 2.6±0.5 hours in the HD and LD protocols, respectively (n= 3 for each group). The differences among the irradiation groups were not statistically significant.

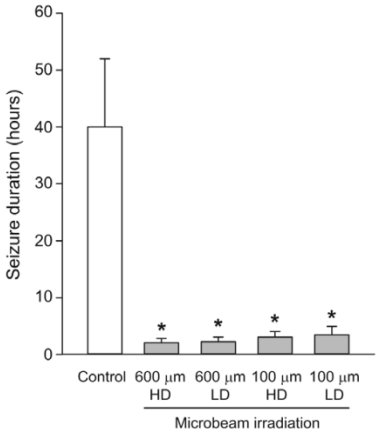


Figure II-31. Microbeams irradiations induce a reduction of seizure duration. Duration of convulsive seizures was significantly reduced in rats undergoing transections either with 100 mm wide (n = 6) and 600 mm wide (n = 6) microbeams. A high dose (HD) and a low dose (LD) protocol were tested for both microbeam groups (n = 3 for each of the four irradiated groups and n = 6 for the non-irradiated control group). *p<0.05 (One-way ANOVA followed by Bonferroni's test) vs. non irradiated rats (Control). The differences among the irradiation groups were not statistically significant.

II.5.4 Microscopic observations

II.5.4.1 Short term effects (2 hours) of convergent and parallel microbeams transections

The effects of parallel and convergent cortical transections were verified immediately after irradiation with immunohistochemistry using pH2AX. In Figure II-32 microbeams paths and signs of radiation induced-DNA damage were evident in pH2AX immuno-labelled sections; ionizing radiation induces DNA double strand breaks which were linearly correlated with the number of pH2AX foci within cell nuclei (Bouquet et al. 2006).

Three months after irradiation, cellular details and the paths of the MBs were identifiable on sections stained with thionin through NISSL staining.

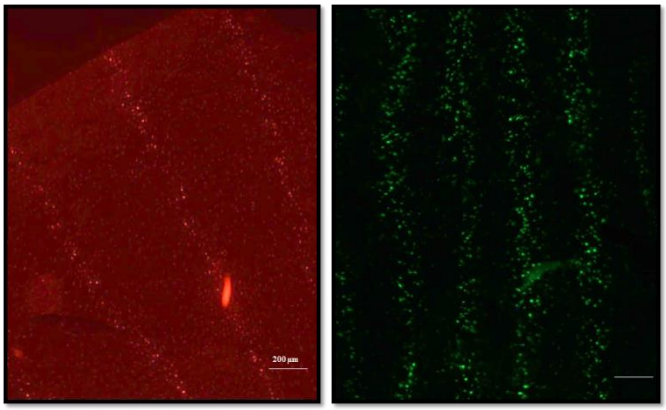


Figure II-32. Immunohistochemistry of pH2AX (red or green) in the cortex of convergent microbeams (detail). On the left, convergent transections, and on the right parallel. Scale bar: 200 µm.

II.5.4.2 Long term microbeams tolerance analysis (3 and 6 months)

Figure II-11 A-D shows clear microbeam cortical transections in brain tissue of normal rats (7 microbeams, each one with a thickness of 100 µm and spaced by 400 µm delivered at 360 Gy skin-entrance dose) assessed by NISSL staining. Figure II-11 E-H shows "thicker" microbeams (or minibeam) transection in normal rats cortex. Along the beam path (not really clear if compare with the previous beams' geometry described) there are apoptotic cells and macrophages.

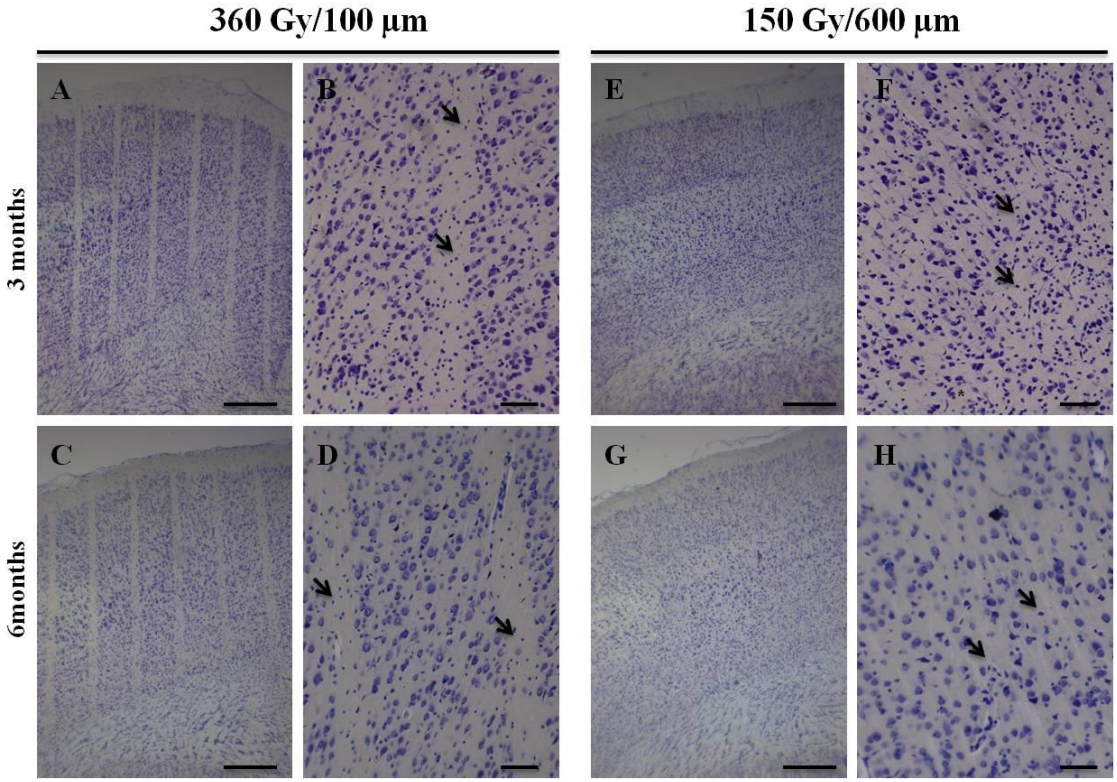
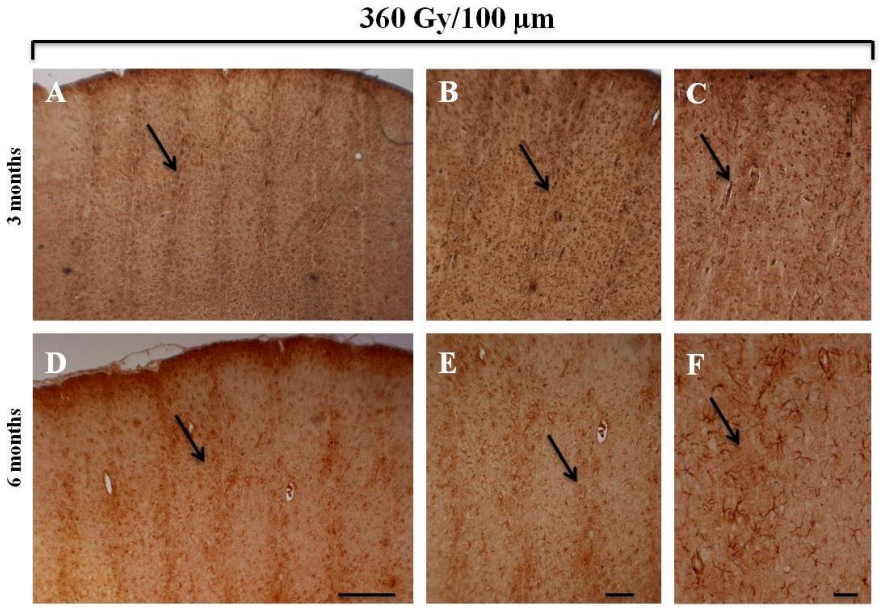


Figure II-33. NISSL staining of sensory motor cortex after 3 and 6 months from irradiation. Normal Wistar adult male rats, were irradiated with A-D) a peak dose of 360 Gy/valley dose of 5.3 Gy, ctc 400 µm and E-H) with a peak dose of 150 Gy/valley dose of 6 Gy, ctc 1200 µm. B,D,F,H represent a higher magnification of A,C,E,G. Black arrows indicate the pycnotic nuclei. Scale bars: 500 µm for A, C, E, G and 100 µm for B, D, F, H.

II.5.4.3 Medium (3 months) and long term (6 months) effects on astrocytes

Immuno-histochemistry GFAP staining showed astrocyte activity after 3 months and 6 months in both irradiated groups (Figure II-12). Staining was found to be higher at 3 months later. GFAP-positive cells after 3 months from irradiation were found to be sparse in the irradiated area. They showed a richer cytoplasm, larger cell size, and dark cytoplasmic staining (Figure II-12 A-C and G-I). In contrast, 6 months after irradiation, the astrocyte in the irradiation target showed a star-ray appearance, with light staining of the cytoplasm, few branches, and long, thin processes (Figure II-12 D-F D-F and L-N).



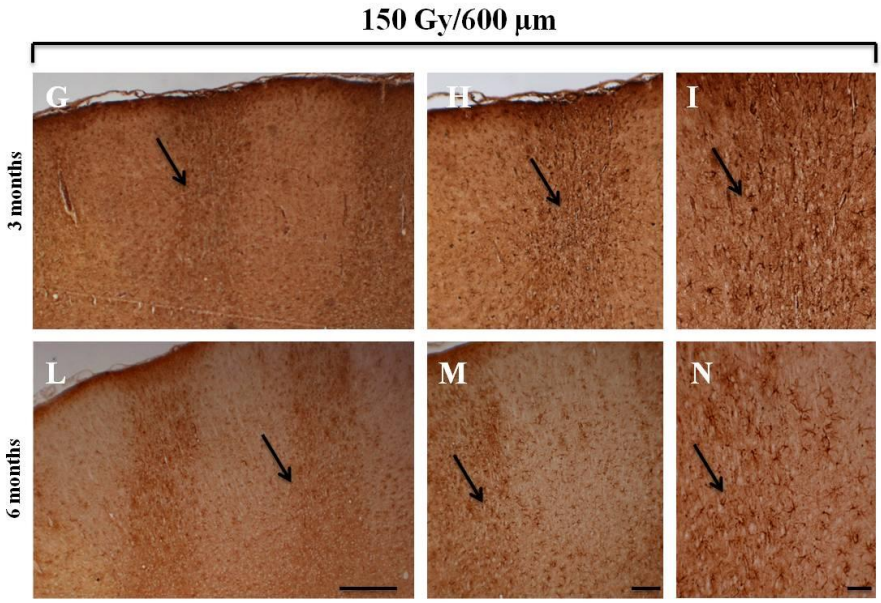


Figure II-34. GFAP staining in cortical sensorimotor cortex after irradiation at 360 Gy. A-C and G-I) After 3 months from irradiation the astrocytes showed a growth pattern with abundant cytoplasm. D-F and L-N). After 7 months the astrocytes showed a star-ray appearance with a light staining of cytoplasm. Black arrows identify the microbeam path. Scale bars are for A, D,G and L of 500 µm, for B, E, H and M of 200 µm, for C, F, I and N of 100 µm.

II.5.4.4 Medium term effects on neurons

Extensive neuronal degeneration was found along microbeam paths (100 µm-width, skin entrance dose of 360 Gy and a valley dose of 5.3 Gy) 3 months after irradiation (

Figure II-35 A-C). At the same time point in the ”thicker” microbeam paths (600 µm-width, skin entrance dose of 150 Gy and a valley dose of 6 Gy) we observed a less extensive degeneration of cells (Figure II-3 D-F).

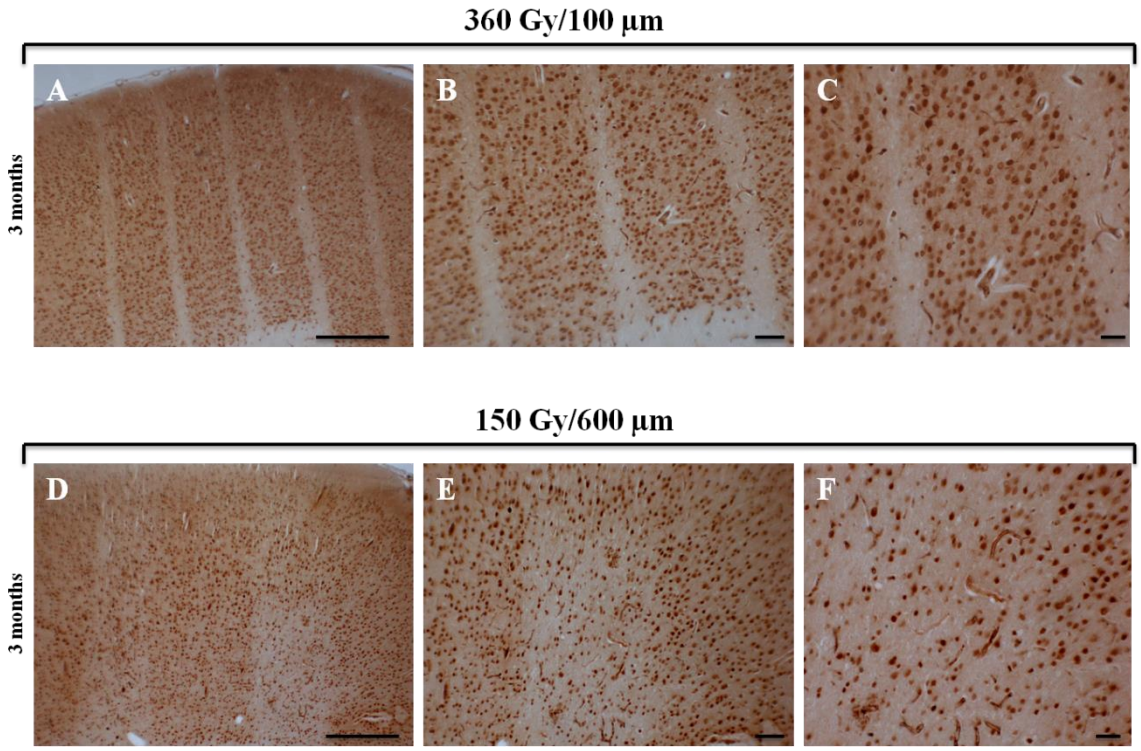


Figure II-35. Neuronal loss assessed by expression of the neuronal protein NeuN in coronal slices of sensorimotor cortex after 3 months from irradiation. A-C) Photomicrographs of the region microbeam-irradiated at 360 Gy as peak dose and D-F) target region at 150 Gy dose. Scale bars are for A, D, 500 µm, for B, E, 200 µm, for C, F, 100 µm.

II.6 Discussion

Image-guided microbeam radiosurgery is an emerging tool to generate cortical transections or to induce precise focal brain lesions. The Central Nervous System (CNS) radiobiology of microplanar beams was first studied at the National Synchrotron Light Source (NSLS) of the Brookhaven National Laboratory (BNL), where the preservation of CNS architecture after incredibly high radiation doses (up to 4000 Gy) delivered to mouse brain by 25 mm wide beams was first described (Zeman et al. 1961; Curtis 1967b; Curtis 1967a). Further work at BNL and the ESRF, investigated the tissue tolerance to microscopic beams at doses tens to hundreds time larger than those allowed by conventional macroscopic beams (Slatkin et al. 1995; Laissue et al. 1998; Dilmanian et al. 2006). Unidirectional irradiation using microbeam arrays has confirmed through several experiments the exceptional resistance of the normal-tissue to high dose microbeam irradiation (Dilmanian et al. 2006; Dilmanian et al. 2002; Dilmanian et al. 2005; Dilmanian et al. 2003; Miura et al. 2006; Zhong et al. 2003; Dilmanian et al. 2007; Blattmann et al. 2005). This phenomenon is referred to as “tissue-sparing effect”, indicating the preservation of the architecture of the tissues traversed by microbeams carrying doses tens to hundreds times higher than those associated with radionecrosis after conventional radiotherapy.

The doses to regenerative normal cells and tissues between microbeams, the ‘‘valley’’ doses, are probably the most important determinants of normal tissue damage in MRT (Romanelli & Bravin 2011). For a given target, the valley dose depends on three main parameters: the microbeam width, the spacing between the microbeams and the dose delivered along each microbeam. For a given peak dose, the valley dose is higher for wider microbeams and for smaller c-t-c distances. In this pilot experiment microbeam sizes and c-t-c distance were here chosen on the basis of the literature describing the tissue sparing effect of X-ray microbeams deposited in the central nervous system (Bräuer-Krisch et al. 2010; Romanelli & Bravin 2011). We have tested combination of microbeam sizes/spacing at the two extremes of the range better documented in the literature, which spans from 25/200 up to 680/1200 micron. After having fixed the size and spacing, the peak dose was chosen in order to determine a valley dose between 4 and 6 Gy that is well tolerated by the brain (Uyama et al. 2011). Immunohistochemical staining using pH2AX show clearly that the neurons hit by the microbeam along its penetration path die almost immediately while the adjacent cells separated by a few micron but outside the high dose volume remain viable. Progressively lower doses (but still much higher than in conventional radiosurgery or radiation therapy) are required to avoid tissue damage if thicker beams (100 to 600 µm) are used.

Submillimetric beams (sized 0.6 to 0.7 mm) appear to retain the tissue sparing effect allowing the delivery of incident doses of 400 Gy to the spinal cord of rats without inducing neurological damage: irradiation of rat spinal cord with four parallel 0.68-mm thick microbeams at 400 Gy in-depth beam dose did not induce paralysis after 6 months in three out of four rats (Dilmanian et al. 2006). This latter study showed not only that a highly radiosensitive structure such as the spinal cord can receive a high irradiation dose through a microbeam array without neurologic sequelae but also that a beam width up to 0.68 mm is well tolerated, substantially maintaining the tissue sparing properties of thinner beams. The ability of microbeam arrays to avoid radionecrosis and to preserve the architecture of the irradiated tissue is mainly attributed to the rapid regeneration of normal microvessels. Only a short segment of the microvascular bed receives ablating doses while the adjacent endothelial cells fall into the valley dose region receiving just a few Gy and can restore quickly the continuity of vascular supply (Blattmann et al. 2005; Romanelli & Bravin 2011; Uyama et al. 2011; Gabbiani et al. 1984). The wide spatial interface between the unhindered tissue placed in the valleys and the tissue irradiated with peak doses within the microbeam paths facilitates a widespread vascular recolonization of the tissue receiving necrotic doses preventing the dissolution of the architecture of the irradiated tissues (Romanelli & Bravin 2011). The self-repair of the normal microvasculature through the migration of unaffected cells surrounding the paths of microbeam penetration is considered the most likely basis for this ability of normal tissue to tolerate high dose microbeam irradiation (Serduc et al. 2006a; Serduc, Christen, et al. 2008; Serduc, Van De Looij, et al. 2008). The

tolerance of the vascular bed to high dose microbeam irradiation has been clearly demonstrated by the lack of extravasation of dyes administered to the experimental animals, which remained confined in the vessels after irradiation from 12 h until three months following 1000 Gy (Serduc et al. 2006a). This radioresistance phenomenon was not observed in 9L glioma microvessels, confirming the presence of a differential response in normal and tumour brain tissues in rodents, an effect that can have significant clinical applications (Dilmanian et al. 2003). The neoplastic vasculature appears to be unable to replicate the fast repair of the segments hit by the peak dose, facilitating the development of radionecrosis over the irradiated tumour (Miura et al. 2006; Smilowitz et al. 2002). A novel way to use microbeam arrays in a quasi-surgical way has been explored here: a microbeam array was placed over selected cortical areas in order hit tangentially and cut the horizontal axons connecting adjacent cortical columns. As described above, cortical transections are a surgical procedure to parcellize an epileptic focus located in eloquent cortex (Morrell et al. 1989; Morrell et al. 1995). Cutting the horizontal axons required for the spreading of epileptic activity is an effective way to control the seizures without inducing neurologic dysfunction. Synchrotron-generated microbeams can be used to create cortical transections in rats offering a chance to study the tolerance of CNS to this technique. This novel experimental application of microbeams, if confirmed by further experiments, can provide a new and attractive tool to modulate cortical function by transecting the fibers connecting the cortical columns. Aside from the tight dosimetry, the low energy of these beams makes them well suited to treat superficial targets such us the cortex. The smallest cortical area capable to sustain synchronous spikes generating seizures is estimated to be around 1 cm^2 in monkeys (Eidelberg et al. 1959) and 0.5 mm^2 in rats (Reichenthal & Hocherman 1977). It has been shown that parallel cortical transections with a thickness of 25 µm delivering doses up to 1000 Gy are not associated with short or long-term injury to the adjacent tissue (Serduc et al. 2006a). Therefore microbeam transections appear to be well suited to parcellize and disconnect an epileptic focus, even in small animals. We found here that larger beam size and higher doses were associated with faster relief from convulsive seizures. However all rats undergoing cortical transections recovered from convulsive seizures compared to non-transected controls. These experiments indicate that submillimetric X-ray beams may be used to modulate seizure spreading in eloquent cortex without inducing evident neurological dysfunction. These results suggest further investigations directed to assess the potential of microbeam transections to modulate cortical functions and to treat focal epilepsy. Microbeam transection, either placed over neocortical seizure foci or through the hippocampus, could prove to be an excellent tool to be added to the current radiosurgical techniques used to control seizures. The development of clinical devices delivering submillimetric beams able to generate cortical transections might add a powerful new tool to the clinical treatment of epilepsy and, more in general, to modulate cortical functions in a wide variety of neuropsychiatric disorders.

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CHAPTER III

Preservation of hippocampal stem cells following microbeam transections in normal rats

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Summary

Cranial radiation therapy is a common treatment modality for brain tumors, but can lead to partial or complete ablation of hippocampal stem cells even at low-medium irradiation doses. Impairment of hippocampal neurogenesis is thought to be an important mechanism underlying cognitive decline. The possibility of hippocampal stem cell preservation after microbeam transections performed at high radiation dose is here described. The tolerance of healthy tissue and the preservation of proliferative cells at different time points is described and experimental results comparing microbeam (600 Gy) with broad beam (10 Gy) irradiation are reported. The comparison is made on behavioral tests on cognitive performance and histology.

