LITHIUM PROTECTS THE JUVENILE BRAIN FROM IONIZING RADIATION

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To Francesco

"Our care of the child should be governed,

Not by the desire to make him/her learn things,

But by the endeavour always to keep burning within him/her that light

Which is called intelligence."

Maria Montessori

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ABSTRACT

Radiotherapy used in the treatment of brain tumours in children results in a range of cognitive dysfunctions that impact the quality of life in the surviving population. In past decades, great strides were made in understanding the cellular and molecular aetiology of these deficits. Postnatal hippocampal neurogenesis is highly vulnerable to irradiation, especially in the juvenile brain, and dysfunction in this structure is recognised as a prominent feature of the radiation-induced neurocognitive sequelae. With these insights, new therapies for cognitive decline after radiotherapy are emerging. Lithium, a long-known mood stabiliser, has been shown to have neuroprotective and neurogenic effects in several disease models, including irradiation, by positively harnessing neural stem/progenitor cell (NSPC) proliferation in neurogenic regions of the brain, such as the hippocampus. Despite several studies focussing on the effects of lithium, little is known about its effects in the developing brain. This is a valid concern when considering lithium as a potential treatment for childhood cognitive and degenerative disorders. In **paper I**, we addressed the radiation-induced electrophysiological changes in the dentate gyrus, which manifested as an increase in synaptic efficacy as well as a shift from long-term potentiation to long-term depression at medial perforant path granule cell synapses. These findings provided evidence that the higher radiation sensitivity of the juvenile brain compared with the adult brain was attributable to the overt disruption of plasticity mechanisms, which likely correlates with the cognitive impairments observed after radiotherapy. Unfortunately, lithium was ineffective in rescuing this particular impaired synaptic plasticity. In **paper II**, we examined the effects of lithium on growth dynamics and cell cycle arrest in irradiated NSPCs. Lithium rescued proliferation in NSPCs, reduced DNA damage, and prevented the propagation of genotoxicity. In **paper III**, we determined the distribution of lithium in the brains of young mice using time-of-flight secondary ion mass spectrometry. This technique demonstrated that lithium regionalised in brain structures with high cell density, such as neurogenic areas, and this spatial distribution was associated with changes in lipids, such as vitamin E, a potent antioxidant. To exclude the potential of lithium protecting tumour cells, in **paper IV,** we examined whether delaying lithium treatment resulted in the same degree of protection as that previously observed using pre-treatment or early treatment. This study determined a safe treatment regimen for use in future clinical practice and showed that even long after radiotherapy, lithium restored neurogenesis and preserved lineage commitment, as long as periods of treatment discontinuation were allowed. Overall, this work demonstrates that the imminent use of lithium is warranted in treating the radiation-induced cognitive impairments that severely impact the quality of life in children who receive radiotherapy and survive cancer.

Keywords: young, lithium, delayed, irradiation, neurogenesis, DNA damage

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Strålbehandling är effektivt mot hjärntumörer hos barn, men resulterar i en rad kognitiva problem som allvarligt påverkar livskvaliteten hos det ökande antal patienter som överlever sin sjukdom. Under senare år har stora framsteg gjorts i förståelsen av de cellulära och molekylära mekanismer som orsakar dessa problem. Stamceller i hjärnan är mycket känsliga för strålning, särskilt i den unga hjärnan. Nybildning av nervceller, så kallad neurogenes, i hippocampus är viktigt för minne och inlärning och problem med dessa funktioner är ett framträdande inslag i strålningsinducerade så kallade sen-effekter. Baserat på denna nya kunskap kan man utveckla nya terapier som motverkar kognitiv nedsättning efter strålbehandling. Litium, som är en etablerad behandling av bipolär sjukdom, har visat sig kunna skydda hjärnan mot skada och öka neurogenesen i flera sjukdomsmodeller. Trots många års studier är vår kunskap rörande litiums effekter på den växande hjärnan idag ytterst begränsad. Det är viktigt att undersöka detta när man nu överväger litium-behandling för barn. I denna avhandling studeras effekter av litium på neurala stamceller i den unga växande hjärnan.