Résumé

La radiothérapie crânienne est une modalité de traitement courant pour les tumeurs cérébrales, mais elle peut conduire à une ablation partielle ou totale des cellules souches de l'hippocampe même à des doses d'irradiation faibles ou moyennes. Il semble que l'altération de la neurogenèse de l'hippocampe soit un mécanisme important impliqué dans le déclin cognitif dans la mémoire et l'apprentissage. La possibilité de conserver des cellules souches hippocampiques après les transections par microfaisceaux à haute dose est décrite ici. La tolérance des tissus sains et la préservation des cellules proliférantes à différents temps après irradiation ainsi que les résultats expérimentaux comparant l'irradiation par microfaisceaux (dose de 600 Gy) et celle par un faisceau homogène (dose de 10 Gy) sont présentés. La comparaison est réalisée à partir de tests comportementaux étudiant les performances cognitives et une analyse histologique.

III.1 Introduction

Radiation therapy (RT) of the brain is an essential therapeutic tool in the treatment of primary and secondary malignancies, but can be associated with a risk for adverse side effects, including cognitive dysfunction (Khuntia et al. 2006) which can severely affect quality of life (Scoccianti et al. 2012). These impairments are hippocampal-dependent functions in both children (Moore et al. 1992; Roman & Sperduto 1995; Tofilon & Fike 2000) and adults (Lee et al. 1989; Surma-aho et al. 2001). Hence, the hippocampus plays a crucial role in learning and memory (Izquierdo & Medina 1997) and considerable data at the moment showing that irradiation leads to impairment of those functions (Raber 2010; Yazlovitskaya et al. 2006; Rola et al. 2004). This situation is due to the fact that irradiation alter the microenvironment where are implicated many cells necessary to give neurogenesis support (Monje et al. 2002; Mizumatsu et al. 2003; Snyder et al. 2005).

Studies in animal models suggest that these deficits may be associated with an increase in hippocampal apoptosis (Tada et al. 2000) and a decrease in hippocampal neurogenesis (Tada et al. 2000; Parent et al. 1999; Snyder et al. 2001). A decreasing in adult hippocampal neurogenesis may result in distinct behavioral impairments related to locomotion and anxiety (Ben Abdallah et al. 2013). A whole-brain irradiation of adult rats with 10 Grays causes apoptosis among proliferating cells in the *dentate gyrus* (DG) of the hippocampus, leaving other cells unscathed (Jeong et al. 1999; Peissner et al. 1999), and a single 5-Gy dose was reported to block adult neurogenesis. In particular, severe structural and functional injury in adult hippocampus of rats generally occur after relatively high radiation doses (Augustine et al. 2005; Hopewell 1979; Dropcho 1991; Tofilon & Fike 2000), while lower doses can lead to cognitive dysfunction without inducing significant morphological changes (Abayomi 1996; Butler et al. 1994; Crossen et al. 1994; Dennis et al. 1991; Kramer et al. 1992; Roman & Sperduto 1995). In juvenile rats moderate irradiation dose (8 Gy) dramatically impaired growth of the DG and the subventricular zone (SVZ) and there was no morphological recovery, as assessed by the size of the neurogenic regions (Fukuda et al. 2005; Palmer et al. 2000).

The near complete ablation of hippocampal stem cells induced by relatively low doses of radiation is the most likely explanation for the dementia seen in an increasing number of cancer patients undergoing RT. For this reason knowing the tissue sparing properties of synchrotron-generated X-ray microplanar beams (microbeams), we transected the hippocampus of adult Wistar male rats to study the effect on stem cells (SC) preservation and on the cognitive and behavior components after irradiation. During these 3 years we

incurred in technical problems that did not allow to complete the project; therefore some results are only partial as it will be highlighted in the discussion session.

III.1.1 Structure and function of hippocampus

The hippocampus is an elongate structure located within the temporal lobe and is divided in dorsal (septal) and ventral (temporal) parts which differ markedly in their anatomical and functional organization (Figure III-36). The structure and function of hippocampus appears to be highly conserved across mammalian species. The structure consists of two major subregions: the *cornu ammonis* (CA) and the *gyrus dentatus* (DG).

Structure

The CA can be further sub-divided into four cellular regions or subfields (CA1-CA4). The regions CA1-CA4 are defined by cell type.

- **CA1** cellular subfield is composed by pyramidal cells, mostly triangular (Mouritzen Dam 1979). The *stratum pyramidalae* layer of the human CA1 is large in contrast to its narrow and dense appearance observed in rats (Stephan 1983).
- **CA2** is composed of large, ovoid, densely packed *somata* making the *stratum pyramidale* dense and narrow (Fanselow & Dong 2010).
- **CA3** refers to the inwardly curving aspect of the *cornu ammonis* as it enters the *gyrus dentatus*. Like CA2, CA3 consists of pyramidal somata but their population is less dense. A feature of CA3 is the presence of thin, non-myelinated fibers that arise from the *gyrus dentatus*. These fibers surround the *pyramidal somata* and are compressed between the *strata radiatum* and *pyramidale* forming an additional layer known as the *stratum lucidum* that is characteristic of the CA3 subfield.
- **CA4** is situated within the cavity of the gyrus dentatus which distinguishes it from CA3. *Somata* in this region are large, ovoid, sparse, and scattered amongst large mossy interweaving myelinated fibers characteristic of this subfield (Fanselow & Dong 2010).

The **DG**, in most coronal and sagittal views, appears as a V-shaped structure enveloping the end of the pyramidal cells of CA3 (Figure III-36).

The DG is a narrow, curved tissue that envelops the CA4 region of the CA. The DG is separated from the CA1-CA3 regions by the hippocampal *sulcus* that disappears early in the developmental process and becomes redundant. Thus the *cornu ammonis* and the *gyrus dentatus* are fused together, separated only by the vestigial hippocampal sulcus. The dentate

gyrus is composed of three regions: crest, lateral blade (suprapyramidal portion) and medial blade (infrapyramidal portion). In the coronal view, the medial blade appears first rostrally, while the lateral blade appears superior and more caudal to the medial blade. Continuing caudally, the blades meet and form a V shaped structure. At the most caudal aspect of the hippocampus, the medial and lateral blades join at each end to form the crest. The dentate gyrus is also divided into three layers: molecular, granule and polymorph (also referred to as the hilus). A relatively cell sparse area with varying cell sizes, the molecular layer (DGmo) is located superficial to the granule cell layer (GCL), which contains an extensive number of small, densely packed and uniformly sized cells. The molecular and granule cell layers partially enclose the polymorph layer (DGpo), which contains sparsely distributed large polymorphic cells. At the interface of the DGsg and the DGpo lies the subgranular zone, one of the few regions in the brain in which adult neurogenesis occurs (Cerbone et al. 1993).

It is well documented that hippocampus has an important role in the formation of memory, but until now, a vast majority of actual experimental work with animals and humans has been done considering the hippocampus as a homogeneous structure with a unified functional role (Eichenbaum 1999). In fact, numerous studies reported the different functions between dorsal (DH) and ventral (VH) hippocampus (Moser et al. 1995; Moser & Moser 1998; Moser et al. 1993; Ferbinteanu & McDonald 2001; Jung et al. 1994; Bannerman et al. 1999). While the DH is involved in the processing of cognitive memories, the VH is mainly associated with the control of behavioural inhibition, stress and emotional memory.

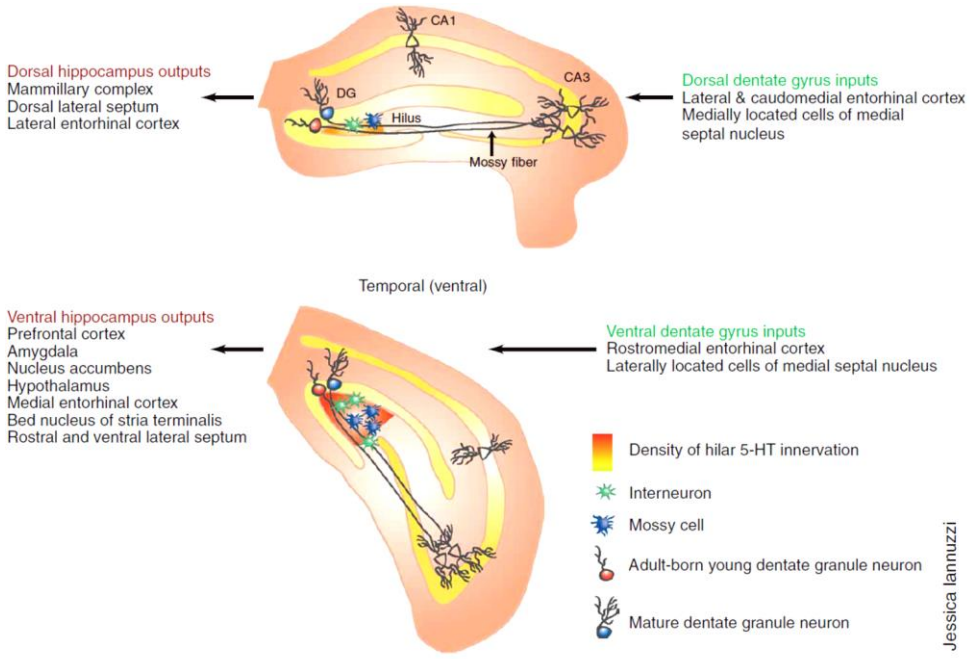

Figure III-36. Distinct roles for dorsal and ventral hippocampal neurogenesis in the regulation of emotion. The sketch illustrates the differences in the afferent and efferent connectivity of the hippocampus along the longitudinal (septo-temporal) axis. Also shown here is the asymmetry in numbers of mossy cells and hilar interneurons and enriched innervation of the ventral hilus by serotonergic projections (see text for references). By virtue of this asymmetry in hippocampal connectivity, neurogenesis in the ventral dentate gyrus may have a distinct role in regulation of emotion from that of neurogenesis in the dorsal dentate gyrus**.** Extracted from Sahay and Hen 2008.

Function

The function of the hippocampus has been extremely well studied and it is well accepted that the regions (CA1-4 and DG) play an important role not just concerning the formation of memories, but it is likely that they play a more general role in information processing and in the subsequent regulation of behavior (Mitchell et al. 2009). All regions are involved in different types of learning and memory, such as contextual memory (Corcoran et al. 2005), spatial memory (Wenk 2004; Morris et al. 1982), configural representations (Sutherland & Rudy 1989). Numerous studies report morphological, neurochemical and functional differences between dorsal and ventral zones of hippocampus (Moser et al. 1993; Jung et al. 1994; Bannerman et al. 1999). According to these findings, it has been reported that the effect of disruption is different depending on the affected site (Moser et al. 1993; Lorenzini et al. 1996; Levin et al. 1999). Additionally, the behavioral effects are supported by other neurochemical and physiological heterogeneity (Bruinink & Bischoff 1993; Jung et al. 1994; Ming & Song 2005).

Simplifying the circuitry activity, the connectivity of the hippocampus is often described in terms of a tri-synaptic circuit. The circuit begins with inputs to the dentate gyrus from the enthorinal cortex via the perforant path. Then, axons from the dentate gyrus granule cells project onto the pyramidal cell of CA3 via the mossy fiber pathway; the CA3 pyramidal neurons synapse onto the CA1 cells via the Schaffer collaterals (Amaral & Witter 1989) (Figure III-37).

III.1.2 Neurogenesis process

Some studies reported that juvenile neurogenesis adds to the final adult complement of granule neurons in olfactory bulb (OB) and DG (Altman & Das 1965), but neither the total numbers of granule neurons added, nor the temporal dynamics of that addition during juvenile development, have been defined. Furthermore, the term “adult” neurogenesis has been applied across studies with substantial differences in the age of the animal. Neuronal

stem cells proliferate and give rise to new granular cells through the following developmental stages: proliferation, differentiation, migration, maturation and synaptic integration (Ming & Song 2005) (Figure III-37). These stages can be identified on the basis of cell morphology, mitotic capability, electrophysiology characteristics, and expression of developmental regulated markers (Ehninger & Kempermann 2008). Cell proliferation occurs when a progenitor cell divides either symmetrically or asymmetrical (Cameron & McKay 1998). Symmetric division can produce either two progenitor cells (expansion of the progenitor cell population) or to two daughter cells (in which case the proliferative capacity is lost). Asymmetric division gives rise to a progenitor cell and an undifferentiated daughter cell. The daughter cell(s) then migrates from the subgranular zone to the inner layers of the granule cell layer. At the same time the daughter cell undergoes a process of fate determination at which point the cell will differentiate into either a neuron or a glial cell (Brown et al. 2003). The new born neurons play an important role in synaptic plasticity (Ming & Song 2005; Snyder et al. 2005) and reduction in the number of these cells impairs learning and memory (Shors et al. 2002).

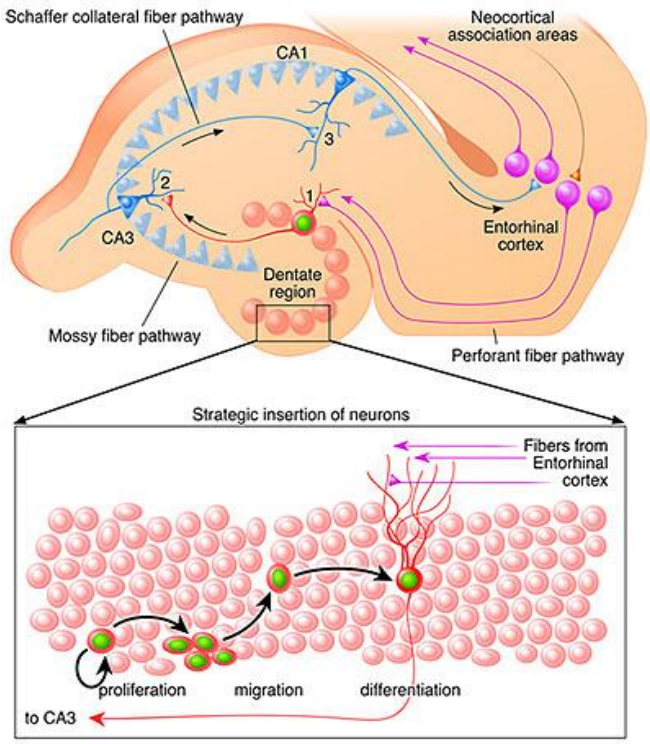

Figure III-37. Neurogenesis in the adult hippocampal dentate gyrus. Proliferation and fate determination: Stem of the dentate gyrus give rise to transit amplifying cells that differentiate into immature neurons. Migration: Immature neurons migrate into the granule cell layer of the dentate gyrus. Integration: Immature neurons mature into new granule neurons, receive inputs from the entorhinal cortex, and extend projections into CA3 (extracted from Lie *et al* 2004).

III.1.3 Evidence for a hippocampal role in place learning and memory: behavioral tests

Behavioral tests of spatial navigation and memory have been particularly illuminating with regard to the hippocampal function. There are several maze tasks that are used to verify cognitive impairments, and they differ by *i*) dimensions, including types of apparatus, which vary from arenas such as water maze (WM) to highly structured routes such as radial-arm maze (RAM); *ii*) availability of visuospatial, associative or sensory cues; *iii*) task requirements ranging from spontaneous exploration to complex sequences of choices; *iv*) motivation which may involve aversive escape, the opportunity to shelter or to discover novel objects or food at particular locations. Given this diversity, it is likely that mazes tap a variety of processes that contribute to, or affect spatial learning. Hence 'spatial' abilities measured in one procedure may not resemble those engaged in another, posing problems for the interpretation of drug- or lesion-induced deficits. For example, in the water maze reference memory task commonly used to assess learning and memory in rodents (Morris 1984; Faes et al. 2010), hippocampal lesions severely limit the rate of learning (Morris et al. 1982; Sutherland et al. 1982). Like the water maze, the radial arm maze tests spatial memory by requiring rodents to return to locations not previously visited to search for food (Olton & Samuelson 1976). However, after extensive training, lesioned rats can gradually learn how to navigate to a particular place, and to search accurately in this location during trials (McGregor et al. 2004; Morris et al. 1986).

III.2 Experimental outline

The aim of this work was to explore the hippocampus tolerance and the preservation of stem cells to microbeam irradiation (MBs, 600 Gy/75 µm) compared to broad beam irradiation (BB, 10 Gy/homogenous) on normal adult Wistar rats. The skin entrance dose for BB (10 Gy) was chosen, based on its ability to reduce proliferation of subgranular zone (SGZ) precursor cells by 78%, without any noticeable side effects at 2 months after irradiation (Snyder et al. 2005; Wang et al. 2005). The objectives were:

- investigating the presences of proliferative cells 24 and 48 h later in dorsal hippocampus by immunohistochemistry;

- evaluating changes in dorsal hippocampal volume induced by the irradiation in adult rats by MR Imaging;
- confirming morphological changes in the adult hippocampus by histological analysis;
- investigating a potential correlation between neurogenesis and impairments in learning and memory in adult hippocampus by performing behavioral cognitive tests 12 months after irradiation;
- verifying the effect of BB and MB irradiation on the proliferation of hippocampal cells by immunohistochemistry 1 month later.

III.3 Materials and Methods

III.3.1 Animal preparation

Adult male Wistar rats (5-6 weeks old; body weight, ~150-200 g) were purchased from Charles River, L'Arbresle (France). All rats were anesthetized in an anesthesia box circulated with a mix of air/isofluorane 5% for 5 minutes, then maintained with intraperitoneal injection of xylazine/ketamine (64.5/5.4 $mg.kg^{-1}$) for irradiation and with 2.5% isoflurane in air for each MRI session.

III.3.2 Irradiation parameters

Each rat was in vertical prone and fixed by ear bars and teeth on a custom-made Plexiglas stereotactic frame and placed on a Kappa- type goniometer (Huber, Germany), by which the rat could be translated and rotated in front to the fixed horizontal X-ray beam.
The irradiation was performed in the dorsal hippocampus (AP -2 mm and ML ± 1.0 mm relative to the bregma) of adult Wistar male rats. The target volume was about 3 mm-wide and 2 mm-height (Figure III-38).

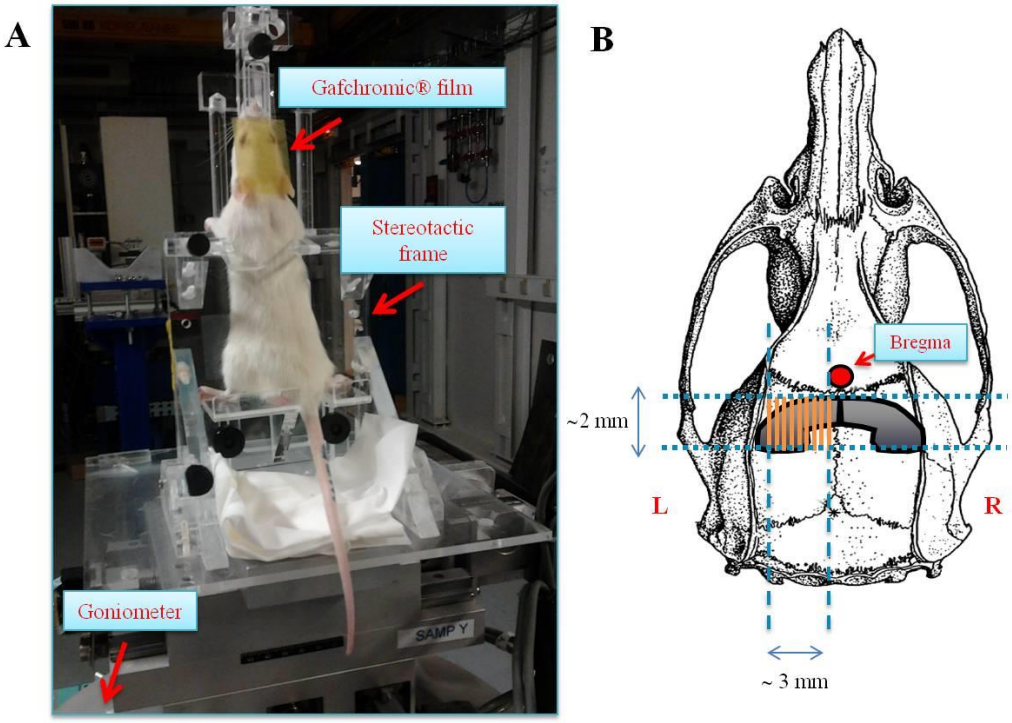

Figure III-38. Representative position of the animal on a custom-made Plexiglas stereotactic frame during the irradiation A) and a skull sketch extracted from rat atlas (Paxinos & Watson 1986) where is depicted the irradiation volume of the target area composed by 9 microbeams, each one of 75 µm-width and spaced by 400 µm. The peak entrance dose was 600 Gy.

We performed two types of irradiation: one with microbeams (MBs) and the other one with broad beam (BB). The animals were either unilaterally irradiated with MBs (left side, *n=5*; right side, *n=5*) or BB (left side, *n=4*; right side, *n=4*), or bilaterally irradiated (BB, *n=10*; MBs, *n=10*), or irradiated with both BB (on the left side) and MBs (on the right side) (*n=6*). Thirteen rats were used as non-irradiated controls. A summary of the animal number used is reported in Table III-5. The exposure time was adjusted by the type of radiation. For the broad beam the dose rate in air at the animal surface was approximately 16 000 Gy.s^{-1}.

Beam properties	MBs	Broad Beam
Beam width	75 µm	Homogenous
Beam number	9	- -
Center-to-center distance	400 µm	- -
Peak dose	600 Gy	10 Gy

Valley dose	~ 4.1 Gy	10 Gy
Number of animals		
~150 g *Same animal irradiated with both irradiation types*	6	
~200 g	20	18

Table III-5. Irradiation parameters for parallel microbeams hippocampal transections and broad beam.

- **Microbeam hippocampal transections:**

Animals where irradiated with an array of 2 mm-high beams composed by 9 microbeams, 75 µm thick, and having a 400 µm center-to-center distance; peak dose of 600 Gy (indicated as skin-entrance at 3.5 mm). The valley dose was about 4.1 Gy calculated with Monte Carlo simulation. Dosimetry calculations are explained in the paragraph III.3.2.1.

- **Broad beam irradiation:**

In order to compare the microbeam transections tissue response to the broad beam, a uniform irradiation was delivered at 10 Gy skin-entrance dose.

III.3.2.1 Monte Carlo simulation and dose calculation

The dose were calculated by means of the Monte Carlo method normally used for MRT dose planning and quality assurance at the ESRF (Siegbahn et al. 2006). Briefly, the calculation was performed for 1 microbeam with a FWHM of 75 micron and a height of 2 mm using the MRT filtered spectrum (Bräuer-Krisch, a. Bravin, et al. 2003) impinging perpendicular the rat head phantom. The lateral dose profile was computed at 3.5 mm depth and the 9 profiles were superposed with a 400 micron center-to-center to compute the PVDR in the center of the field. At 3.5 mm depth the peak entrance dose results with a PVDR of 146.5 to a valley dose of 4.1 Gy. Further details of the dosimetry protocols are described in the second chapter (II.4.3.3).

III.3.3 Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) was performed at the imaging platform of the Grenoble Institute of Neuroscience (GIN). Images were acquired using a 7T Bruker Avance III system using a quadrature volume coil. Three months after irradiation four adult rats for each group were chosen. Radiation-induced anatomical changes were assessed on T2-weighted images (TurboRARE sequence. TR: 4000 ms, effective TE: 33 ms, FOV: 30x30 mm, matrix: 256x256, slice thickness: 0.5 m, Naverage = 2, Duration 4min16s) and RARE sequence. Brain vessel permeability was then characterized using a T1-weighted MR sequence (TurboRARE sequence. TR: 1300 ms, effective TE: 7.7 ms, FOV: 30x30 mm^2, matrix: 256x256, slice thickness: 0.5 m, Naverage = 4, Duration 4min4s) acquired 5 min after an intravenous injection of Gd-DOTA (200 $\mu mol.kg^{-1}$, Dotarem, Guebert).

III.3.3.1 Imaging of the hippocampus

Hippocampus volumes were accurately calculated from MRI scans (

Figure III-39). Several studies have tried to evaluate the volume in either normal or pathological hippocampi, with the aim of correlate the volume variations with pathologies. The typical imaging used for this aim is characterized by the combination of optimal imaging parameters as T1- weighted that maximizes the grey and white matter contrast (Jack et al. 1992).

III.3.3.2 Anatomical Structure Delineation (ROI Analysis)

The manual volume measurements on the T1 weighted MR images of the dorsal hippocampus were processed using the ImageJ software® (NIH)[2]. Every MRI slice has a unique code that can be found in the header of the DICOM file. The regions of interest (ROI) were manually segmented following the corresponding section (in this case the dorsal hippocampus) of the stereotaxic atlas (Paxinos & Watson 1986). ROIs measurement of the right and left hippocampus were manually delineated. The volumes of cerebral hemispheres and white matter were estimated by multiplication of the total sectional surface area with the section interval (0.5 mm) following the Cavalieri's principle (Roberts et al. 2000):

$$\mathbf{V=\Sigma a \times t}$$

where **V** is the volume, **Σa** is the total sectional area of the structure and **t** is the space between the examined sections. The interested area was measured from -2.12 mm to -4.52 mm with respect to the bregma (Paxinos & Watson 1986). Sectors CA1 trough CA4 of the hippocampus, the dentate gyrus, and the *subicumlum* were included in the in-plane boundaries. To reduce manual tracing errors, the coronal T1-W MR images were magnified three times and trilinear interpolation was performed.

[2] *Image J is available at the site: http://rsb.info.nih.gov/ij/*

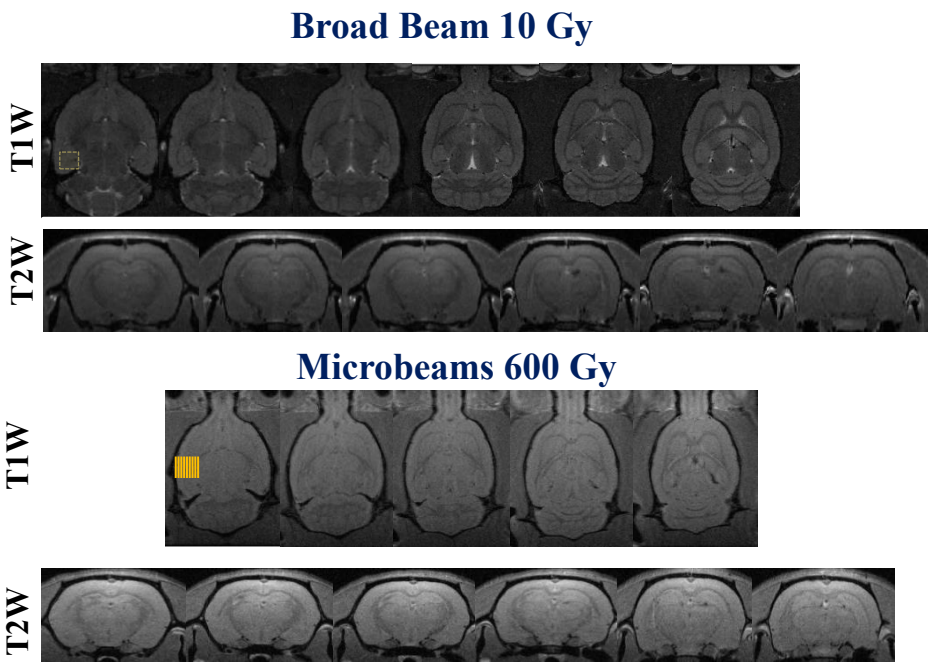

Figure III-39. MRI of rat brain after 3 months from irradiation.. MR characterization (T1-weighted images 5 min after Gd-DOTA injection) of brain rats irradiated with BB on the top and with MBs on the bottom.

A black radial eight arm maze (ViewPoint Sa, France) with 8 arms (long 55 cm) originating from a central platform (47 cm octagon; Figure III-40) was used. This labyrinth is used to test a variety of memory tests including short-term working memory and long-term reference memory by examining the number, sequence of arms, and the time necessary to complete the task (Shif et al. 2006). However, this task relies on animals being motivated to receive a food reward for performance. The maze was placed on the floor in an adapted room surrounded by one desk, geometric designs on the walls to permit the animal to orient himself. During testing, the room was dimly lit with diffuse white light. Each rat was placed on the center of the platform into a cardboard black cylinder to avoid the immediate escape into an arm. Rat

behavior was monitored by a videocamera using the software VideoTrack V3 (ViewPoint Sa, France).

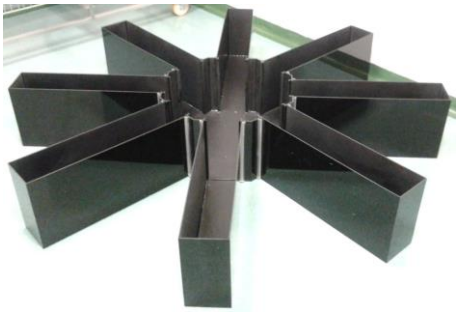

Figure III-40. Standard radial eight-arm maze with eight arms originating from a central platform (ViewPoint Sa, France) used in this study.

III.3.4.1 Animals

Behavioral tests on learning and memory impairments were performed 12 months after irradiation. Before to perform radial maze tasks, 14-months-old Wistar male rats were kept on a restricted diet and body weight was maintained at 85% of their free-feeding weight over 24-days period, with water available *ad libitum*. The effect of food deprivation was monitored three times per week. Food deprivation continued throughout the experiment. To maximize exploratory behavior, irradiated and not-irradiated rats were handled for 10 minutes every day for one week prior to testing. After handling was completed, each rat was allowed to individually explore the test apparatus. All tests started at 9:00 and each animal received one trial a day for 8 days during a 12 h light/cycle.

III.3.4.2 Procedure and phases of the behavioral tests

In the black radial eight arm maze each rat was placed on the centre platform into a black cardboard cylinder to avoid the immediate escape into an arm. The test began with the removal of the black cardboard cylinder, in this way the rat was allowed to explore the maze for 10 min. The behavioral parameters were the horizontal activity (HA, as number of alley visits) and the vertical activity (VA, as number of rearing, rise its head). We scored the number of arms visited before the first repetition occurred (first error, FE); the position of first repetitive arm entry), the number of working (WME) and reference (RME) memory

errors and retention of WME and RME. This study was divided in 3 phases: i) habituation to the new environment; ii) habituation to the reward; iii) training of 8 days following the paradigm of 4-arms baited described by Olton and Papas (Olton & Papas 1979) in a radial maze. The same arms baited were used throughout all sessions for each rat for each day of the training.

1st trail: habituation to the testing environment

This first trial was characterized by habituation to the environment (maze). It began with acclimatization of each animal to the maze without food pellets. The day after each animal allowed to freely explore the maze, and these attempts to considered behavioral parameters like HA and VA.

2nd trail: Habituation to the reward

In the second trial each animal received a 10-min habituation to the food. We placed on the entire platform honey pops (Kellogs®).

Food preference test:

We choose this kind of reward because we performed a food-preference test on three rats of the same age not food-deprived (out of the animals used for the test). The test started when each rat was placed in the cage, and the time when a rat started and stopped eating each chocolate, pellets (40 mg) and honey pops was noted (data not showed). Eating was denned as picking up food, biting and chewing it, and ingesting it. After 10 min rats were removed from the cage, and the time spent eating each type of food was determined. Each rat was tested only once (Rolls & Rolls 1973).

3rd step: Training 8 days

Animals are trained one session per day for seven consecutive days. Each animal was placed individually in the center of the maze where the same four arms (i.e. 1, 3, 4 and 7) baited. For each daily training trial, one piece of reward is placed at the end of each arm. The other four arms (2, 5, 6 and 8) were never baited (Figure III-41). This version of radial eight arm maze (Jarrard 1983) was done to test working and reference memory at the same time. The same maze arms were baited each day, and across sessions, the rats learn to ignore the remaining four arms, which never contain reward. This is the reference memory component of the task, and entry into a never-baited arm is considered a reference memory error. Within a training session, re-entry into one of the four baited arms would be considered a working memory error. The visited arms are registered: an arm entry is counted when all four limbs of the rat were within an arm. The animal was allowed to choose arms in any order. The trail ended when all four baited arms were visited, or after 5 over 10 minutes elapses. Rats with

hippocampal lesions are expected to exhibit working memory errors with this kind of task (Olton & Papas 1979).

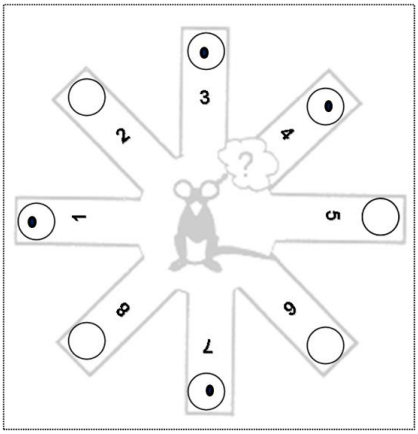

Figure III-41. Schematic of a radial eight-arm maze illustrating an example of 4 baited reward arm location (1, 3, 4 and 7) and the 4 respective non-reward arms (2, 5, 6 and 8).

Retention

After 5 and 9 days from the last day of training (day 8th) we performed another experiment on two irradiated groups of animals. This step was added to verify for how long the information persist in the brain of BB and MBs irradiated animals.

III.3.4 Histology and immunohistochemistry

Anti-**NeuN** (Neuronal Nuclei) is a marker used extensively to specifically detect post-mitotic cells (Lind et al. 2005). In general, NeuN reactivity is predominantly nuclear, although it can also be detected in the cytoplasm of many neuronal cell types (Weyer & Schilling 2003; Van Nassauw et al. 2005). It has been hypothesized that NeuN may be required for the maintenance of the post-mitotic state, or during the process of axonogenesis[3] (Weyer & Schilling 2003).

Anti-**GFAP** (Glial Fibrillary Acidic Protein) is a marker for astrocytes. In the adult, newly generated neuronal cells integrate a pre-existing network, where the glial cells are already in place and are considered as support cells. These data suggest that astrocytes in the adult brain may play a role in neurogenesis (Hellström et al. 2009).

[3] Axogenesis is a subfield of neural development concerning the process by which neurons send out axons to reach the correct targets. Axons often follow very precise paths in the nervous system.

Concerning cell proliferation markers, the common used in this thesis, are Ki-67 and BrdU (Kee et al. 2002) (Figure III-42). **Ki-67** is a nuclear protein expressed in dividing cells for the entire duration of their mitotic process (Scholzen & Gerdes 2000). Several studies reported that Ki-67 is expressed in all phases of the cell cycle except the resting phase and at the beginning of the G_1 phase(Scholzen & Gerdes 2000; Van Rijn et al. 2010). Because of its short half-life of about 1 h, it is rarely detectable in cells in the G_O phase (Zacchetti et al. 2003). Ki-67 is not detectable during DNA repair processes and is mainly absent in quiescent cells (Zacchetti et al. 2003). **BrdU** (5-Bromo-2-deoxyuridine) is an exogenous thymidine analogue that can be injected into an animal to label cells in S phase (synthesized DNA) (Gratzner 1982). BrdU has a bioavailability time of approximately 2 hours (Packard et al. 1973) during which time it will become incorporated into cells that are synthesizing new DNA. Then BrdU could be detected in the tissue using specific primary antibodies. BrdU can be used as a marker of cell proliferation but is not a specific marker of neurogenesis.

III.3.5.1 BrdU injection

Bromodeoxyuridine (BrdU, Sigma-Aldricht, France) was dissolved in 0.9% saline. The solution was prepared immediately before use and injected intraperitoneally using a volume of 10 ml/kg. For cell proliferation analysis, 50 mg/kg BrdU was administered three times at a two hour interval. Rats were killed 24 hours later.

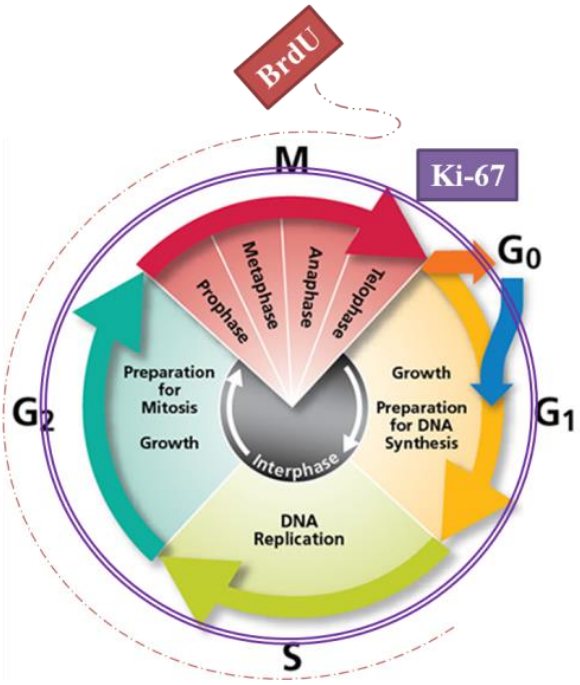

Figure III-42. Cell cycle with BrdU and Ki-67 involvement. The cell cycle has two major phases: interphase, the phase between mitotic events and the mitotic phase, where the mother cell divides into two genetically identical daughter cells. Interphase has three distinct successive stages. During the first stage called G1, cells "monitor" their environment, and when the requisite signals are received, the cells synthesize RNA and proteins to induce growth. When conditions are right, cells enter the S stage and "commit" to DNA synthesis and replicate their chromosomal DNA. Finally, in the G2 phase, cells continue to grow and prepare for mitosis (M). Ki-67 is positive in all phases except G0, BrdU is positive during much more restricted portions of the cycle. Image adapted from the web site: w3.bdbiosciences.com.

III.3.5.2 Tissues collection and immuno/histolabeling

In this work we used 2 different protocols to collected sections of brain tissues: frozen tissue and tissue embedded in paraffin

Tissue embedded in paraffine

Rats were killed by intraperitoneal injection with Doléthal®. Brains were dissected out and immediately fixed in a solution composed of ethyl alcohol (60%), acetic acid (10%), and chloroform (30%). Twenty hours later, brains were placed in 70% ethanol until they were included in paraffin. Ten micrometers of serial sections were cut through a microtome (RM2245 semiautomatic Leica, Milan, Italy) and used for immunohistochemical analysis

- *Brightfield immunohistochemistry for BrdU*: tissues were first incubated at room temperature in 0.3% H_2O_2 for 30 minutes by three rinses in 10 mM PBS (phosphate buffered saline) (composed by NaCl, KCl, Na_2HPO_4, KH_2PO_4) prior to a 30 minutes incubation at 37°C in 1 N hydrochloric acid in PBS, used to denature DNA. The

tissue was then rinsed in 0.1 M tetraborate buffer for 10 minutes (pH 8.5) followed by three more rinses in 10 mM PBS. Sections were incubated with primary antibody, mouse anti-BrdU, (1:10, Becton Dickinson, San Jose, CA) overnight at 4°C. The next day sections were incubated in secondary biotinylated anti-mouse (1:200, Vector) followed by avidin-biotin complex (Vectastain ABC Kit, Vector, Burlingame, CA), then 3-3'-diaminobenzidene (DAB SigmaFast tablets, Sigma-Aldricht, France). Mounted sections were stained with 0.1% cresyl violet in order to visualize the entire granule cell layer. Sections were then dehydrated in progressively increasing concentrations of ethanol, and finally in toluene. Slides were coverslipped with Permount™.

Frozen tissue

Twenty-four hours, 48 hours and 3 months after irradiation, rats were randomly chosen and killed by Dolethal overdose. The brain was rapidly excised, frozen in precooled isopentane at −50 °C and stored at −80°C. Fifteen micron coronal brain sections were obtained at −20 °C using a cryotome (Microm HM560, France). The slices were mounted onto Superfrost® Plus glass slides (Fisher Scientific, Pittsburgh, PA).

- *NISSL* staining: tissue slices were stained for 8 minutes with 0.1% thionin and then were dehydrated in alcohol series, cleared in toluene, and coverslipped with Pertex (Medite, Burgdorf, Germany). In order to minimize variability, sections from all animals were stained simultaneously.

- *Fluorescence immunohistochemistry for pH_2AX, NeuN and Ki67*: sections were fixed with PFA 4% for 15 min and blocked with donkey normal serum (DNS, Interchim) diluted in 10 mM (PBS) for one hour (10 mM PBS/DNS 5%). The primary antibodies used were pH2AX (1/500, 05636, Upstate) and Ki-67 (1/200, Clone S6, Lab Vision Corporation, Fremont) diluted in 10 mM PBS/NDS 1%. Sections were washed 4 times with PBS, then incubated with the secondary antibodies Alexa Fluor-conjugated donkey F(ab9)2 (1:200, Invitrogen, CA, USA) for 2 hours at room temperature. Finally, the tissue was rinsed three times in 10 mM PBS and the sections were then mounted on glass slides and coverlipped with DAPI mounting medium (Vectar Shield, Vector Laboratories, Burlingame, CA, USA). In order to minimize variability, sections from all animals were stained simultaneously.

Frozen tissue with preliminary intracardiac perfusion of rats

Rats were intraperitoneal injected with Doléthal® and transcardiac perfused with 4% paraformaldehyde (PFA) in PSB (pH=7.4) one year after irradiation. Brains were rapidly removed, postfixed at + 4 °C in the same solution used for the perfusion, cryoprotected in 30% sucrose in PBS at + 4 °C for 48 hours, frozen, and kept at -80 °C until used. For immunostaining, brains were cryosectioned in 20 µm slices, sampled on Superfrost/Plus® (Fisher Scientific, Pittsburgh, PA) slides.

- Fluorescence immunohistochemistry for Ki-67 and GFAP: the protocol was as above In this case we used as primary antibodies GFAP (1/1000, Sigma-Aldricht, France) and Ki-67 (1/200, Clone S6, Lab Vision Corporation, Fremont) diluted in PBS/NDS 1%.

III.3.5.3 Stereological count

The stereological count was performed at the Neuromed Institute, Pozzilli, Italy on brightfield sections. The number of BrdU positive cells in the dentate gyrus of animals irradiated with BB on the left side and with MBs on the right side, was assessed by stereological technique and an optical fractionator using a Zeiss Axio Imager M1 microscope equipped with a motorized stage and focus control system and with a digital video camera. The software Image-Pro Plus 6.2 for Windows (Media Cybernetics, Inc., Bethesda, MD) equipped with a Macro was used for the analysis of digital images. The Macro was obtained by ‘‘Immagini e Computer’’ (Bareggio, Italy). The characteristics of this Macro are published (King et al. 2002). The analysis was performed on sections of 20 µm, sampled every 380 µm on the rostrocaudal extension, in which the dentate gyrus was identified and outlined at a 2.53 magnification. BrdU-positive cells were counted at 100X magnification as described (Gundersen & Jensen 1987). For stereological analysis, we used a grid of dissectors (counting frame of 150 X 150 µm; grid size 100 X 50 µm), with 1.3 as numerical aperture of the lens. The total number BrdU positive cells in the dentate gyrus was computed using the formula:

$$\mathrm{N} = \mu(\mathbf{n}) * \frac{1}{SSF} * \frac{1}{ASF} * \frac{1}{TSF}$$

where **n** is the total number of cells counted on each dissector; **SSF** (fraction of sections sampled) the number of regularly spaced sections used for counts divided by the total number of sections across the dentate gyrus; **ASF** (area sampling frequency) the dissector

area divided by the area between dissectors (7500 μm^2 * dissector number/region area); and **TSF** (thickness sampling frequency) the dissector thickness divided by the section thickness (20 mm).

III.3.5.4 Microscopy

Fluorescent sections were analyzed by an Olympus BX51 microscopy equipped for epifluorescence and objectives X10, X20, X40. The slices were photographed with a video camera connected to the microscope. The images were then processed by the software Cell^B (Olympus microscope, Life science instrument, France).

III.3.5 Statistical analysis

In this work were applied both the one-way and the two-way analysis of variance (ANOVA) tests. One-way ANOVA measures significant effects of one factor only, two-way ANOVA tests (also called two-factor analysis of variance) measure the effects of two factors simultaneously. For example, an experiment might be defined by two parameters, such as treatment and time point. One-way ANOVA tests would be able to assess only the treatment effect or the time effect. Two-way ANOVA on the other hand would not only be able to assess both time and treatment (*i.e.* irradiation) in the same test, but also whether there is an interaction between the parameters. We did not performed post-hoc test on individual factors if the ANOVA said the factor is not significant; the level of significance was set up at $p<0.05$. All values are expressed by mean ± standard error of the mean (SEM). As statistical software we used a GraphPad prism (Version 5). The statistical analysis applied were:

- For the hippocampal volume measured through MRI scan, we analyzed data by one-way ANOVA.
- For behavioral tests two-way and one way ANOVA were carried out. In particularly, HA, VA, WME, RME, retention for WME and for RME were analyzed by two-way ANOVA followed by Bonferroni's multiple comparison; FE results occurred in the first habituation day came out from one-way ANOVA.