I experimentella djurmodeller har vi visat att strålning ger upphov till elektrofysiologiska förändringar i hippocampus. Vi fann en ändring från långtidspotentiering till långtidsdepression, vilket sannolikt kan förklara de problem med inlärning som uppträder hos djuren. Dessa resultat visar att den unga hjärnan har en högre känslighet och reagerar annorlunda på strålning jämfört med den vuxna hjärnan, vilket sannolikt förklarar de mer uttalade kognitiva funktionsnedsättningar som observerats hos barn och ungdomar efter strålbehandling. Tyvärr observerade vi ingen skyddande effekt av litium mot dessa strålningsinducerade förändringar.

Det har tidigare visats att litium inte skyddar cancerceller, snarare tvärtom, vilket är betryggande. Vi undersökte effekterna av litium på tillväxt och celldelning i bestrålade neurala stamceller. Intressant nog fann vi att litium skyddade neurala stamceller mot strålning, såtillvida att de celler som stannat upp i sin delning kunde sättas igång igen. Litium minskade DNA-skadorna, och tycktes enbart rädda de celler som inte har för mycket DNA-skador. Dessa resultat stödjer således användning av litium för att förhindra skador på hjärnans normala celler.

Vi fann att litium ackumuleras i områden av hjärnan med hög celltäthet, såsom områden med stamceller, och att denna rumsliga fördelning korrelerade med förändringar i lipider, t ex vitamin E, en potent antioxidant.

Slutligen undersökte vi om litiumbehandling skulle kunna vara effektiv även om man väntar tills långt efter att strålbehandlingen avslutats. Anledningen till detta är att det ej är möjligt att introducera litium i samband med strålning i de befintliga behandlingsprotokollen för patienter, utan att detta kan sättas in först då strålbehandlingen är avslutad. Behandling med litium efter avslutad strålbehandling resulterade i samma grad av skydd som det vi tidigare observerat vid förbehandling eller behandling under själva strålningen. Denna djurexperimentella studie talar således för att det bör vara såväl säkert som effektivt att använda litium i framtida klinisk praktik även långt efter strålbehandling. Litium återställde nybildningen av nervceller, men för att detta skulle ske krävdes perioder av behandlingsuppehåll.

Sammanfattningsvis presenterar vi i detta arbete resultat som stödjer och uppmuntrar framtida användning av litium vid behandling av strålningsinducerade kognitiva funktionsnedsättningar hos barn som överlever sin cancer.

LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers:

I. **Irradiation of the Juvenile Brain Provokes a Shift from Long-Term Potentiation to Long-Term Depression.** Giulia Zanni, Kai Zhou, Ilse Riebe, Cuicui Xie, Changlian Zhu, Eric Hanse and Klas Blomgren. *Developmental Neuroscience, DOI: 10.1159/000430435 (2015)*

II. **Lithium Increases Proliferation of Hippocampal Neural Stem/Progenitor Cells and Rescues Irradiation-Induced Cell Cycle Arrest *in vitro*.** Giulia Zanni *, Elena Di Martino *, Anna Omelyanenko, Michael Andäng, Ulla Delle, Kecke Elmroth and Klas Blomgren. *Oncotarget, DOI: 10.18632/oncotarget.5191 (2015)*

III. **Spatial Lithium Dynamics in the Juvenile Brain Elucidated using High Resolution Ion Imaging.** Giulia Zanni, Wojciech Michno, Elena Di Martino, Anna Tjärnlund-Wolf, Klas Blomgren & Jörg Hanrieder. *In manuscript*

IV. **Delayed Lithium Treatment after Irradiation of the Juvenile Brain Positively Modulates Neurogenesis.** Vinogran Naidoo *, Giulia Zanni *, Gabriel Levy, Elena Di Martino, Klas Blomgren. *In manuscript*

* These authors contributed equally to this work

CONTENT

ABBREVIATIONS

AHP	Adult hippocampal progenitors
Akt	Serine/threonine- specific protein kinase
ALS	Amyotrophic lateral sclerosis
ANP	Amplifying neural progenitors
AP-1	Activator protein 1
ATM	Ataxia telangiectasia-mutated protein
bcl-2	Cytoprotective B-cell lymphoma protein-2
BDNF	Brain derived neurotrophic factor
Bmp	Bone morphogenic protein
BPAD	Bipolar affective disorders
BrdU	Bromodeoxyuridine
CA	Cornu Ammonis
C3	Complement component 3
CCND1	Cyclin D1 gene
CDK	Cyclin-dependent kinase
CNS	Central nervous system
cPLA2	Cytosolic phospholipase 2A
CREB	Cyclic AMP-responsive binding element
DAG	Diacylglycerol
DCX	Doublecortin
DDR	DNA damage response
DG	Dentate gyrus
DNA-PK	DNA-dependent protein kinase
DSB	Double strand break
EC	Entorhinal cortex
EGF	Epidermal growth factor
EPSP	Excitatory postsynaptic potential
FGF2	Fibroblast growth factor 2
GABA	Gamma aminobutyric acid