- For the cell count we applied One-way ANOVA analysis, taking the number of BrdU positive cells in the dorsal hippocampus in each group, BB-, MBs- and sham-irradiated.

III.4 Results

III.4.1 MB and BB irradiation and the hippocampal volume

MRI performed 3 months after irradiation showed that MB and BB irradiation did not affect the volume of dorsal hippocampus as compared with non-irradiated control rat hippocampus. This result was observed in rats irradiated in the left hippocampus with MB or BB or in rats irradiated in the left hippocampus with MBs and in the right hippocampus with BB (

Figure **III-43**).

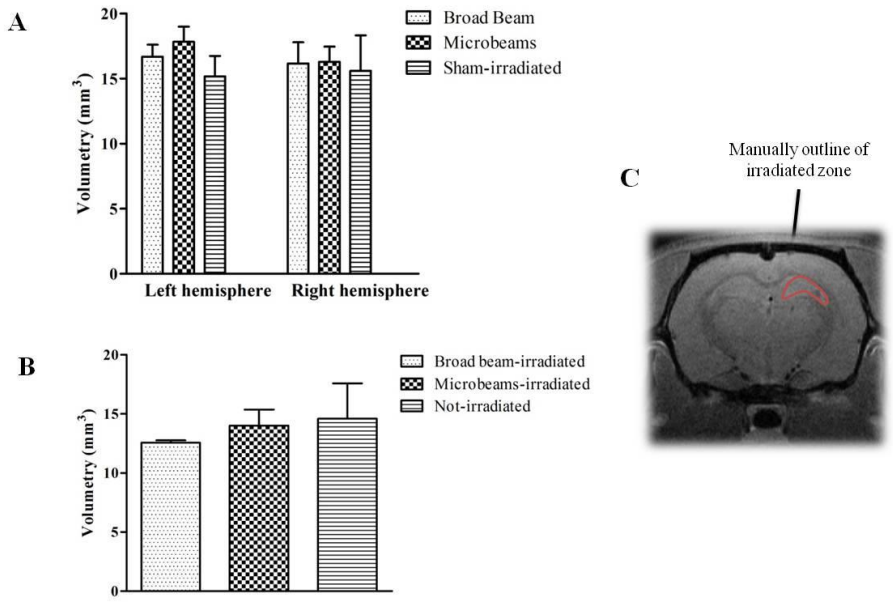

Figure III-43. Hippocampus volumetry in irradiated and not-irradiated adult rats 3 months later. Effects of microbeam (75 µm wide, spaced by 400 µm at 600 Gy entrance dose) and broad beam (homogenous at 10 Gy) irradiations on the volume of both hippocampi (A) and on the volume of the irradiated hippocampus with MB or BB (B) C) Coronal MR image showing the manual outlining of the left hippocampus for MR volumetry.

III.4.2 Studying learning and memory tasks one year later

Horizontal activity

Two-way ANOVA revealed significant main effects between different types of irradiations ($p<0.05$) but not during 5 minutes ($p>0.05$). Post-hoc analysis showed that the MBs irradiated group more interested to visit the maze compare to the activity of BB- or sham-irradiated rats, mainly in the first 5 minutes of the 10-min testing period. The development of HA as a function of time was similar in each group but not between groups (Figure III-44 A).

Vertical activity

Two-way ANOVA indicated that MBs irradiation could influence the HA in this aged animals. Rats irradiated with MBs are more "curious" than sham or BB-irradiated rats (Figure III-44A).

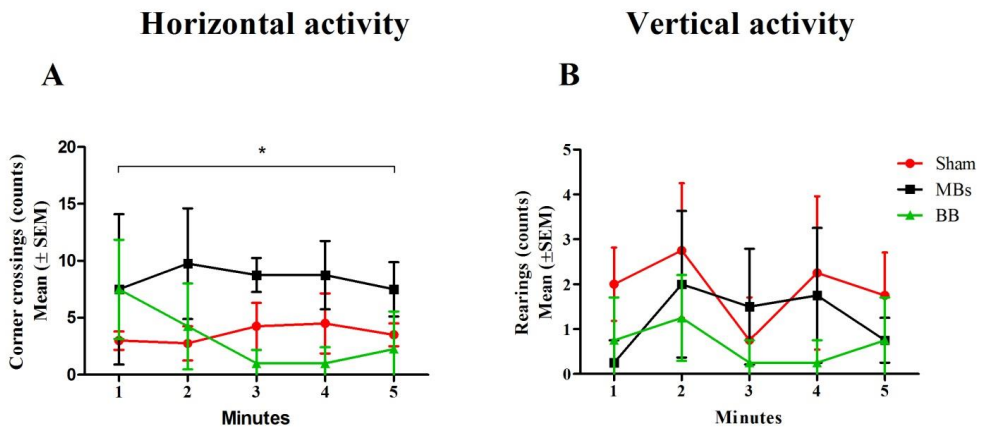

Figure III-44. Excitability rats in the eight arm radial maze. A) Horizontal activity (motor activity); B) vertical activity (frequency of rearings); those activities are represented by graph plotted in the first five minutes exposure in one minute blocks. Data represents mean+SEM. Two-way ANOVA followed by Bonfferoni's posttest. Means are significative different at $p < 0.05$.

Spatial orientation: First error

An efficient rat explore each of the eight arms only once; thus, the best possible score for these index would be 9 (8 arms plus the center of the maze). The results showed that MBs-irradiated rats need more time to reach the four baited arms. One-way ANOVA test indicated no significant differences irradiated and not-irradiated groups the first day of trial (Figure III-45).

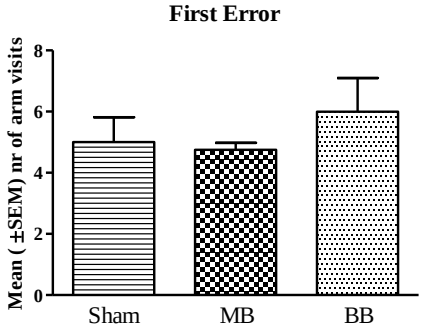

Figure III-45. Effects of broad beam and microbeam irradiation upon selective spatial attention (SSA) in eight arm radial maze. The position of first repetitive arm visit (first error) were scored in irradiated and not-irradiated adult Wistar male rats. One-way ANOVA was carried out.

Working (WME) and reference memory errors (RME)

Analysis of memory errors revealed that MBs irradiated rats made more errors than BB- or sham-irradiated rats at 3, 5 and 7 days of training. These results in general are not statistical significative. They shows eventual impairment in learning (double entries into baited places) and memory (entering never baited arms) but it could be explained not by the irradiation type (MBs or BB) but by the aging and the strain of these rats. These conditions are explained in details in the discussion section. MBs-irradiated rats need more days training to make fewer errors (

Figure III-46).

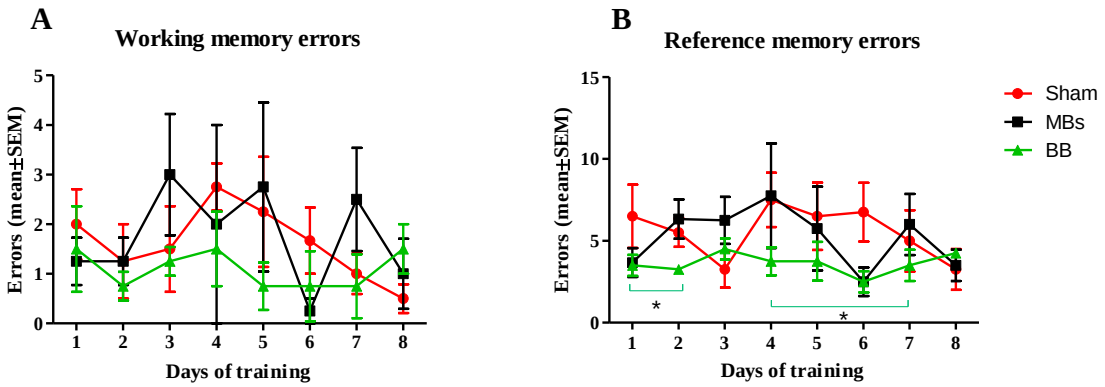

Figure III-46. Working A) and reference B) memory errors after microbeam and broad beam irradiation on adult Wistar male rats. The graphs present the mean number of working or reference memory errors committed during 8 days**.**

Retention in WME and RME

The first retention session was carried out 5 and 9 days after the last training session (day 8th of training) for both, working and memory errors. Mann Whitney test showed that there is no significant difference between the two irradiated groups (Figure III-47).

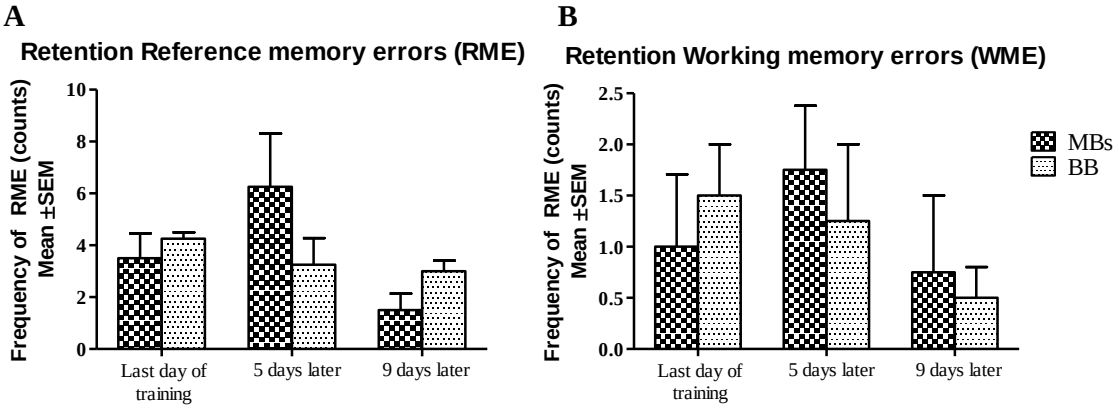

Figure III-47. Retention testing 5 and 9 days after the last day of training (day 8th). Two-way ANOVA showed no significant differences were observed between the two irradiated groups.

III.4.3 Microscopic observations

III.4.3.1 Short-term effects of MBs and BB on healthy tissue of adult normal rats

The effects of MRT are detectable already after 2h after irradiation. As shown in Figure III-48 pH2AX foci are well correlated with MBs paths that cross the hippocampus one day after 600 Gy exposure.

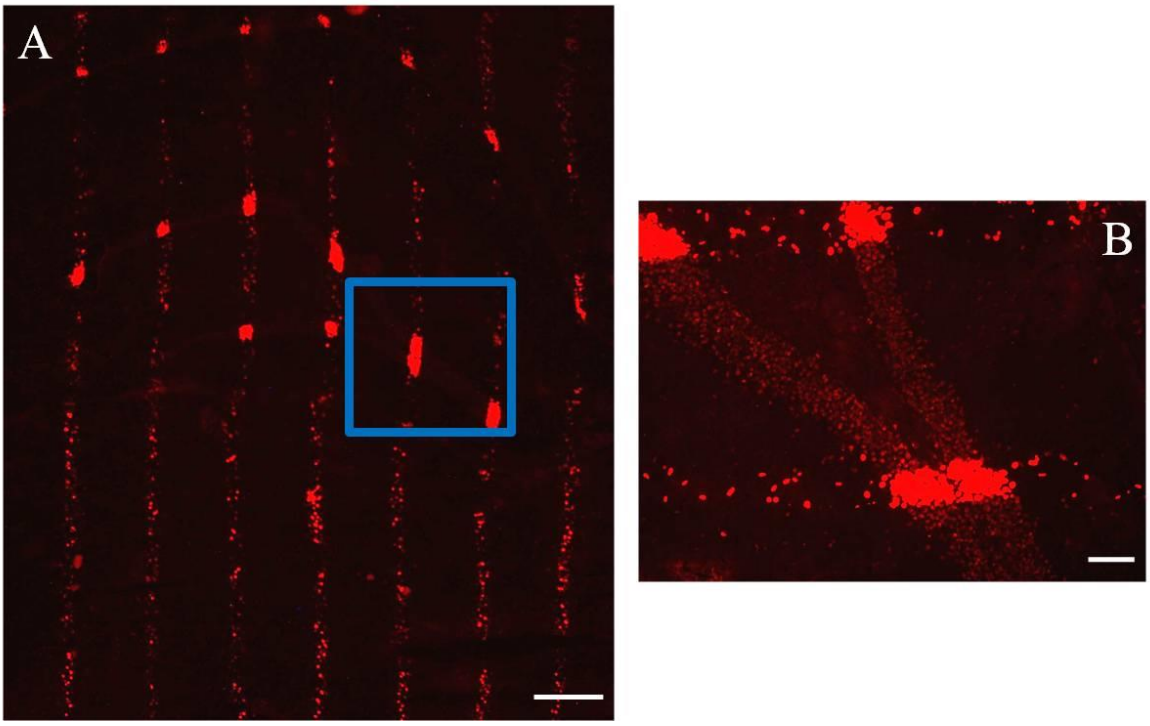

Figure III-48. Immunohistological verification of MRT-treated Wistar rats. A-B) pH2AX immunohistochemistry performed 24 hours after irradiation. Figure B is a magnification of the square in figure A. Scale bars:400 µm (A) and 50 µm (B).

III.4.3.2 MB irradiation and cell proliferation 48 hours later

Figure III-49 showed that is still clear the microbeam paths after 2 days from irradiation, marked with pH2AX and also proliferative cells, marked with Ki67-positive labeling was observed between MBs path, then in the granular cell layer and the molecular layer of *dentatus gyrus* (DG) and in the *hilus* of hippocampus, as well as in the subgranular zone (SGZ).

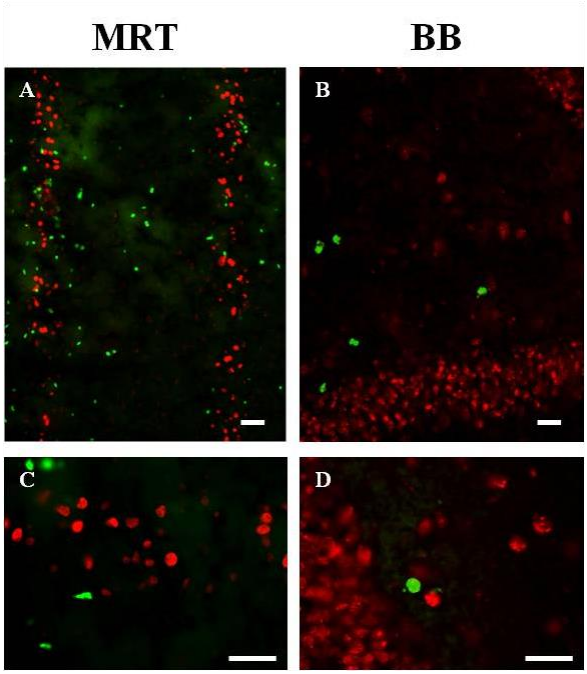

Figure III-49. Detection of the Ki-67 and pH2AX proteins by immunofluorescence 48 hours after hippocampus irradiation. A-C) Unilateral MRT-treated (9 microbeams of 75 µm wide spaced by 400 µm center-to-center, peak dose of 600 Gy). B-D) Unilateral BB-treated (10 Gy). In red pH2AX (DNA damage) and in green Ki67 (proliferation cells). Scale bar: 50 µm (A-C-E) and 100 µm (B-D).

These results suggest that there are several proliferative cells in the valley zone, but those cells marked only with Ki-67 do not show only proliferation of neurons but also they could identify other type of cells (*i.e.* endothelial cells, or macrophages/monocytes, or astrocytes) involved in the vascular hypothesis after irradiation. Qualitative difference in cell proliferation between broad beam and microbeams irradiation are evident in Figure III-49, whereas in Figure III-50 is evident a high number of proliferative cells in MBs irradiated hippocampus compared to not-irradiated hippocampus in the same animal.

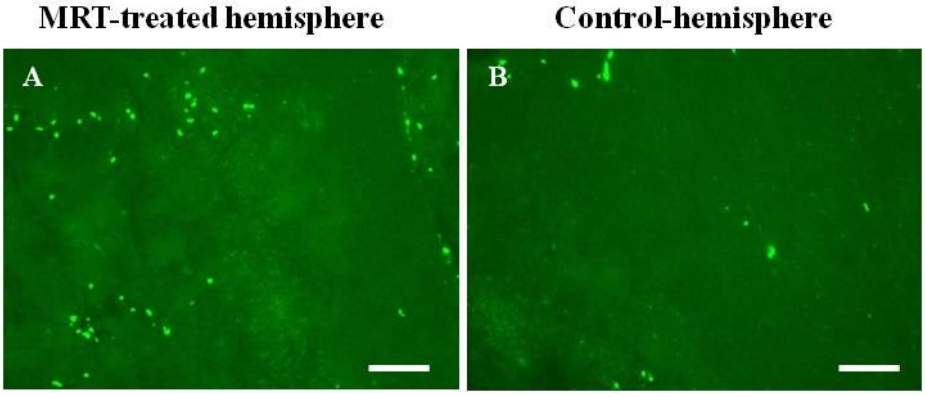

Figure III-50. Ki67 (green) of coronal brain section of two hippocampi in the same animal obtained 48 h post-irradiation. On the left panel (A) the irradiated hemisphere and on the right (B) the not-irradiated hemisphere. Scale bars: 100 µm.

III.4.3.3 Proliferative cells in the dorsal hippocampus 1 month after MBs and BB irradiation on the same animal

Two irradiations were performed in the dorsal hippocampus of a series of animals, BB (10 Gy) on the left side and MBs (600 Gy) on the right side. Figure III-51 shows a significant decrease of BrdU-positive cells in both irradiated hippocampi compared to not-irradiated hippocampus. One-way ANOVA followed Bonferroni post-test did not show a significant difference in MBs-irradiated hippocampus compare to BB-irradiated hippocampus. Mean are considered significativetly different for $p<0.05$.

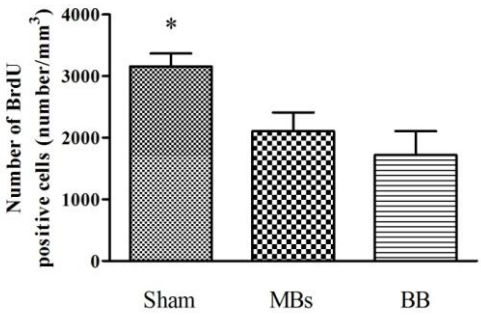

Figure III-51. BrdU positive cells in dorsal hippocampi of Wistar male rats irradiated with both, BB (broad beam, 10 Gy) on the left side and MBs (microbeams, 600 Gy, thickness: 75 µm and c-t-c: 400 µm) on the right side. One-way ANOVA followed by Bonferroni post hoc test. Errors bars represent SEM. *p<0.05.

III.4.3.4 Analysis of morphological changes in the hippocampus 3 months after MBs and BB irradiation

Results showed by MRI scan images were confirmed by histology technique, where the only architecture abnormalities, concerning hippocampi irradiated with MBs, where was evident neuronal losses in the "striae" at low magnification, but much better at high magnification (Figure III-52 A3, B2 and C2). Furthermore, at high magnification it was evident that the striae (each one of 75 µm), which were separated by approximately 400 µm, correspond to the irradiated microslices (Figure III-52A3). Otherwise, hippocampal areas irradiated with BB did not show any alteration by a microscopic point of view if compared with not irradiated hippocampi (Figure III-52 B1-B2 vs C1-C2).

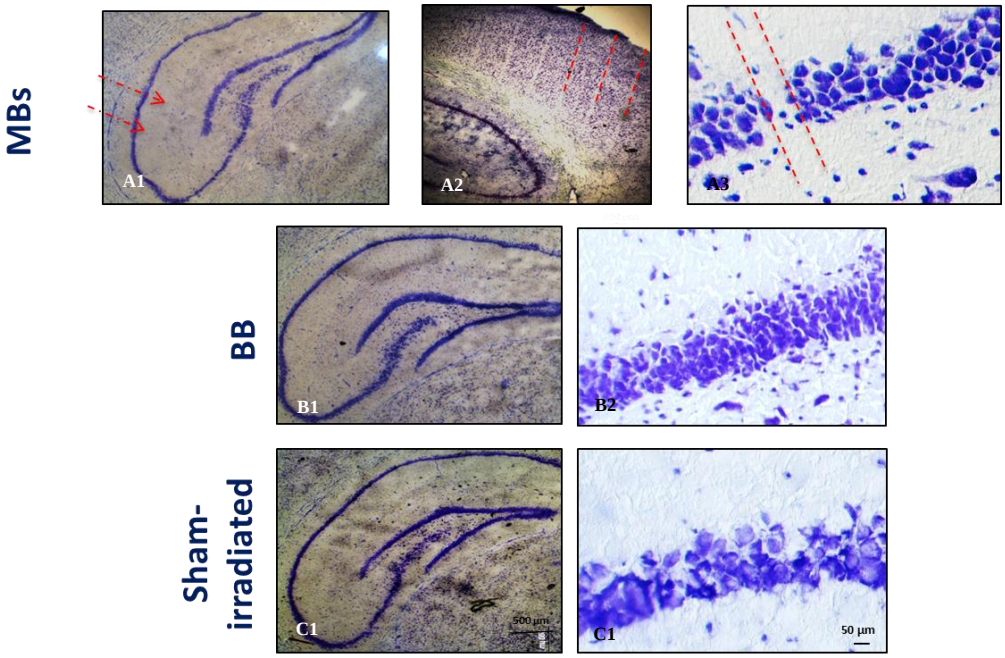

Figure III-52. Morphology of irradiated and not-irradiated hippocampus 3 months after exposure of coronal frozen brain slices. A1-A3 shows MBs irradiated-hippocampus within neuronal loss in the microbeam path (red lines); B1-B2 shows BB irradiated hippocampus within not neuronal loss and no neuronal ectopia and C1-C3 shows intact hippocampus in sham-irradiated rats. DG1: one part of *denatus gyrus* region corresponding to the larger images in A2, B2 and C2. Scale bars for A1, B1, C1: 500 µm; A2, 100 µm; A3, B2, C2: 50 µm.

III.4.3.5 Mature neurons after 3 months from irradiation

The most widely used marker for mature neurons is NeuN, which typically strongly marks the post-mitotic state of cells. NeuN was detected in all sections examined after 3 months from MBs irradiation (Figure III-53).

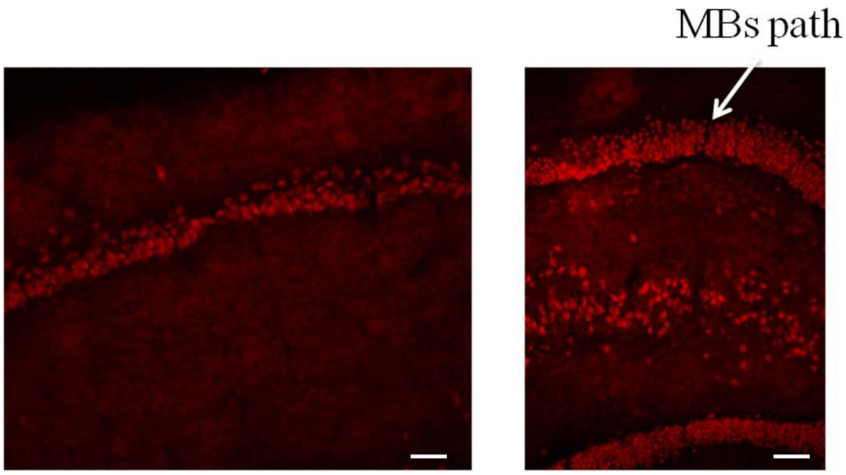

Figure III-53. Photomicrographs showing rat DG labeled with NeuN after 3 months from microbeam (MB) irradiation. Mature neurons show expression of NeuN (red) in the area surrounding MB paths. Arrow indicates the microbeam path. Scale bar: 100 µm.

III.4.3.6 Analysis of the reactive glyosis one year after irradiation

The experiment was performed to identify reactive glyosis in three main hippocampal regions (DG, CA1 and CA3) irradiated by MBs and BB. Using GFAP immunofluorescence, the astrocytes were observed in both irradiated groups and no difference was shown if compared with sham-irradiated rats. In particular, it seems that in the dentate gyrus there is more reactive glyosis in MBs-irradiated hippocampus than in the BB- and sham-irradiated group (Figure III-54 A-C); in the CA1 region it seems that there was no change in quantity of $GFAP^+$ cells (Figure III-54 D-F); in CA3 region it seems that there is no difference among the groups and in the CA3 region it seems that in BB-irradiated hippocampus there is more high reactive glyosis with respect to the MBs and sham (Figure III-54 G-I) groups.

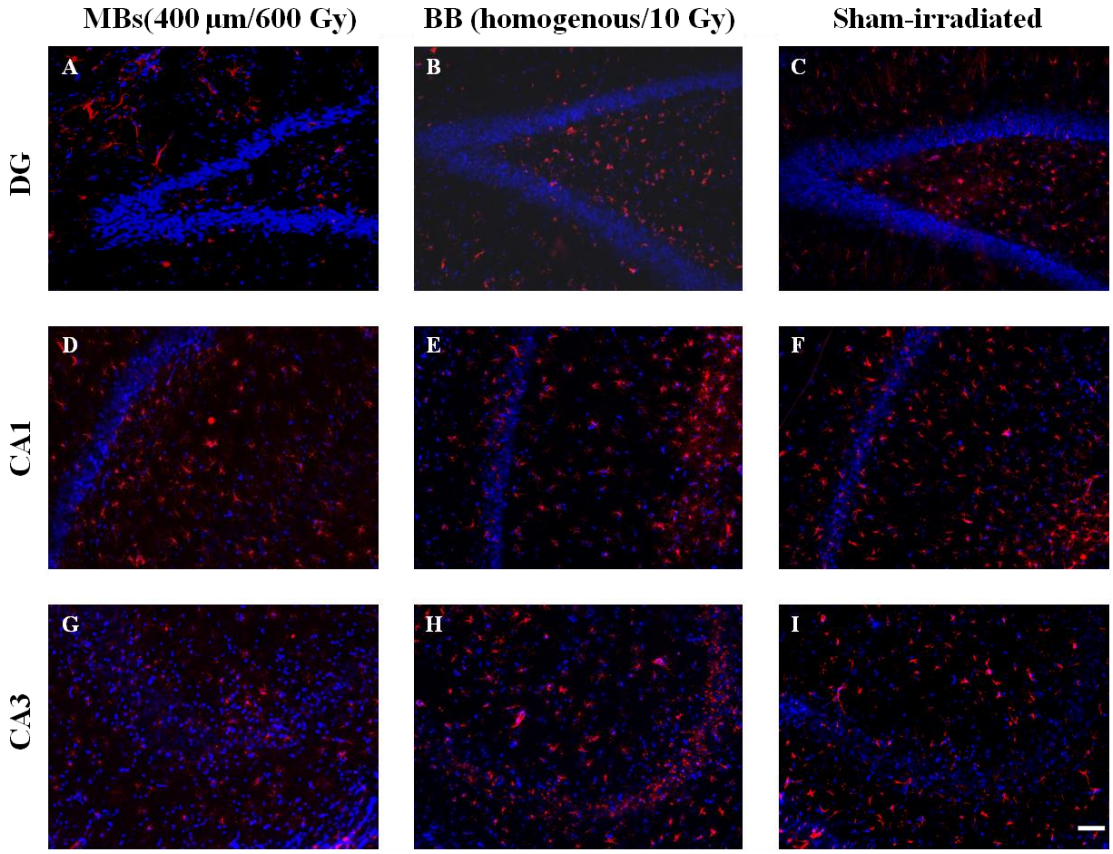

Figure III-54. Immunofluorescence for GFAP in perfused frozen rat brain coronal sections. This images show the presence of active glyosis after 1 year from irradiation. CA= *Cornious ammonium*, DG=*dentatus gyrus*. Scale bar: 50 µm.

III.5 Discussion

Cranial RT is an essential therapeutic tool in the treatment of various brain tumors but at the same time, the ionizing radiation, kills dividing cells in non-cancerous tissue. Even if mature neurons are postmitotic and therefore not directly affected by radiation, the brain's actively dividing neural stem cells (NSC) are largely wiped out, even by very low doses (Mizumatsu et al. 2003). One of the most important neurogenetic areas in the brain is the hippocampus that plays an important role in cognitive functions such as learning, memory and spatial information process.

Assuming that the synchrotron X-ray generated-microbeams are well tolerated by tissues at high doses, the purpose of this study was to investigated the effects of hippocampal

transections at 600 Gy, as skin-entrance dose, on the preservation of stem cells and cognitive impairments. In all experiments we compared the MBs irradiation with the BB irradiation dose (10 Gy). This BB radiation was chosen because other works have shown that it is able to eliminate neuronal stem cells in the hippocampus and SVZ of adult rats or mice (Mizumatsu et al. 2003; Tada et al. 2000; Parent et al. 1999).

Our first objective was to visualize microbeam transections 1 day after irradiation, for this reason we used pH2AX immunostaining. Immunocytochemical detection of the phosphorylation of the histone H2AX to form pH2AX represent the most common method to visualize and quantify ionizing radiation-induced DNA double- strand breaks (Rothkamm et al. 2012; Dickey et al. 2009). This method gave the possibility to combine this marker with Ki-67 antibody, a nuclear protein involved in cell mitotic process (Scholzen & Gerdes 2000). Our results (Figure III-14) suggest that there are proliferative cells between microbeam paths after 2 days from irradiation. Ki-67 labeled proliferation cells such as neurons, endothelial cells (EC), macrophages/monocytes, and astrocytes. All these cells are involved in the effect-response of microbeam radiation. For instance, ECs are involved in the vascular biology, in fact Sabatasso and coworkers (Sabatasso et al. 2011) found that vascular toxicity and physiological effects of microbeam radiation depend on the stage of capillary maturation. Then, the presence of macrophages and astrocytes is possible as response to radiation injury (Serduc et al. 2006a; Slatkin et al. 1995; Calvo et al. 1988). The preservation of proliferative cells in tissue irradiated with MBs compared with the one irradiated with BB after 2 days confirm the high tolerance of tissue to microbeam radiation (Figure III-49) at very high dose (600 Gy). Assuming that mature neurons are terminally differentiated, whereas stem or progenitor cells have a prominent proliferative capacity and an highly vulnerability to irradiation; our future perspective is to investigate how MB irradiation would affect stem/progenitor cells in the hippocampus adopting the strategy of co-labeling. This strategy would be necessarily to explore the potential of migration and differentiation of the precursors detected by BrdU incorporation, the migration marker for neurons (DCX) and the specific marker for mature neurons (NeuN), should be used to count the BrdU-positive cells in the DG of hippocampus after MBs tansections following time table. Therefore, would be possible characterized the neuronal or astrocyte proliferation with Ki-67^{+}/NeuN^{+} or Ki-67^{+}/GFAP^{+}, respectively.

To verify the preservation of proliferative cells we performed an experiment in which we irradiated the same animal with both BB on the left side and MBs on the right side. Our findings showed that after 1 month from irradiation there was no quantitative difference of

proliferative cells marked with BrdU between the two hemispheres (Figure III-51). In the literature it is reported that a single exposure to a dose of 15 Gy eliminated 85% of BrdU positive proliferating cells in the SVZ while inducing little noticeable histological damage (McGinn et al. 2008). In literature, is shown that X-ray irradiation ablate proliferating populations of neural stem and progenitor cells in embryonic, juvenile and adult rats (Tada et al. 2000; Parent et al. 1999) as well as mice (Mizumatsu et al. 2003; Raber et al. 2004). Hence, our results showed that no differences in BrdU positive cells population between microbeams and broad beam irradiated hemispheres, this could be probably explained by a migration of cells through the *corpus callosum* (Hoehn et al. 2002; Flexman et al. 2011) from MB-irradiated hemisphere to BB-irradiated one. Comparing these quantitative results with other ones obtained from unilaterally irradiation (BB or MBs) at the same time course (1 month) (Figure III-49 and Figure III-50) it could give an answer on a possible cross-reaction between the two different irradiated-side modes.

MR Imaging and NISSL histology, both performed at 3 months from irradiations, identified no oedema (Figure III-43) and no cytoarchitecture abnormalities in the dorsal hippocampi irradiated with MBs (Figure III-52). This investigation has demonstrated differences in the tissue response between BB and MB irradiations. In fact, one key aspect of this study is that after MBs irradiation, proliferative cells in the hippocampus are partially affected differently from the BB case. This indicates the high tolerance of healthy tissue to array of MBs delivered at very high doses.

Our results showed what is reported in literature, starting from Laissue and coworkers (J.-A. Laissue et al. 2001) that verified the radioresistence of healthy tissue using the larger brain of the piglet as a surrogate for human pediatric MRT. The cerebellum (1.5 cm x 1.5 cm) received an irradiation of 625 Gy with microbeams of ~28 µm-wide and spaced by 210 µm. Then, after 15 months from irradiation, histology was performed and it showed evidence of cell destruction along the beam path only ("stripes"). In a parallel study (Slatkin et al. 1995) a rat brain was irradiated using parallel beams of 37 µm-wide separated by 75 µm on center–beam spacing. MBs were delivered at 312, 625, 1250, 2500, 5000, or 10000 Gy as skin-entrance dose. The result was that at 10000 Gy the brain tissue developed necrosis in and around the irradiated slices. All the other animal group irradiated at 5000 Gy, 2500 and 1250 Gy showed loss of neuronal and astrocytic nuclei and their perikarya in the stripes. While, brain tissues between skin entrance doses of 312 and 625 Gy appeared normal. Proceeding in the radiotolerance research way, another research group (Dilmanian et al. 2005) showed temporarily demyelitation 2 weeks post irradiation without axonal and vascular damage,

followed by a remyelization 3 months post-irradiation. Same tolerance after three months was confirmed by the same research group (Anschel et al. 2007) that showed irradiation of spinal cord with 270 µm-thick planar beam at 740 Gy as entrance dose. Then, Serduc and collaborators (Serduc et al. 2006a) demonstrated clearly the tolerance of the vascular bed to microbeam irradiation at very high doses (1000 Gy), looking to the lack of extravasation of dyes administered to the experimental animals.

Until now in our discussion we spoke about the high tolerance of healthy tissue to the MBs delivered at high irradiation dose using immuo-histology techniques and MRI scan images to see edema eventually expose results came out from our experiments and compared to the literature. Since that our experiments were performed in the hippocampus and knowing that hippocampus play an important role in memory and learning involvement in cognitive performance, we performed after one year from irradiation a behavioral test called radial eight arm maze (RAM).

As it is well known the nervous system is very sensitive to DNA damage, particularly in comparison with other non-replicating cell types (*i.e.* skeletal and cardiac muscle); it is often profoundly affected by DNA repair deficiency, which can result in neurodegeneration (McKinnon 2009). Unrepaired DNA damage in the nervous system can be sufficient to cause cognitive decline (Borgesius et al. 2011).

After 1 year from MBs- and BB-radiation on normal rats no significant differences in memory and learning were showed. Unfortunately, these results have to be taken very cautiously for several reasons, such as the environmental conditions in the room (*i.e.* silence); rat strain (Wistar strain is not very common for radial arm maze test), and their age. In particularly, the animal age is important in the regulation of neurogenesis process, because is a factor that influences whether deficits in spatial learning after reduction of neurogenesis (Martinez-Canabal et al. 2012).

Therefore, in literature is evident that RT at relative low-dose (lower than 10 Gy) doesn't affect cognitive function. However, high dose of radiation (20 Gy) may harm the nervous system, resulting in poorer spatial learning and memory (Zhang et al. 2013). This last result was previously showed by Snyder and co-workers (Snyder et al. 2005) using radiation doses such as 10 Gy, this irradiation caused block of the neurogenetic process and a long-term spatial memory deficits assessed by Morris water maze.

Our perspective is to deeply understand the cognitive impairments on animal irradiated with microbeams at different time points using a RAM and compare them with rats irradiated with broad-beam at 10 Gy, used as rat model of convential radiotherapy.

After behavioral test we performed immunohistology on the same animals to verify reactive gliosys. By our results seems that there is the same level of reactive gliosys in MBs- and BB-irradiated rats. Our findings are in agreement with the literature, means that at later stages of irradiation there are evidences of increasing glyosis and demyelitation (Tofilon & Fike 2000).

This work has a promising future and further synchrotron experiments are foreseen to investigate memory and learning after irradiation and the amount and type of proliferative cells. It should be possible to study neurogenesis process affected by MBs, and its relationship memory and learning tasks using various behavioral tests such as radial eight arm maze, water arm maze and object recognition memory task.

Hence, this work suggests that much work needs to be done to understand the effectiveness of MBs transections on the hippocampus.

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Chapter IV
Conclusion and future directions

Microbeam radiosurgery is under continuous development since the 1990s. The advantages of this technique over currently employed radiosurgical techniques include a higher therapeutic index due to the higher sparing of healthy tissues, a sharp dose fall-off which can contribute to highly circumscribe the induced damage and the possibility to deliver the dose with very high precision. This methodology could be used in the treatment of malignant brain tumours and of no-cancerous diseases such as epilepsy.

In fact, the aim of this dissertation was to explore in a rat model the effects of microbeam radiosurgery to treat a brain disorder such as epilepsy and to study the preservation of proliferative cells in the hippocampus after irradiation.

We performed cortical and hippocampal transections using microbeam arrays delivered at very high doses with minimal damage in the tissue surrounding the irradiated area. Data showed in this Thesis work provides the evidence that cortical transections, made by microbeam radiosurgery, can control seizures in epileptic animal model and no paralysis or minimal paralysis were evident in normal rats. Moreover, after hippocampal transections at very high doses proliferative cells are present between microbeams paths.

- **Cortical transections on normal and epileptic rats**

In the second Chapter we showed the high tolerance of the motor cortex to MBs delivered at different radiation doses and its power on an epileptic rat model. In fact, our starting hypothesis was to use microscopic and localized incisions delivered by synchrotron radiation to stop the propagation of seizures in a low invasive way.

Our aims were to verify both, the preservation of cortical architecture following high-dose microbeam irradiation and the ability to induce non-invasively the equivalent of a surgical cut (MST) over the cortex either, in epileptic and normal rats.

As reported in literature, MST can help to reduce or to stop the spreading of seizures arising from functional cortical areas. The side effect is that the epileptic activity may recur after a period of 2 to 20 months. It is uncertain whether this procedure can achieve long-term seizure control.

For this reason we used an epileptic rat model, created by injection of kainic acid, to study the effect of microbeams transections on seizures. Our findings showed a reduction of seizures after MBs irradiation. Performing different types of irradiation (MBs size from 25 to

600 µm, center-to-center distance from 200 to 1200 µm, peak entrance doses from 600 to 150 Gy), we established a different biological effects of cortical transections performed through microbeams with a thickness between 100 and 400 µm-wide, and those performed through "thick" microbeams (or minibeams) having a thickness of 500 µm-wide or larger. These cortical transections were performed using a parallel or convergent geometry. As mentioned before, parallel and convergent transections were performed on sensorimotor cortex and, for this reason, motor performance was assessed by a Rotarod® test. No paralysis was observed in normal animals undergoing cortical transections. Therefore, in most of all the groups irradiated, the motor performance was reordered as normal.

Our results are in agreement with previous studies that revealed the different biological effects on healthy tissue of microbeams and minibeams without showing neurological deficit in rats (Uyama et al. 2011; Dilmanian et al. 2008). We think that this biological effect could be explained by the different cross-talk connections between the vascular network (during angiogenesis process) and neurons (during the neurogenesis process), hence between the seamless irradiated area (valley dose zone) and the transected area (peak dose zone) (Anschel et al. 2010; Slatkin, Blattmann, Wagner, Glotzer & Laissue 2007; Serduc et al. 2006a; Van De Looij et al. 2006; Slatkin et al. 1995).

This was a pilot study that demonstrated the feasibility of microbeams transections towards the sensorimotor cortex but further studies are necessary to better understand the methodology which will be useful for future animal experiments and for the treatment of epilepsy in humans as future perspective.

- **Hippocampal transections on normal rats**

In this chapter we showed that MBs irradiation can partially affect cells in proliferation compared with conventional radiotherapy. In fact, our starting hypothesis was the delivery of microbeams at high radiation doses (600 Gy) to the dorsal hippocampus minimally (or does not) effect the hippocampal morphology and neurogenesis process. In particular we tested microbeam transections (9 beams of 75 µm-width separated center-to-center by 400 µm, with skin entrance dose of 600 Gy and 4.1 Gy as valley dose) delivered to the hippocampus of adult Wistar rats. Neurogenesis process is linked to cognitive performances, and therefore, for this reason, we developed a behavioural test to study learning and memory performances comparing MBs *vs* and BB (delivered at 10 Gy, considered as conventional radiotherapy rat model) (Soussain et al. 2009).

Through an immunohistochemical analysis we found that there is a partial alteration of proliferative cells in the pathway of MBs irradiated-hippocampi compared with

homogeneous beam (measured 2 hours later). Also, by gadolinium-enhanced MRI we verified the absence of oedema at 3 months from irradiation.

Hence, we found that microbeam transections delivered to the hippocampus did not induce significant behavioural impairment, but we must consider that this test was performed not under perfect conditions as already discussed. To the best of our knowledge, this type of behavioural test was never performed on X ray-irradiated Wistar rats; the literature reported use of other kinds of memory and learning tests, such as open field and Morris water arm maze (Zhang et al. 2013). We decided to use this kind of instrument because in this way we were able to measure both, reference and working memory, at the same time. As formerly delineated, results showed the preservation of hippocampal structure with evidence of highly precise transections that divide the hippocampus in columns was evident without induce any cytoarchitecture alteration.

This second part of the work confirms the safe delivery of high doses of radiation to specific and radiosensitive part of the brain, if performed using microbeams. The next step is to quantify all the cells involved in the neurogenesis process at different time points to verify the microbeam impact and also develop new behavioural tests to understand at which level learning and memory are involved.

In conclusions, our findings lead us to investigate the potential use of microbeam transections to treat focal epilepsy or situation which in stem cells preservation is required to understand cognitive problem during radiation therapy.

Besides, our future plans foresee to continue studying the effects of microbeams delivered at very high doses to very small target brain region such as the *Substantia nigra pars compacta* (SNc) or the *Subthalamic nucleus* (STN). We performed a pilot study on few animals with the aim to ablate the STN, because it is a highly effective surgical target of PD, but is associated to severe complications such as brain oedema or radionecrosis. Stereotactic delivery of microbeams towards the SNc or STN could mimic a parkinsonian syndrome. In this way it could be possible create a new experimental animal model of parkinsonism to study the pathophysiology of PD. Furthermore, with this method should eliminate both mechanical damage and bleeding belonging to the stereotactic surgery necessary to induce symptoms of Parkinson disease through injection of drugs such as 6-OHDA or MPTP.

Hence, the development of clinical devices delivering submillimetric beams able to generate cortical or hippocampal transections might become a new powerful tool for the clinical treatment of functional brain disorders.

La radiochirurgie par microfaisceaux connaît un développement continu depuis les années 1990 motivé par la mise en évidence de phénomènes de radiotolérance des tissus sains. La reconstruction des tissus irradiés par ces rayonnements de grande précision et de haute intensité, est attribuée à la capacité de régénération des tissus sains entourant les lésions et constitue un avantage par rapport aux techniques de radiochirurgie conventionnelles. Cette méthode est utilisée dans le traitement des tumeurs cérébrales malignes mais aussi pour les maladies non-cancéreuses telles que l'épilepsie.

L'objectif de ce travail était d'étudier les effets de la radiochirurgie par microfaisceaux dans le cadre de l'épilepsie sur un modèle de rat épileptique. La préservation des cellules souches de l'hippocampe, après irradiation à des doses très élevées, a aussi été étudiée dans le cas des rats normaux.

Dans le cadre de cette étude, des transsections au niveau du cortex et de l'hippocampe ont été effectuées en utilisant des microfaisceaux de doses élevées (de 150 à 600 Gy). Un minimum de dommages dans les tissus entourant la zone cible a été observé. Les données acquises lors de cette thèse ont fourni la preuve que les transsections corticales par microfaisceau permettent de contrôler les convulsions sur le modèle animal épileptique étudié sans générer de dommages moteurs chez les animaux normaux. Par ailleurs, une prolifération de cellules entre les zones irradiées après transsections de l'hippocampe a été observée, soutenant les observations comportementales..

- **Transsections corticales chez rats normaux et épileptiques**

Dans le deuxième chapitre nous avons montré, pour les rats épileptiques et normaux, la préservation de l'architecture corticale après irradiation de microfaisceaux de haute dose. Nous avons montré par ailleurs la capacité des microfaisceaux à induire une incision chirurgicale non invasive (MST) au niveau du cortex.