GSK3β	Glycogen synthase kinase 3 beta
γH2AX	Phosphorylated histone 2AX
HFS	High frequency stimulation
HI	Hypoxia ischemia
IL-1β	Interleukin 1 beta
IMP	Inositol monophosphatase
iNOS	Inducible nitric oxide synthase
IPSC	Inhibitory postsynaptic current
IPPase	Inositol polyphosphate 1-phosphatase
IR	Ionising radiation
JAK	Janus kinase
LEF	Lymphoid enhancer binding factor
LiCl	Lithium chloride
LTD	Long term depression
LTFU	Long term follow-up
LTP	Long-term potentiation
MARCKS	Myristoylated alanine-rich C kinase substrate
MPP	Medial perforant pathway
NeuN	Neuronal nuclei
NHEJ	Non-homologous end joining
NF-kB	Nuclear factor kB
NMDA	N-methyl-D-aspartate receptor
NSC	Neural stem cell
NSPC	Neural stem progenitor cell
Olig2	Oligodendrocyte lineage transcription factor
$p16^{Ink4a}$	Cyclin-dependent kinase inhibitor
$p21^{Cip1}$	Cyclin-dependent kinase inhibitor
Pax6	Paired box protein
PCA	Principal component analysis
PI3K	Phospoinositide 3-kinase
PKC	Protein kinase C

PND	Postnatal day
PSA-NCAM	Polysialated form of neural cell adhesion molecule
PSD95	Post-synaptic density protein
PV	Parvalbumin
ROI	Region of interest
S100β	Calcium binding protein
STAT	Signal transducer and activator of transcription
SGZ	Subgranular zone
SVZ	Subventricular zone
SSB	Single strand breaks
TCF	T-cell specific transcription factor
ToF-SIMS	Time of flight secondary ion mass spectrometry
Wnt	Int/Wingless

1 INTRODUCTION

Cranial radiotherapy, alone or in combination with chemotherapy and surgery, is used as the gold standard treatment for primary and metastatic brain tumours. Advancements in modern intervention therapies and healthcare management ([1,2]) are exemplified by the nearly 80% survival of children (aged 0–14 years) diagnosed with central nervous system tumours ([3]). Despite these encouraging results, a rising population of long-term survivors often experience negative outcomes of the radiotherapy and will likely be subjected to a suboptimal quality of life ([4,5]). Indeed, nearly 40% of childhood cancer survivors are at high risk of serious morbidity ([2]) and often develop, alone or in combination, severe complications, such as neurocognitive dysfunction, cardiovascular diseases, infertility, hormonal imbalance, growth retardation, and psychological problems ([2]). Although certain adverse effects may be attenuated by preventive risk-based care ([6,7]), systematic screening for long-term follow up of the cancer survivor population is challenging ([2,8]). Some complications occur concomitantly with radiotherapy, whereas others require years to manifest ([9]). This is particularly true for neurocognitive deficits, which may remain unnoticed for several months or years before becoming clinically apparent ([10-12]). Cognitive declines are irreversible, progress, display a linear dose-response relationship with radiation, and are generally more pronounced in females than males ([13-17]). The neurological implications of these sequelae encompass impairments in the speed of information processing, executive function, and memory formation that ultimately impact scholastic achievements, the likelihood of employment, and the ability to participate in normal social life ([4,16,18]). The severity of these late-appearing sequelae depends on several factors, including age at the time of cranial radiotherapy, dose of radiotherapy, and grade and primary site of the tumour ([17,19,20]). The aetiology of neurocognitive decline is largely attributed to overt changes in the brain vasculature, altered gliogenesis, increased

inflammatory drive, loss of white matter volume, early cellular senescence, and impaired neurogenesis ([17,21-23]). Several pre-clinical and clinical studies targeting the aforementioned pathways are generating new methods for preventing or treating the observed cognitive declines ([22,24,25]).