Dans la littérature il a été démontré qu'une MST pouvait aider à réduire, voir arrêter, la propagation des crises provenant des zones corticales fonctionnelles. Malheureusement il est possible qu'une récidive de l'activité épileptique apparaisse après une période de 2 à 20 mois. En conséquence cette procédure ne permet certainement pas un contrôle des crises à long terme.

Pour cette raison, nous avons utilisé un modèle de rat épileptique, créé par injection d'acide kaïnique (KA) pour étudier l'effet des transsections par microfaisceaux sur les crises épileptiques. Nos résultats ont montré une réduction des convulsions après irradiation. Les transsections ont été effectuées en utilisant différents types d'irradiation (taille des

microfaisceaux de 25 à 600 µm, la distance pic à pic de 200 à 1200 µm, les doses d'entrée de 600 à 150 Gy). Une différence des effets biologiques entre les faisceaux compris entre 100 et 500 µm et les plus épais a été observée (minifaisceaux). Les transsections corticales ont été effectuées en utilisant soit une géométrie parallèle soit convergente. Comme mentionné précédemment, ces transsections ont été réalisées sur le cortex sensori-moteur. Les performances motrices ont été évaluées par le test du Rotarod®. Aucune paralysie n'a été observée chez les animaux normaux et tous les groupes irradiés ont montré une performance motrice physiologique.

Nos résultats sont en accord avec les études antérieures qui ont montré les différents effets biologiques sur tissus sains entre micro et mini faisceaux sans causer de déficit neurologique chez les rats (Uyama *et al* 2011 Dilmanian *et al* 2008) entre la zone vallée (partiellement irradié) et la zone pic (chemin des microfaisceaux).

Cette étude pilote a ainsi démontré la faisabilité des transsections par microfaisceaux sur le cortex sensori-moteur. De plus amples études sont nécessaires afin de mieux comprendre la méthodologie qui sera utile pour le traitement de l'épilepsie chez le petit animal ou l'homme.

- **Transsections hippocampe sur des rats normaux**

Dans le troisième chapitre, nous avons montré la capacité de régénération des cellules souches de l'hippocampe, chez les rats Wistar adultes en bonne santé, après irradiation par microfaisceau de doses très élevées.

Ainsi que décrite dans la littérature, la neurogenèse au niveau de l'hippocampe est un mécanisme important impliqué dans la fonction cognitive et son irradiation peut provoquer des déficiences dans les tâches d'apprentissage et de mémoire (Soussain *et al* 2009).

C'est donc en nous basant sur l'état de l'art que nous avons décidé de soumettre cette structure clef à un traitement par microfaisceaux (9 faisceaux de 75 µm de largeur séparés centre-à-centre de 400 µm, avec la dose d'entrée de la peau de 600 Gy et 4,1 Gy en dose vallée).L'étude effectuée sur une population de rats adultes, basée sur l'observation des fonctions cognitives et des structures histologiques, a permis de mettre en évidence une conservation des cellules prolifératives permettant de reconstituer l'intégrité des zones traitées. Cependant, l'absence de troubles notables du comportement doit être tempéré puisqu'il faut considérer que ce test n'a pas été effectué dans des conditions optimales ainsi qu'il en a été discuté dans le chapitre III. En nous basant sur une étude approfondie de la littérature, il semble que ce type de test de comportement n'a jamais été réalisé sur des rats Wistar irradiés par microfaisceaux par rayonnement synchrotron. La littérature rapporte néanmoins l'utilisation d'autres types de tests de mémoire et d'apprentissage, tels que le champ ouvert et le labyrinthe de bras d'eau de Morris (Zhang *et al* 2013). Les résultats ont

montré la préservation de la structure hippocampique et la précision des transsections au niveau de l'hippocampe. Cette seconde partie confirme la radiorésistance du tissu cérébral irradié par microfaisceaux.

L'étape suivante consistera à quantifier les cellules impliquées dans le processus de neurogenèse à différents points dans le temps pour vérifier l'impact des microfaisceaux et également développer de nouveaux tests de comportement permettant une meilleur compréhension de l'implication des processus de l'apprentissage et de la mémoire.

D'après les conclusions de ces travaux, l'utilisation de microfaisceaux au niveau du cortex moteur et de l'hippocampe semble être un outil potentiel à développer pour traiter l'épilepsie focale ou autre trouble du cerveau. De plus, la préservation des cellules permettra de comprendre les problèmes de mémoire et de l'apprentissage observable après une radiothérapie.

Nous prévoyons, dans un futur proche, de continuer à étudier les effets des microfaiseaux de très hautes doses sur de petites régions cibles telles que la *Substantia nigra pars compacta* (SNc) ou du *Subthalamic nucleus* (STN). Nous avons réalisé une étude pilote sur un petit nombre d'animaux (rats adultes) dans le but de pratiquer l'ablation du STN qui est une cible chirurgicale très efficace de PD. ECette intervention est néanmoins associée à des complications graves telles que l'œdème cérébral ou les radionécroses. Le traitement stéréotaxique par microfaisceaux de la SNC ou du STN pourrait imiter le syndrome parkinsonien. De cette façon, il pourrait être possible de créer un nouveau modèle animal expérimental de parkinsonisme et donc, d'étudier la physiopathologie de la maladie de Parkinson. De cette façon, avec cette méthode, les dommages mécaniques et les saignements liés à l'opération de chirurgie stéréotaxique seraient éliminés, rendant possible l'administration de drogues telles que le 6-OHDA ou MPTP, pour induire les symptômes de la maladie de Parkinson.

Donc le développement de dispositifs cliniques délivrant des faisceaux submillimétriques générant des transsections corticales ou de l'hippocampe pourrait devenir un nouvel outil pour le traitement clinique des troubles fonctionnels du cerveau.

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Synchrotron-Generated Microbeam Sensorimotor Cortex Transections Induce Seizure Control without Disruption of Neurological Functions

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Abstract

Synchrotron-generated X-ray microplanar beams (microbeams) are characterized by the ability to deliver extremely high doses of radiation to spatially restricted volumes of tissue. Minimal dose spreading outside the beam path provides an exceptional degree of protection from radio-induced damage to the neurons and glia adjacent to the microscopic slices of tissue irradiated. The preservation of cortical architecture following high-dose microbeam irradiation and the ability to induce non-invasively the equivalent of a surgical cut over the cortex is of great interest for the development of novel experimental models in neurobiology and new treatment avenues for a variety of brain disorders. Microbeams (size 100 μm/600 μm, center-to-center distance of 400 μm/1200 μm, peak entrance doses of 360-240 Gy/150-100 Gy) delivered to the sensorimotor cortex of six 2-month-old naïve rats generated histologically evident cortical transections, without modifying motor behavior and weight gain up to 7 months. Microbeam transections of the sensorimotor cortex dramatically reduced convulsive seizure duration in a further group of 12 rats receiving local infusion of kainic acid. No subsequent neurological deficit was associated with the treatment. These data provide a novel tool to study the functions of the cortex and pave the way for the development of new therapeutic strategies for epilepsy and other neurological diseases.

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Introduction

Epilepsy is a common neurological disorder with an incidence of 0.3–0.5% in different populations throughout the world. Approximately 20–30% of patients with epilepsy continue to have seizures despite efforts to find an effective combination of antiepileptic drugs. Epilepsy surgery provides an effective tool to ameliorate seizures through the epileptic focus surgical ablation. The resection of large amounts of cortical or hippocampal tissue can be associated to adverse neurological events. The risk to induce severe neurological deficits is particularly high when the epileptic focus involves the eloquent cortex, such as speech or primary motor areas. A non resective surgical technique called multiple subpial transections (MSTs) was developed to treat patients with medically-refractory epilepsy involving eloquent cortex [1–2]. This technique requires the placement of vertical incisions through the epileptic cortex in order to cut the horizontal axons responsible of the propagation of seizures while preserving the vertical axons subserving neurological functions [1–9]. The vertical columns described by Mountcastle as the basic unit of cortical function [7–9] are disconnected but not injured by this approach, allowing the treatment of epileptic foci located over sensorimotor or language cortex not amenable to surgical resection. Several studies have shown the ability of MSTs to stop the propagation of epileptogenic activity and improve seizure control without inducing devastating neurological deficits [1–2,10–12]. Synchrotron-generated X-ray beams (microbeams) can induce the equivalent of a surgical cut through the brain tissue by delivering very high doses of radiation (hundreds to thousands of Grays) to tissue slices of microscopic thickness [13–14]. In synchrotrons, X-ray beams are tangentially emitted by relativistic electron bunches circulating in a storage ring. The X-ray source is a wiggler (a magnetic structure of alternating poles positioned on a straight section of the storage ring) producing a wide spectrum of photons with an energy range up to several hundreds of kilo-electronvolts (keV). The quasi-laminar and minimally divergent beam can be spatially fractionated into an array of rectangular microbeams of variable size (typically 25–600 μm). Due to an X-ray fluence thousands of times higher than that of standard linear accelerators used in conventional radiotherapy, a dose as high as several hundreds of Grays can be delivered in a fraction of a second. The microbeam ability to reproduce the effects of a

surgical cut originates from their peculiar dosimetric characteristics. The X-ray dose delivered to the thin strip of irradiated tissue is reduced by about two orders of magnitude within a submillimetric distance (Fig. 1) [15–16]. Therefore, neurons and axons along the penetration path die due to overwhelming doses of radiations (over 100 Gy) while the immediately adjacent tissue is exposed to low doses unable to induce histologically evident tissue damage [17], thus generating vertical incisions separating adjacent cortical columns. This new radiosurgery technique is therefore an attractive experimental tool to induce non-invasive cortical transections.

Results

We evaluated whether microbeam cortical transection could be performed on eloquent cortex without induction of neurological damage in an animal model. We also assessed the efficacy of this approach on seizure control. Microbeam cortical transections were generated in the left motor cortex of 6 healthy two month-old Wistar rats. We explored the effect of two different X-ray microbeam arrays: the sensorimotor cortex of 3 rats was transected by an array of 7 microbeams having a thickness of 100 µm, center-to-center (c-t-c) distance of 400 µm with a skin entrance dose of 360 Gy along the beam trace (peak dose). A second group of 3 rats underwent 4 cortical transections made with 600 µm wide beams spaced by 1.2 mm and depositing a skin entrance dose of 240 Gy (peak dose). The doses delivered to the tissue volume in the middle between the beams (valleys) were limited to 5.3 Gy and 5 Gy, respectively. Beam delivery was done using a rat atlas-based image guided system in anesthetized rats (Fig. 2). Fluorescence immunostaining for phosphorylated H2AX, carried out 24 hours after irradiation, showed a clear-cut microbeam path through the motor cortex (Fig. 3). No acute or chronic neurological or behavioral abnormality was observed after irradiation. Long-term behavioral and neurological monitoring was carried out until sacrifice 7 months after irradiation. These rats showed a regular weight gain and no signs of motor behavior impairment as assessed by the rotarod test (Fig. 4). Histological analysis revealed the absence of neurons along the irradiation path and preservation of the cortical architecture in the immediately adjacent tissue (Fig. 5). To test the hypothesis that cortical column disconnection by microbeam irradiation can provide an effective barrier against the spreading of seizures, we used a rat model of Kainic Acid (KA)-induced convulsive epilepsy. KA is an exogenous agonist of KA ionotropic glutamate receptors which induces severe convulsive seizures following local injection into the sensorimotor cortex [12]. All rats (n = 18) injected with KA developed convulsive seizures (forelimb contralateral or bilateral jerks) within 26±12 min, followed by generalized tonic-clonic seizures and status epilepticus (within 2 hours). Rats were then divided into two groups: control rats (n = 6) and rats receiving microbeam transections (n = 12). Using the same irradiation geometry previously described for healthy rats, transections were induced using either 100 µm wide (n = 6) and 600 µm wide (n = 6) beams. A high dose (HD) and a low dose (LD) protocol were tested for both microbeam groups: peak-valley doses of 360-6 Gy (HD) versus 240-4 Gy (LD) were delivered to rats irradiated with 100 µm wide microbeams, whereas peak-valley doses of 150-6 Gy (HD) and 100-4 Gy (LD) were delivered to rats irradiated with 600 µm wide beams. Dosimetric calculations were performed by using Monte Carlo simulations.

Seizure duration was significantly reduced in rats undergoing transections as compared to control rats (Fig. 6) ($p<0.05$ -One-way ANOVA+Bonferroni's test- vs. non irradiated control rats). In control non-irradiated rats convulsive seizures disappeared after 40±12 hours. In the group receiving 100 µm transections, convulsive seizures disappeared after an average of 3.0±0.5 hours and 3.3±0.5 hours in the HD and LD protocols, respectively (n = 3 for each group). A similar result was observed in the group of rats treated with 600 µm beams, with seizures disappearing after an average of 2.2±0.2 hours and 2.6±0.5 hours in the HD and LD protocols, respectively (n = 3 for each group). The differences among the irradiation groups were not statistically significant.

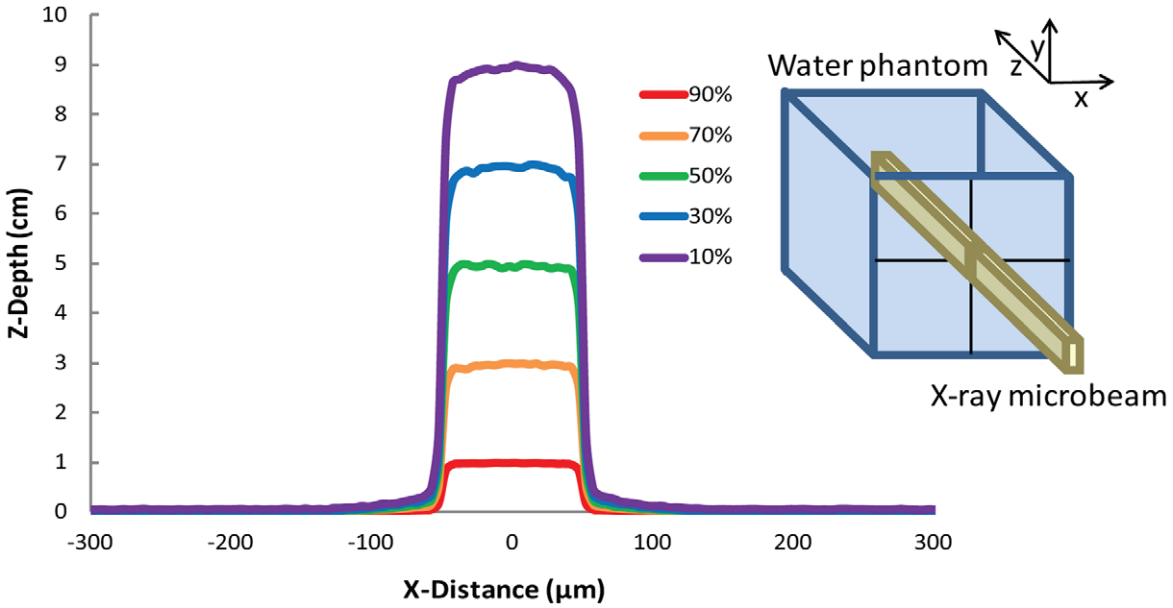

Figure 1. Isodose curves deposited by a microbeam. Dose profiles deposited in a 16×16×16 cm^3 water phantom by a single 100 µm wide and 10 mm high X-ray microbeam of 100 keV (Monte Carlo simulations), i.e. of energy similar to the median energy of the used spectrum (inset), propagating along the z direction The 50% of the dose is deposited at about 5 cm depth. Objects are not to scale.
doi:10.1371/journal.pone.0053549.g001

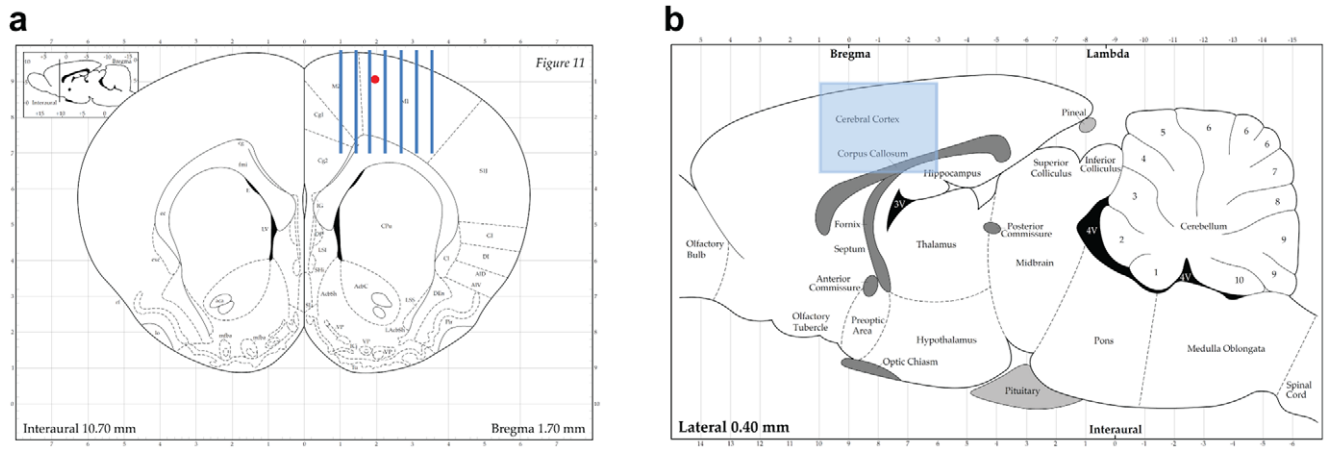

Figure 2. Schematic representation of microbeam irradiation geometry. Coronal (a) and sagittal (b) section of the Paxinos and Watson's rat brain atlas. Arrays of 100 or 600 µm thick microbeams (here represented the 100 µm case, minimum center-to-center spacing: 400 µm) were delivered perpendicular to the sensorimotor cortex using an atlas-based image guided X-ray setup. In (a) is indicated the point where the kainic acid injection was performed to create status epilepticus in rats. Copyright 1998 with permission from Elsevier.
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Discussion

Image-guided microbeam radiosurgery is an emerging tool to generate cortical transections or to induce precise focal brain lesions. The Central Nervous System (CNS) radiobiology of microplanar beams was first studied at the National Synchrotron Light Source (NSLS) of the Brookhaven National Laboratory (BNL), where the preservation of CNS architecture after incredibly high radiation doses (up to 4000 Gy) delivered to mouse brain by 25 µm wide beams was first described [18–20]. Further work at BNL and the ESRF, investigated the tissue tolerance to microscopic beams at doses tens to hundreds time larger than those allowed by conventional macroscopic beams [21–24].

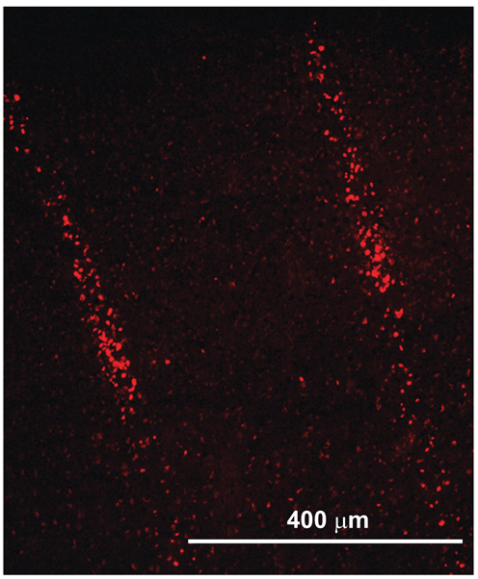

Figure 3. Immunohistochemistry of phosphorylated Gamma-H2AX in the cortex of an irradiated rat. Image shows the microbeam paths across the sensorimotor cortex of healthy rats: apoptotic neurons hit by microbeams (size: 100 µm, c-t-c 400 µm, incident dose 360 Gy) are evident.
doi:10.1371/journal.pone.0053549.g003

Unidirectional irradiation using microbeam arrays has confirmed through several experiments the exceptional resistance of the normal-tissue to high dose microbeam irradiation [24–32], a radiobiological phenomenon referred to as "tissue-sparing effect", indicating the preservation of the architecture of the tissues traversed by microbeams carrying doses tens to hundreds times higher than those associated with radionecrosis after conventional radiotherapy.

The doses to regenerative normal cells and tissues between microbeams, the "valley" doses, are probably the most important determinants of normal tissue damage in MRT [33]. For a given target, the valley dose depends on three main parameters: the microbeam width, the spacing between the microbeams and the dose delivered along each microbeam. For a given peak dose, the valley dose is higher for wider microbeams and for smaller c-t-c distances. In this pilot experiment microbeam sizes and c-t-c distance were here chosen on the basis of the literature describing the tissue sparing effect of X-ray microbeams deposited in the central nervous system [14,33]. We have tested combination of microbeam sizes/spacing at the two extremes of the range better documented in the literature, which spans from 25/200 up to 0.68/1200 micron. After having fixed the size and spacing, the peak dose was chosen in order to determine a valley dose between 4 and 6 Gy that is well tolerated by the brain [34]. Immunohistochemical staining using pH2AX show clearly that the neurons hit by the microbeam along its penetration path die almost immediately while the adjacent cells separated by a few micron but outside the high dose volume remain viable (Fig. 3). Progressively lower doses (but still much higher than in conventional radiosurgery or radiation therapy) are required to avoid tissue damage if thicker beams (100 to 600 µm) are used. Submillimetric beams (sized 0.6 to 0.7 mm) appear to retain the tissue sparing effect allowing the delivery of incident doses of 400 Gy to the spinal cord of rats without inducing neurological damage: irradiation of rat spinal cord with four parallel 0.68-mm thick microbeams at 400 Gy in-depth beam dose did not induce paralysis after 7 months in three out of four rats [24]. This latter study showed not only that a highly radiosensitive structure such as the spinal cord can receive a high irradiation dose through a microbeam array without neurologic sequelae but also that a beam width up to 0.68 mm is well tolerated, substantially maintaining the tissue

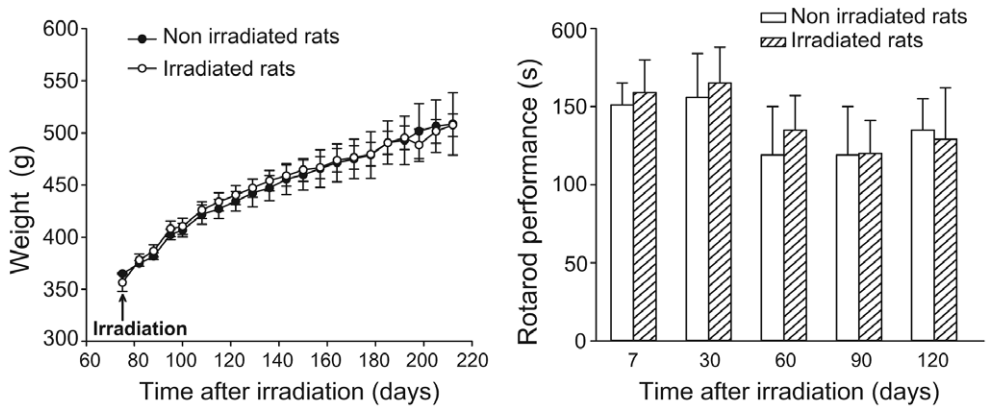

Figure 4. Weight trend and rotarod performance of healthy non-irradiated and irradiated rats. Data are means+S.E.M. (n = 3 for non-irradiated rats and n = 12 for irradiated rats).
doi:10.1371/journal.pone.0053549.g004

sparing properties of thinner beams. The ability of microbeam arrays to avoid radionecrosis and to preserve the architecture of the irradiated tissue is mainly attributed to the rapid regeneration of normal microvessels. Only a short segment of the microvascular bed receives ablating doses while the adjacent endothelial cells fall into the valley dose region receiving just a few Gy and can restore quickly the continuity of vascular supply [32–35]. The wide spatial interface between the unhindered tissue placed in the valleys and the tissue irradiated with peak doses within the microbeam paths facilitates a widespread vascular recolonization of the tissue receiving necrotic doses preventing the dissolution of the architecture of the irradiated tissues [33]. The self-repair of the normal microvasculature through the migration of unaffected cells surrounding the paths of microbeam penetration is considered the most likely basis for this ability of normal tissue to tolerate high dose microbeam irradiation [17,36]. The tolerance of the vascular bed to high dose microbeam irradiation has been clearly demonstrated by the lack of extravasation of dyes administered to the experimental animals, which remained confined in the vessels after irradiation from 12 h until three months following 1000 Gy [17]. This radioresistance phenomenon was not observed in 9L glioma microvessels, confirming the presence of a differential response in normal and tumour brain tissues in rodents, an effect that can have significant clinical applications [28]. The neoplastic vasculature appears to be unable to replicate the fast repair of the segments hit by the peak dose, facilitating the development of radionecrosis over the irradiated tumor [29,37].

A novel way to use microbeam arrays in a quasi-surgical way has been explored here: a microbeam array was placed over selected cortical areas in order hit tangentially and cut the horizontal axons connecting adjacent cortical columns. As described above, cortical transections are a surgical procedure to parcellize an epileptic focus located in eloquent cortex [1,2]. Cutting the horizontal axons required for the spreading of epileptic activity is an effective way to control the seizures without inducing neurologic dysfunction. Synchrotron-generated microbeams can be used to create cortical transections in rats offering a chance to study the tolerance of CNS to this technique. This novel experimental application of microbeams provides a new and attractive tool to modulate cortical function by transecting the fibers connecting the cortical columns. Aside from the tight dosimetry, the low energy of monochromatic beams makes them well suited to treat superficial targets such us the cortex.

The smallest cortical area capable to sustain synchronous spikes generating seizures is estimated to be around 1 cm^2 in monkeys [38] and 0.5 mm^2 in rats [39]. It has been shown that parallel cortical transections with a thickness of 25 μm delivering doses up to 1000 Gy are not associated with short or long-term injury to the

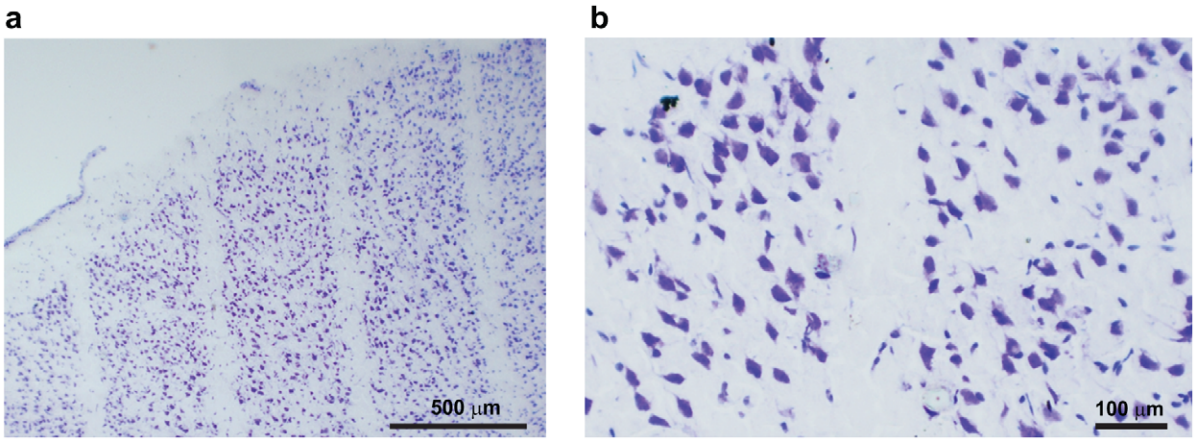

Figure 5. Nissl staining of sensory motor cortex. Nissl analysis performed 7 months after microbeams irradiation shows the lack of neurons along the microbeam path whereas neurons are spared in between microbeams (5b is a higher magnification of 5a).
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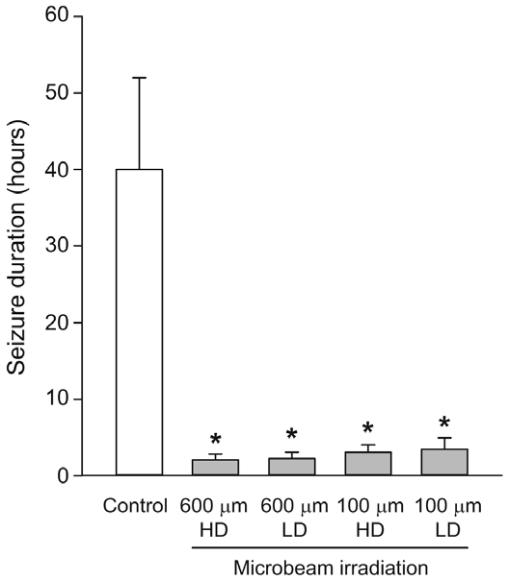

Figure 6. Microbeams irradiations induce a reduction of seizure duration. Duration of convulsive seizures was significantly reduced in rats undergoing transections either with 100 μm wide (n = 6) and 600 μm wide (n = 6) microbeams. A high dose (HD) and a low dose (LD) protocol were tested for both microbeam groups (n = 3 for each of the four irradiated groups and n = 6 for the non-irradiated control group). *$p<0.05$ (One-way ANOVA+Bonferroni's test) *vs.* non irradiated rats (Control). The differences among the irradiation groups were not statistically significant.
doi:10.1371/journal.pone.0053549.g006

adjacent tissue [17]. Therefore microbeam transections appear to be well suited to parcellize and disconnect an epileptic focus, even in small animals. We found here that larger beam size and higher doses were associated with faster relief from convulsive seizures. However all rats undergoing cortical transections recovered from convulsive seizures compared to nontransected controls.

These experiments indicate that submillimetric X-ray beams can modulate seizure spreading in eloquent cortex without inducing evident neurological dysfunction. These results suggest further investigations directed to assess the potential of microbeam transections to modulate cortical functions and to treat focal epilepsy. Microbeam transection, either placed over neocortical seizure foci or through the hippocampus, could prove to be an excellent tool to be added to the current radiosurgical techniques used to control seizures. The development of clinical devices delivering submillimetric beams able to generate cortical transections might add a powerful new tool to the clinical treatment of epilepsy and, more in general, to modulate cortical functions in a wide variety of neuropsychiatric disorders.

Materials and Methods

Animals

All operative procedures related to animal care strictly conformed to the guidelines of the French Government with licenses 380324 and A3818510002. Male Wistar rats (250–275 g) were purchased from Charles River Laboratories (L'Asbresle, France). Rats were kept under environmentally controlled conditions (ambient temperature = 22°C, humidity = 40%) on a 12-hour light/dark cycle with food and water ad libitum.

Irradiation source

Irradiations were performed at the ID17 biomedical beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Details on the technique and of the related instrumentation are reported in [14]. Briefly, synchrotron X-ray radiation originates tangentially from relativistic electron bunches circulating in a storage ring. The source (wiggler) produces a wide spectrum of X-rays with a median energy of 90 keV and range extending, after filtration using Be (0.5 mm), C (1.5 mm), Al (1.5 mm) and Cu (1.0 mm), from 50 to about 350 keV [15]. The quasi-laminar beam obtained so far is collimated into an array of rectangular microbeams of variable size depending on the chosen collimator slits, which are placed about 42 m from the photon source and 1 m upstream from the head of the experimental animals [40]. At ESRF the X-ray dose rate is about 16000 Gy/s.

Geometry of the beam arrays and irradiation set up of healthy rats

Rats were placed vertically and fixed by ear bars and teeth on a custom-made Plexiglas stereotactic frame and placed on a Kappa-type goniometer (Huber, Germany), by which the rat could be translated and rotated in front to the fixed horizontal X-ray beam. The beam height was defined by a (520±5) μm tungsten slit, placed at 1 m upstream the animal. Microbeams of different widths (100±5 and 600±5 μm) and number (respectively, 7 and 4) were used to irradiate different groups of rats. The c-t-c spacing was (400±5) μm center-to center (c-t-c) for the (100±5) μm beams and (1200±5) μm for the 600 μm beams. These beams are shaped by a hard X-ray tungsten collimator which allows producing arrays of microbeams with a ±2 μm precision in width [40]. Irradiated cortical field was 4 mm on the antero-posterior direction (1 mm anterior to −3 mm posterior to the bregma) and, respectively, 2.5 mm and 4.2 mm on the lateral direction (starting 1 mm lateral to midline in the first case and from the midline itself in the second case [41]). The X-ray dose rate was directly proportional to the instantaneous electron current circulating in the storage ring, which was continuously monitored. The dose was delivered by vertically moving the Kappa goniometer, at a speed that was inversely proportional to the dose to be delivered. Each irradiation lasted less than 1 s. The vertical irradiation field was determined by the opening-closing of fast shutters, located 7 m upstream the rats and synchronized with the movement of the kappa goniometer [40]. All movements were remotely controlled and irradiation values were preset by the operator before the treatment. The animal immobility during exposure was checked on 3 control screens located in the control hutch. The incoming spatially non-fractionated dose was measured using an ionization chamber and the mid-valley doses were calculated with Monte Carlo simulations.

Monte Carlo simulations and dose calculations

The Monte Carlo code PENELOPE 2006 [42–43] was used for calculating the deposited dose. PENELOPE simulates the coupled transport of photons, electrons and positrons in the energy interval from 50 eV to 1 GeV, and in arbitrary material systems. PENELOPE has been widely used in the medical physics field [43–45] and in MRT dose assessment [15,46–47]. The main advantage of this code is a careful implementation of accurate low energy electron cross sections, which are of particular importance in this work. The simulation algorithm is based on a scattering model that combines numerical databases with analytical cross section models for the different interaction mechanisms and is applicable to energies (kinetic energies in the case of electrons and

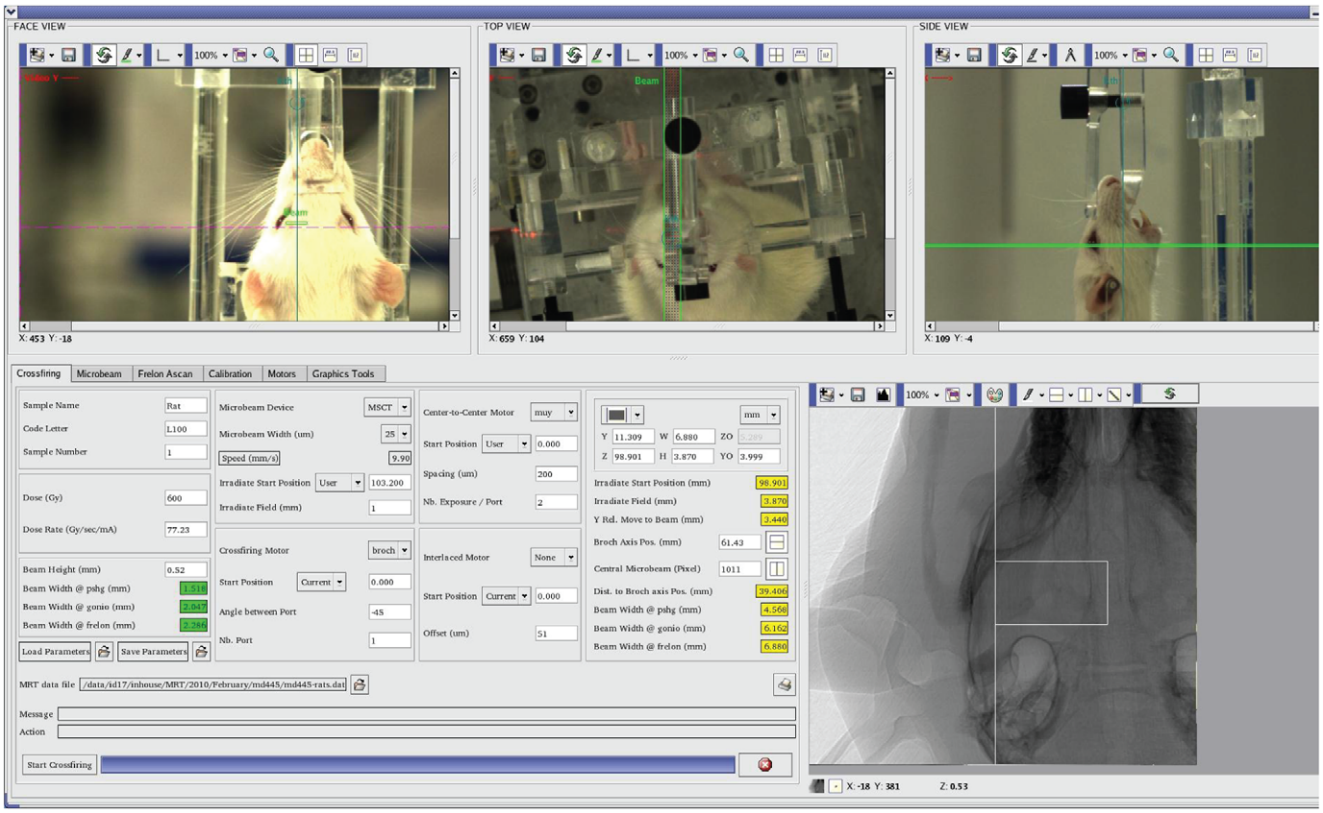

Figure 7. The control panel of the rat irradiation and imaging systems. After an optical prepositioning of the target performed through 3 remote controlled video cameras (three upper panels), high resolution target determination is performed by X-ray imaging. A computer-guided robotic arm (Kappa goniometer, below the rat and not visible in the Figure) moves the stereotactic headframe to acquire radioscopic image necessary to individuate the bregma (radiographic image, bottom right) from which stereotactic coordinates allow the irradiation.
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positrons) from a few hundred eV to 1 GeV. It uses a mixed simulation scheme in which hard interactions are simulated collision by collision and small angular deflections and energy losses are treated in a grouped manner. Since the working energy range is a few hundreds of keV, the most relevant interactions are photoelectric effect and Compton scattering. The photoelectric cross sections used in PENELOPE are obtained by interpolation in a numerical table that was extracted from the Lawrence Livermore National Laboratory Evaluated Photon Data Library [48]. Regarding Compton scattering, PENELOPE considers bounding effects and Doppler broadening when simulating Compton interactions. In this work the number of primary photon stories was 2×10^8 in all the calculations.

Dose deposition simulation geometry

A rat head phantom was constructed with the geometry package in PENELOPE, as described in [15]. It consists of three concentric ellipsoids whose volumes are 13.00 cm^3 for the brain, 3.13 cm^3 for the skull, and 7.15 cm^3 for the skin; these values have been extracted from MRI images acquired on the rats of the same strain and weight. The use of realistic geometries, instead of water phantoms is essential for a correct assessment of the dose deposition. Peak and valley dose calculation was performed following the different irradiation geometries (microbeam number, spacing and width) used in the *in vivo* experiments.

Induction of seizures by local kainic acid injection and irradiation parameters

Male Wistar rats were implanted with intracerebral guide cannulas (Bilaney GmbH, Schirmerstr, Germany), under isoflurane (2%) anesthesia, in a Kopf stereotactic frame. The site of implantation was the left cerebral cortex (coordinates: 1.0 mm anterior to the bregma, 2.0 mm lateral to the midline, 1.0 mm ventral from the outer surface of dura mater [41], Fig. 2). KA (20 µg/1 µl) was slowly injected by a microcannula into the left sensorimotor cortex. The cannula was left in place for 2 min before being withdrawn. Rats were allowed to recover in a large heated box for 1 hour and then were transferred to their home cages. Two hours after seizure induction, epileptic activity spread maximally, leading to status epilepticus. At this point the rats were anesthetized and placed on a plexiglas stereotactic frame fixed on the Kappa goniometer to undergo microbeam sensorimotor cortex transections under general anesthesia (ketamine, 100 mg/kg, +xylazine, 10 mg/kg, i.p.). The alignment of the target was performed by in-situ X-ray image guidance [49]. High resolution (pixel size 47 µm) radiographs were performed on rats placed on the goniometer using attenuated X-rays issued from the wiggler source. The bregma was identified on the radiographs allowing positioning the target following the rat atlas [41] (Fig. 7, bottom right). The total dose per image (one image/animal) was <5 mGy and the total imaging time procedure lasted <5 min. The brain cortex was transected using the same irradiation modalities (coordinates, image guidance, microbeam sizes and spacing, doses) described for healthy rats. The transected rats emerged

from anesthesia shortly after the procedure. The presence of convulsive seizures allowed to rely on behavioral observation for assessment. Electrocorticography was carried out as well in 3 further rats to verify the feasibility of this technique but, given the possibility of dosimetric distortions induced by the interaction of X-ray beams with the EEG electrodes in the brain, we preferred to rely, during this preliminary experiment, on behavioral observation.

Evaluation of post-irradiation behavior in normal rats undergoing motor cortex microbeam transections

Twelve rats underwent left motor cortex irradiation. Rats were placed in large transparent Plexiglas boxes hosting two animals and observed daily for the week following the irradiation and then weekly. Body weight and neurological observation (in particular signs of contralateral hemiparesis) were performed once a week (Fig. 4). Motor behavior was assessed by the rotarod test. The rotarod apparatus consisted of a rotating horizontal cylinder (30 mm) and a motor driver control unit (Ugo Basile, Varese, Italy). The cylinder was divided into five separate rotating compartments and fully enclosed to ensure that the rats did not jump out of their area. Rats were placed on the rod, which was rotating at an accelerating speed from 5 to 15 rpm. Automatic timers recorded the time (in seconds) the rats remained on the rod. Control (non-irradiated) and irradiated rats were assessed 7 days, 1, 2, 3 and 4 months after irradiation. In each day of testing rats were placed on the rotarod apparatus three times.

Evaluation of post-irradiation behavior in rats undergoing motor cortex microbeam transections after local kainic acid injection

Rats were observed for signs of seizures. Seizures were classified as non-convulsive limbic seizures (staring, automatisms) and convulsive seizures (unilateral forelimb clonus, bilateral forelimb clonus, generalized tonic-clonic seizures). After KA injection continuous observation was performed by 3 skilled observers in alternating blocks of 8 hours during the first 24 hours. During the second and third day time, continuous observation was done in blocks of 6 hours. Then the rats underwent observation for 1 hour once a day for 4 days and, after the first week, weekly observations of 1 hour. The period between the first and last observed convulsive seizure was annotated.

Immunohistological evaluation of the short and long term effects of cortical transections in naïve rats

Two days after irradiation, 2 out of 12 naïve rats receiving motor cortex transections were randomly chosen and sacrificed. Brains were rapidly dissected out and 20 μm coronal sections were cut at −20°C on a cryostat (Microm HM560, Walldorf, Germany). For immunohistochemistry, sections were fixed with PFA 4% for 15 min and blocked with donkey normal serum (DNS) diluted in phosphate-buffered saline (PBS) for 1 hour (PBS/DNS 5%). As marker of DNA damage, brain sections were pretreated with alcohol 50% in PBS for 1 hour at room temperature and then incubated with the primary antibody against phosphorylated H2AX (1:500, Upstate Biotecnology, Lake Placid, NY, USA). Sections were washed 4 times with PBS, then incubated with the secondary antibodies Alexa Fluor-conjugated donkey F(ab′)2 (1:200, Invitrogen, Carlsbad, CA, USA) for 2 hours at room temperature. The sections were examined with a Nikon Eclipse E600 microscope equipped for epifluorescence. The remaining rats were killed by decapitation and brains were dissected out and immediately frozen at −80°C. Ten micron thick serial sections were cut and used for histological analysis after having been stained with cresyl violet for Nissl.

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Author Contributions

Developed the irradiation control software: CN. Performed Monte Carlo dosimetry for the experiment: YP. Performed Monte Carlo dosimetry to produce results at Figure 1: JS. Conceived and designed the experiments: PR EB-K EF YP HR CN DJA AB. Performed the experiments: PR EB-K EF YP HR CN DJA AB. Analyzed the data: GLD GB. Wrote the paper: PR GB AB.

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Curēus

A Concise Review of the Emerging Applications of Synchrotron-Generated Microbeams in the Treatment of Brain Disorders

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Abstract

Synchrotron-generated X-ray microplanar beams (microbeams) are characterized by the ability to avoid widespread tissue damage following delivery of doses ranging from hundreds to over a thousand Gray. The preservation of tissue architecture following high-dose microbeam irradiation is known as "tissue-sparing effect" and is strictly related to the ability of microbeams to restrict spatially these exceedingly high doses to the beam path with minimal doses spreading outside to the adjacent tissue. Image-guided microbeam radiosurgery has been recently used to generate cortical transections or to induce deep-seated lesions in the rat brain. The ability to generate focal lesions or microscopic transections over the eloquent and non-eloquent cortex in experimental animals is of great interest for the development of experimental models in neurobiology, opening new treatment avenues for a variety of neuropsychiatric disorders originating from focal brain dysfunction. This paper reviews the current state of research on the radiobiological properties of synchrotron-generated microscopic X-ray beams and their emerging microradiosurgical application, with special reference to the treatment of a variety of brain disorders.