This work reviews the most recent developments in the knowledge of adverse radiation-induced cognitive effects and explores hippocampal neurogenesis and the effects of lithium as a promising neuroprotective and neuroregenerative agent in the developing brain after radiotherapy.

1.1 History of lithium

Lithium was discovered in 1817 by the Swedish chemist Johan August Arfwedson ([26]). It is a highly reactive alkali metal, with atomic number 3 in the periodic table and two isotope forms present in nature, ^{6}Li and ^{7}Li. In 1859, Garrod found that the most remarkable property of lithium was its power of imparting solubility to uric acid, thus revealing the potential of this ion in treating patients with gout ([27]). A likewise serendipitous finding led John Cade, in 1949, to initiate a lithium clinical trial with ten adult patients having bipolar affective disorder (BPAD) in an open-label uncontrolled study; he observed marked improvements in lithium-treated manic individuals ([28]). However, due to general distrust, it was not until the second half of the 20th century that lithium became a recognised treatment for BPAD ([29]). The United States Food and Drug Administration approved lithium for the treatment of BPAD in 1970, long after other countries had issued approval, including France (1961) and the United Kingdom (1966) ([29]). Lithium is now considered the gold standard therapy for BPAD and is administered to patients in the form of a salt. The therapeutic spectrum is very narrow, ranging from 0.6 to 1.2 mmol/L in serum and the therapeutic index (the ratio of toxic to therapeutic levels) is low,

indicating that regular monitoring of plasma concentrations in patients is crucial ([30]).

The notable adverse effects of lithium treatment include hand tremor, dizziness, dehydration, diarrhoea, nausea, nystagmus, hypercalcemia, nephrogenic diabetes insipidus, and lithium-induced nephropathy ([31,32]). Hypothyroidism, weight gain, and a reduced ability to concentrate urine are also common in patients treated with lithium ([33,34]).

1.2 Lithium pharmacokinetics

Lithium shares properties with both Na^+ and K^+, and it has been shown to alter the countertransport of salts as well as to upregulate the Na^+-K^+ pump. Therefore, the presence of lithium in serum can unbalance electrolyte equilibrium or, in the case of bipolar disorders, act as an electrolyte stabiliser ([35-39]). Brain lithium concentrations do not equal those of the plasma until after 14 days of administration ([40]), and this may be the reason the effects of lithium on the brain are exerted only after 3−4 weeks of treatment ([41]). It was previously observed that lithium is regionally distributed in the brain, with the highest lithium concentrations primarily found in high cell density regions ([42-44]). The intracellular lithium concentrations are higher than those in the serum ([45]), and transport occurs through three pathways common to erythrocytes and nerve cells, the Na^+-K^+ pump, Na^+-Li^+ countertransport, and a leak ([46]). A fourth pathway, the bicarbonate-stimulated lithium flux, is present only in erythrocytes ([47]). Lithium is not subjected to metabolic transformation, meaning that its clearance is a function of glomerular filtration, and the elimination half-life is dependent on both the volume of distribution and clearance rate. The volume of distribution of lithium depends on an individual's age, comorbidities, and body mass composition ([48]). One study reported that lithium displayed a shorter half-life and a higher clearance rate in children than in adults, suggesting that a

steady state is reached sooner in children ([49]). These finding also indicate that adjusted lower doses and continuous monitoring of the lithium plasma levels may be required in children.

1.3 Lithium pharmacodynamics

Lithium and magnesium ions share a diagonal relationship, with similar atomic and ionic radii. Lithium is thought to behave as a second messenger in the cell, inhibiting or promoting a broad range of enzymatic reactions ([50-52]). This suggests that numerous signalling pathways are targeted by lithium, and these pathways in turn modulate a complex and intertwined intracellular enzymatic cascade **Fig. 1**. The therapeutic benefits of lithium are time- ([40]) and dose-dependent ([53]), and they occur at post-transcriptional, post-translational, and transcriptional levels.