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Review Article
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Introduction And Background

Stereotactic radiosurgery aims to attain growth-control through the ablation of a neoplastic lesion, to induce the obliteration of vascular malformations or to restore the correct functioning of a neural circuitry by modulating or ablating selected brain nuclei or fiber bundles. The ablation of the tissue contained within a well-defined target is achieved through the precise delivery of several hundreds of ionizing radiation beams sized 4 to 60 mm sent from a wide array of directions and intersecting over the volume selected for ablation with 3 to 5 mm 80% to 20% dose fall-out from the volumetric boundaries of the target. There is growing evidence that synchrotron-generated microscopic beams (microbeams) beams can carry much higher doses to the target as compared to conventional high-energy photons or gamma irradiation. Microbeams beams are characterized by the ability to carry extremely high doses without inducing damage to surrounding tissue. This paper aims to offer a concise review of the state of microbeam research applied to the CNS with special reference to the potential new clinical applications that can originate from this novel approach.

Review

Synchrotron-generated X-ray beams are tangentially emitted by relativistic electron bunches circulating in the storage ring of a synchrotron radiation facility. The X-ray source is a wiggler (a magnetic structure of alternating poles positioned on a straight section of the storage ring) producing a wide spectrum of photons with an energy range up to several hundreds of kilo electronvolts (keV). The quasi-laminar beam can be spatially fractionated into an array of rectangular microbeams of variable size by means of a multislit collimator [1]. The X-ray fluence is thousands of times higher than that of standard linear accelerators used in conventional radiotherapy. At the European Synchrotron Radiation Facility (ESRF, Grenoble, France), shown in Figure 1, the dose rate is around 16.000 Gy per second. This dose output is much higher than those available using conventional linear accelerators delivering up to 6 Gy per second. The minimal beam divergence of synchrotron X-rays allows the delivery of extremely high doses to the cells along the penetration path with minimal dose dispersion over the adjacent cells. Depending on the microbeam size, typically between 25 to 75 microns, a quasi-surgical cut involving a width comprising up to 3-4 neuronal cell bodies can be generated along the tissue penetration path (see figure 2). The delivery of an array of parallel beams generates alternating volumes receiving peak doses of several hundreds of Grays and valley doses of a few Grays (see figure 3). The beam distance center-

to center is variable from 100 to 400 gm, depending on the collimator choice and settings. The biological properties of larger beams measuring up to 0.7 mm (also known as minibeams) have been investigated lately. Minibeams could be generated without a synchrotron and can facilitate clinical use [2, 3]. For an overall description of the dosimetric methods, definitions, and calculations, please refer to the references [4, 5].

Figure 1: The European Synchrotron Radiation Facility (ESRF), Grenoble, FR. In foreground: the biomedical beamline ID17, external to the synchrotron radiation storage ring.

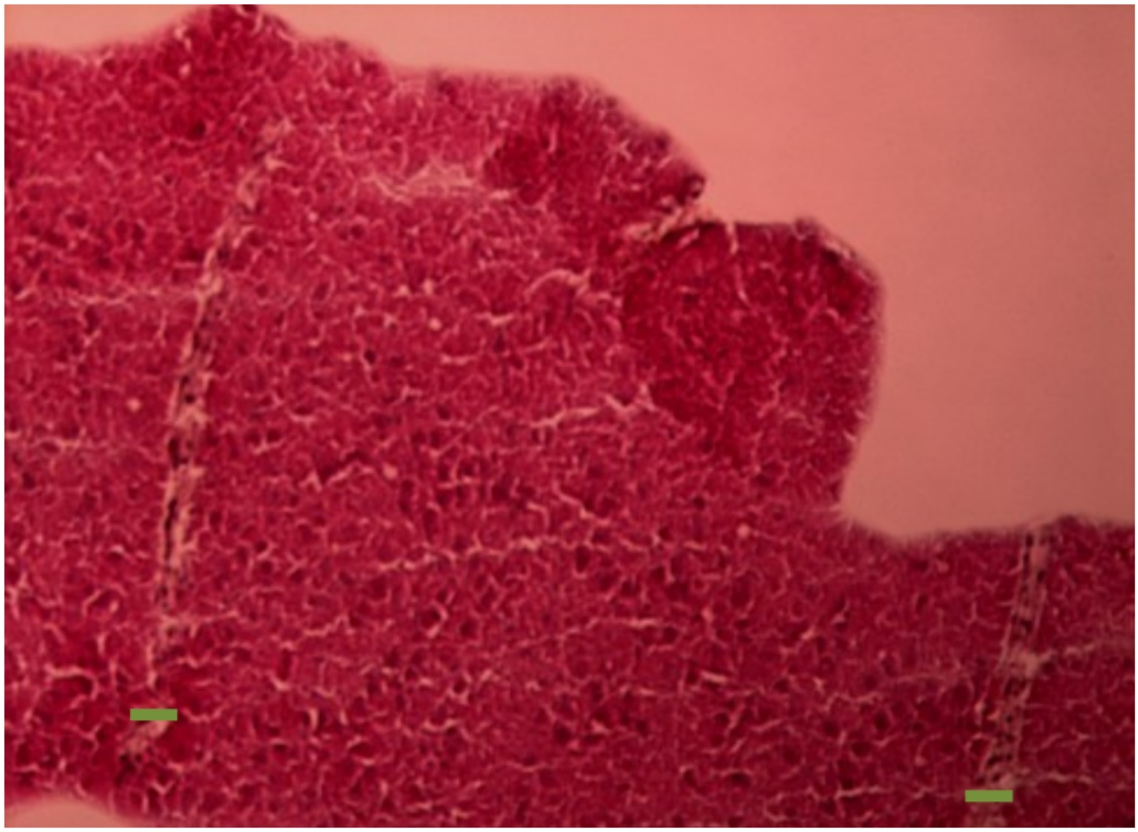

Figure 2: Haematoxylin-Eosin staining showing a quasi-surgical cut through the rat cortex 3 months after the delivery a microbeam array (incident dose of 600 Gy, beam thickness: 75µm, spacing: 400 µm).
Scale bars (placed at the bottom of the microbeam paths) : 75 µm

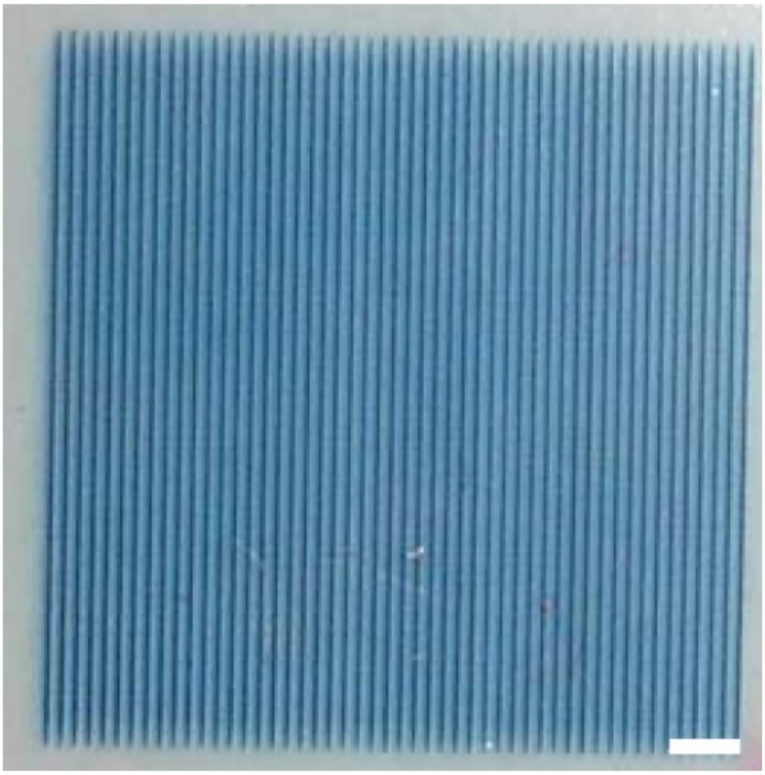

Figure 3: A microbeam array dosimetry on gafchromic film.
Dose distribution is characterized by volumes with extremely high peak doses (in the range of several hundreds Gy, color blue) and very low valley doses (less than a few Gy, color white). The fall-out from high to low dose requires few microns. Scale bar: 1 mm

Radiobiology of Central Nervous System microbeam irradiation

The Central Nervous System (CNS) radiobiology of microplanar beams was first studied about 50 years ago at the Brookhaven National Laboratory (BNL) by Curtis and coworkers. This group was the first to describe the preservation of CNS architecture after incredibly high radiation doses delivered by microscopic deuterium beams. An incident dose of 4000 Gy delivered to the mouse brain by a 25 gm-thin cylindrical microbeam failed to induce radionecrosis, which instead appeared after an incident dose of 140 Gy delivered as a 1-mm thick cylindrical beam [6, 7, 8]. Further work performed in the late nineteen at the National Synchrotron Light Source (NSLS) of the BNL using synchrotron-generated X-ray microbeams, and, later on, at the ESRF, investigated further the tissue tolerance to microscopic beams at doses tens to hundreds time larger than those allowed by conventional macroscopic

beams [9, 10, 11, 12].

Unidirectional irradiation using microbeam arrays has been delivered to adult rat brain [11, 13, 15, 16], suckling rats cerebellum [16], piglets cerebellum [1], duckling embryo brain [18], skin and muscle of the mouse leg [19, 20], rat leg [21] and rat spinal cord [12]. The exceptional resistance of the normal-tissue to high dose microbeam irradiation with no evidence of late tissue effects [12, 22, 23, 24] lead to the development of a new concept in radiobiology, the tissue sparing effect, described in the next paragraph.

The tissue-sparing effect

Microbeam irradiation is characterized by minimal side effects on normal tissue adjacent to the irradiated volume. While the cells along the path of penetration of the beams are completely destroyed, the nearby cell bodies remain unaffected with substantial preservation of the tissue architecture. In essence, microbeam irradiation acts like a surgical cut, leaving a scar extending up to the size of the penetrating beams. The lack of normal tissue damage following high dose unidirectional irradiation using arrays of microbeams is referred to as "tissue sparing effect". The tissue sparing effect is bound to the microbeams size: very high doses (up to 4000 Gy) can be delivered through microscopic beams sized 25 to 60 gm with no histological evidence of widespread radionecrosis outside the penetration path. Immunohistochemical studies using pH2AX show clearly that the neurons hit by the microbeam along his penetration path die almost immediately while the adjacent cells separated by a few microns but outside the high dose volume remain viable (unpublished personal data). Progressively lower doses (but still much higher than conventional radiosurgical or radiotherapeutic doses) are required to avoid tissue damage if ticker beams (100 to 600 gm) are used. Submillimetric beams (sized 0.6 to 0.7 mm) appear to retain the tissue sparing effect allowing to deliver incident doses of 400 Gy to the spinal cord of rats without inducing neurological damage: irradiation of rat spinal cord with four parallel 0.68-mm thick microbeams at 400 Gy in-depth beam dose did not induce paralysis after 7 months in three out of four rats [12]. This study showed not only that a highly radiosensitive structure such as the spinal cord can receive high dose irradiation through a microbeam array without neurologic sequelae but also that a beam width up to 0.68 mm is well tolerated, substantially maintaining the tissue sparing properties of thinner beams.

Microbeam radiosurgery

The ability of microbeam arrays to avoid radionecrosis and to preserve

the architecture of the irradiated tissue is mainly attributed to the rapid regeneration of normal microvessels. Only a short segment of the microvascular bed receives ablating doses while the adjacent endothelial cells fall into the valley dose region receiving just a few Gy and can restore quickly the continuity of vascular supply [24]. The wide spatial interface between the unhindered tissue placed in the valleys and the tissue irradiated with peak doses within the microbeam paths facilitates a widespread vascular recolonization of the tissue receiving necrotic doses preventing the dissolution of the architecture of the irradiated tissues [3]. The self-repair of the normal microvasculature through the migration of unaffected cells surrounding the paths of microbeam penetration is considered by most as the basis for this ability of normal tissue to tolerate high dose microbeam irradiation [25, 26]. The tolerance of the vascular bed to high dose microbeam irradiation has been clearly demonstrated by the lack of extravasation of dyes administered to the experimental animals, which remained confined in the vessels after irradiation from 12h until three months following 1000 Gy [27]. This radioresistance phenomenon was not observed in 9L glioma microvessels, confirming the presence of a differential response to between normal and tumour brain tissues in rodents, an effect that can have significant clinical applications [19]. The neoplastic vasculature appears to be unable to replicate the fast repair of the segments hit by the peak dose, facilitating the development of radionecrosis over the irradiated tumor [12, 20, 28].

The unidirectional delivery of microbeam arrays to parts of the body harbouring neoplastic tissue is known as Microbeam Radiation Therapy (MRT). MRT irradiation has been carried out to deliver very high radiation doses into tumours in a single fraction through an unidirectional approach [11, 15]. Most of the experimental activity performed until now has been focused on the study of the effects of arrays of parallel microbeams [4, 5, 9-24, 28-30]. MRT has been applied to several tumor models including intracerebral gliosarcoma (9LGS) in rats [11, 13, 30], murine mammary carcinoma (EMT-6) [19] and human squamous-cell carcinoma (SCCVII) in mouse and rat [9, 20]. Rats bearing the intracranial 9L gliosarcoma irradiated anteroposteriorly with arrays composed of 27 gm thick microbeams spaced 100 gm on-center showed a clear survival advantage after MRT: 4 out of 14 rats irradiated at 625 Gy incident doses were long-term survivors with little brain damage revealed in histopathology [11]. The introduction of stereotactic techniques to deliver arrays of microbeams that interlace over a selected target volume [31, 12] has recently opened a new field of research that is by most considered as an extension of MRT but is much closer to the stereotactic radiosurgery experience.

The authors prefer to name this technique characterized by the stereotactic delivery of microbeams to a selected target volume as Microbeam RadioSurgery (MRS). MRS is currently performed at the ESRF biomedical beamline by: 1) directing beams to the target in a convergent isocentric fashion (creating a hot spot where the dose is enhanced by the overlapping of the beams) or 2) by interlacing 2 to 4 microbeam arrays over the target. MRS has been used at ESRF to ablate selected volumes (such as the subthalamic nucleus, the substantia nigra and the caudate nucleus) into the rat brain using both approaches. The ability to induce precise lesions in the rat brain using an image-guided non-invasive approach opens new ways to create experimental models of disease. For example MRS nigrotomies could offer a novel experimental model to study Parkinson's disease (PD): experiments are underway to assess if MRS can replace or complement other PD experimental models of PD based on the induction of chemical lesioning of the Substantia nigra. A novel way to use microbeam arrays in a quasi-surgical way is currently being developed at ESRF: convergent or parallel arrays of microbeams carrying high doses are placed over selected cortical areas in order to hit tangentially and cut the horizontal axons connecting adjacent cortical columns. Cortical transections are a surgical procedure to parcellize an epileptic focus located in eloquent cortex [32, 33]. Cutting the horizontal axons required for the spreading of epileptic activity is an effective way to control the seizures without inducing neurologic dysfunction. Synchrotron-generated microbeams can be used to create cortical transections in rats offering a chance to study the tolerance of CNS to this technique.

This novel experimental application of microbeams provides a new and attractive tool to modulate cortical function by transecting the fibers connecting the cortical columns. Aside from the tight dosimetry, the relative low energy of the microbeams (continuous X-ray spectrum ranging from 50 to —350 keV, mean energy —100 keV) makes them well suited to treat superficial targets within the cortex. Microbeam transections, either placed over neocortical seizure foci or through the hippocampus, could prove to be an excellent tool to be added to the current radiosurgical techniques used to control seizures. A series of experiments are currently planned at ESRF aiming at the development of an experimental model of microbeam cortical transections in epileptic rats with the goal of verifying the ability of microbeam cortical transections to control seizures without damage to the eloquent cortex irradiated. Figures 4 and 5 illustrate the irradiation room and stereotactic set-up at ESRF.

Figure 4: Irradiation room at ESRF, located at about 45 meters from the synchrotron radiation wiggler source.
The sample positioning system is a Kappa-goniometer, that combined with an X-ray on-line X-ray detection system, allows for a submillimetric identification of the target. On the picture, a water tank for dosimetry is positioned on the Kappa goniometer.

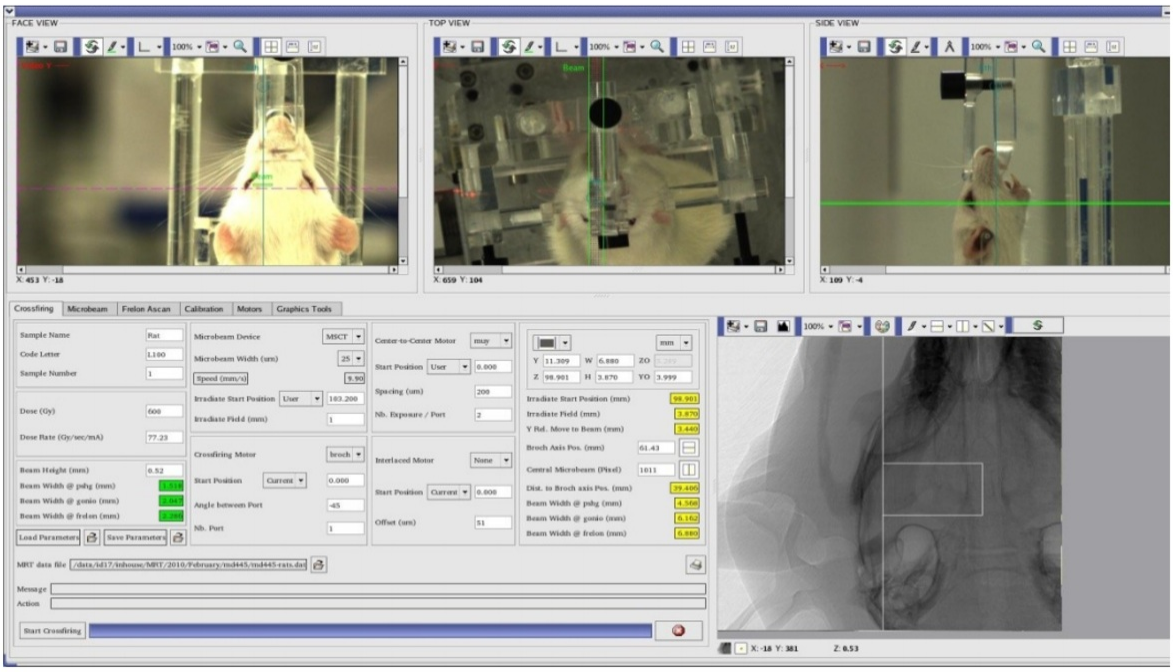

Figure 5: Stereotactic set-up used for rat brain irradiation.
For the prepositioning of the target,based on external markers, 3 high resolution video-cameras are installed. Bottom right: the X-ray radiography, taken just before the irradiation, allows for the submillimetric identification of the target.

Conclusions

The irradiation of normal brain and spinal cord with microbeam arrays is characterized by a distinct tissue-sparing effect. Peak doses between 300 and 600 Gy are in most cases well tolerated by the CNS with little or no histological evidence of brain damage. Fast sprouting and recovery of the microvascular bed has been observed in normal CNS tissue while the inability of neoplastic vascular network to repair itself exposes neoplastic tissue to an enhanced tumoricidal effect. Microbeam radiosurgery is able to induce non-invasively microradiosurgical lesions in the rat brain, thus offering a new way to develop experimental models to study PD, Huntington's disease and many other CNS disorders. A recent development of microbeam research is the use of arrays of microbeams to induce cortical transections aiming to modulate cortical function through the selective cutting of horizontal axons connecting adjacent columns.

Additional Information

Disclosures

The authors have no conflict of interest to disclose.

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A new application of microbeam radiation therapy (MRT) on the treatment of epilepsy and brain disorders

Microbeam radiation therapy (MRT) is a form of radiosurgery which delivers high doses of spatially fractionated X-rays generated by a synchrotron. Normal tissues show very high resistance to radiation doses in the hectogray range delivered by MRT. Until now, MRT has been applied for the management of brain tumors in animal models but other central nervous system pathologies, such as epilepsy, might be potentially treated with this technique. In this Thesis we used microplanar beams to transect the rat cortex and hippocampus. Cortical transections were performed on the sensory motor cortex of healthy and epileptic rats. No neurological deficit was observed in healthy animals. Following transections, convulsive seizure duration was markedly reduced in epileptic animals. Microbeam hippocampal transections were performed in healthy rats without inducing significant behavioral impairment. Also the neurogenesis does not seem to be impaired by MRT as conventional brain radiotherapy. This Thesis shows that submillimetric transections might become a powerful tool for the treatment of functional brain disorders.

Key words: Microbeam radiation therapy, radiosurgery, epilepsy, neurogenesis, brain

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Nouvelle application de la radiothérapie par microfaisceaux (MRT) pour le traitement de l'épilepsie et des troubles cérébraux

La radiothérapie par microfaisceaux (MRT) est une forme de radio-chirurgie basée sur l'utilisation de hautes doses de rayons X, fractionnés dans l'espace, générés par un synchrotron. La capacité des tissus sains à résister à plusieurs centaines de Gray, a rendu possible l'évaluation de la MRT dans le traitement de tumeurs cérébrales sur des modèles animaux. D'autres applications de cette technique, notamment pour le traitement de pathologies du système nerveux central comme l'épilepsie, sont envisagées. Dans cette thèse, les microfaisceaux ont été utilisés pour effectuer des transsections dans les structures de l'hippocampe et du cortex de rats. Les transsections corticales ont été effectuées au niveau du cortex sensoriel moteur chez des rats sains et épileptiques. Dans le cas des animaux sains, le traitement par microfaisceaux s'est révélé être sans incidence neurologique alors qu'une baisse de la durée des crises de convulsion a été observée chez les animaux épileptiques. Au niveau de l'hippocampe, les transsections par microfaisceaux ont été pratiquées sur des rats sains, et comparées à un traitement de radiothérapie conventionnelle. Consécutivement au traitement par microfaisceaux de l'hippocampe aucun trouble du comportement n'a été observé. La conservation de l'intégrité neurologique et de la neurogénèse lors d'un traitement par MRT observé durant cette thèse, tend à prouver que l'utilisation de transsections submillimétriques pourrait devenir un outil puissant dans la prise en charge des troubles fonctionnels cérébraux.

Mots clés: Radiothérapie par microfaisceaux, radiochirurgie, épilepsie, neurogénèse, cerveaux