One well-described target of lithium is the inhibition of inositol monophosphate (IMP) metabolism through the inhibition of two key enzymes necessary for recycling and synthesising IMP, inositol monophosphatase and inositol polyphosphate 1-phosphatase ([54,51]). Inhibiting these enzymes results in a depletion of IMP levels and an accumulation of products ([51]), such as diacylglycerol, an endogenous activator of protein kinase C (PKC), which ultimately leads to a decrease in lipid synthesis ([55]). It has been postulated that the downstream lithium effects of depleting inositol levels result in activating autophagy-mediated processes and enhancing clearance of autophagic substrates ([56]). The lithium-mediated increase in PKC activity affects (with opposing effects in neurons and astrocytes) the MEK/ERK pathway, which plays significant roles in synaptic plasticity, long-term potentiation (LTP), and cell survival ([57,58]). Additionally, lithium appears to downregulate the expression of the PKC substrate myristoylated alanine-rich C kinase substrate, a protein associated with long-term neuroplasticity in developing and adult brains

([59,60,61,62,63]). Chronic lithium treatment also affects, via PKC, cytosolic phospholipase 2A, arachidonic acid metabolism, and adenylate cyclase, all of which are thought to function in synaptic transmission and neuronal signal transduction ([64,65]). Previous studies have also shown that lithium increases the level of cytoprotective B-cell lymphoma protein-2 ([66,67]), a regulatory protein that exerts major anti-apoptotic effects ([68,69]), while at the same time reducing the expression of p53, a tumour suppressor protein, and *Bax,* a pro-apoptotic protein.

The most investigated mechanism for the effects of lithium remains through direct inhibition of glycogen synthase kinase 3β (GSK3β) and the downstream activation of its canonical (Wnt) pathway ([53,70,71,72,73]). The soluble protein Wnt binds to Frizzled receptors and inactivates GSK3β, which causes accumulation of β-catenin that translocates into the nucleus and subsequently induces transduction of its targets, such as T-cell-specific transcription factor and lymphoid enhancer binding factor (LEF) ([74]). A subsequent target of LEF is the cyclin D1 gene ([75]). Interestingly, cyclin D1 is involved in cell cycle progression, driving the G_1/S phase transition ([76]). Lithium appears to mediate, via cyclin D1, the mitogenic activity of neural stem/progenitor cells (NSPCs) both *in vivo* and *in vitro* ([77]). Among other effects, lithium was found to reduce the expression of the microtubule-associated protein tau and enhance the binding of tau to microtubules, promoting microtubule assembly, which is essential for axonal growth, through the direct and reversible inhibition of GSK3β ([78,74]). In neurons, lithium upregulates transcriptional activators, such as cyclic AMP-responsive binding element and activator protein 1, again suggesting that lithium ultimately alters gene expression ([79]). Additionally, the following anti-inflammatory effects of lithium have been observed ([80]): potent inhibition of pro-inflammatory nuclear factor kB ([81,82]), positive modulation of microglial complement component 3 ([83]), decreased production of interleukin IL-1β-

mediated nitric oxide, decreased expression of inducible nitric oxide synthase protein ([84]), and prevention of microglia activation ([85,86]).

The lithium-mediated effect on GSK3β has been further linked to the modulation of serine/threonine-specific protein kinase (Akt) signalling ([87]), the upregulation of brain-derived neurotrophic factor ([88]), and the elongation of the telomere ([89,90]). Lithium has also been shown to inhibit the JAK/STAT3 pathway independently of GSK3β, resulting in the suppression of astrogliogenesis, which may explain the lack of a lithium effect on carcinogenicity ([91]). Recent studies have proposed lithium as a potential treatment both for the late adverse effects of radiotherapy and for enhancing the therapeutic window during radiotherapy ([92,93]). These studies showed that lithium protects adult hippocampal progenitors, in contrast to cancer cells, during radiation exposure by decreasing γH2AX foci, a DNA damage marker, and increasing the nonhomologous end joining DNA repair efficiency. Compelling evidence has shown that lithium efficiently targets cancer cells by reducing the growth rate of the medulloblastoma through inhibition of β-catenin/Gli1 nuclear interaction ([94]). Further studies corroborated the lithium-sensitising potential of a p53 mutant medulloblastoma to radiation ([95]), thereby providing evidence that lithium may be used to enhance the radiotherapy therapeutic window.

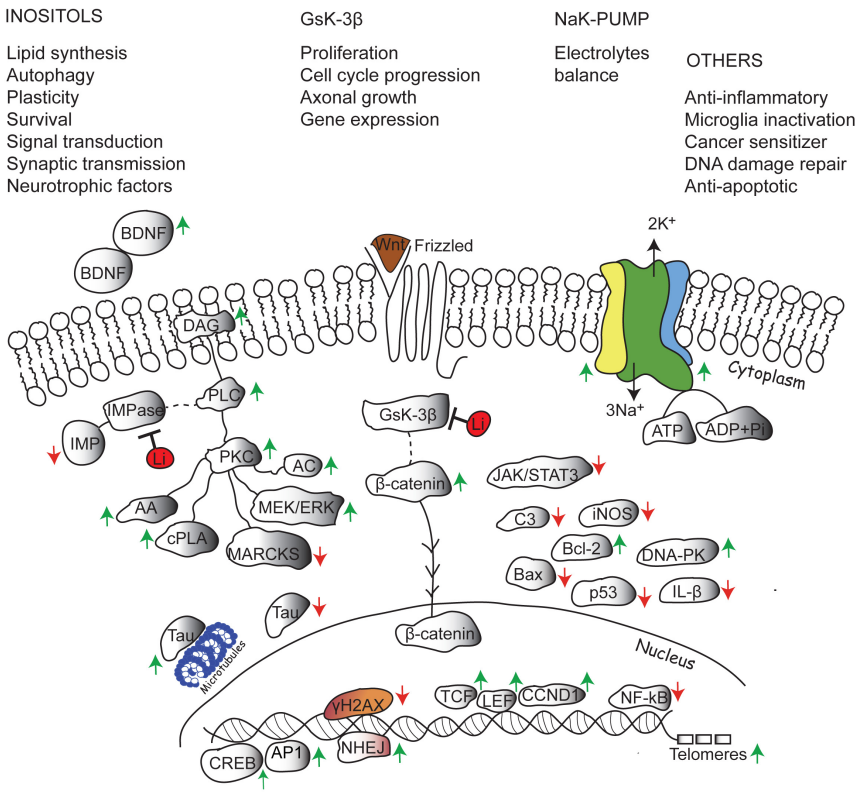

Figure 1. Lithium-responsive signal transduction pathways and targets. Lithium directly inhibits IMP metabolism and GSK3β. The red and green arrows indicate down-regulation and up-regulation of lithium targets, respectively. Author: Giulia Zanni.

1.4 Lithium treatment of diseases

As previously mentioned, lithium is the gold standard treatment for BPAD, but it is also used clinically for treating several other diseases, including aplastic anaemia ([96]), granulocytopenia ([97,98]), hepatitis-associated agranulocytosis ([99]), hyperthyroidism-associated agranulocytosis ([100]), clozapine-induced neutropenia ([101]), radiation-induced neutropenia ([102]), and childhood neutropenia ([103,104]). Lithium increases neutrophil counts in both children and adults, causing leucocytosis ([101]) without impairing neutrophil migration into skin lesions ([105]).

Pre-clinical studies demonstrated that chronic lithium handling protects against the neurodegenerative effects of hypoxia–ischemia in neonatal rodents through its pro-autophagic, anti-inflammatory, and anti-apoptotic effects ([106-108]). Additional compelling evidence has shown that lithium enhances hippocampal neurogenesis in adult mice ([109]) as well as in young rats and mice after hypoxic-ischemic injury ([110,107]). The beneficial effects of lithium on synaptic plasticity in a Down syndrome mouse model have also been investigated ([111]) as well as other pre-clinical studies, demonstrating the efficacy of lithium in preventing neural degeneration and restoring to basal levels altered synaptic networks in conditions such as Parkinson, Alzheimer, and Huntington diseases ([112,113,114]). Limited published clinical data support lithium as a neuroprotective or neuroregenerative agent, but clinical trials are currently being conducted examining lithium treatment for a wide range of brain-related disorders, including stroke ([115]), Alzheimer disease ([116]), spinal cord injury ([117,118]), and amyotrophic lateral sclerosis ([119,120]). Thus, future evidence may support the clinical use of lithium for preventing the neurocognitive sequelae caused by cranial radiation therapy in children. The outcomes of current clinical trials in adults ([121,122,123]) will be valuable in the planning and safety assessment of paediatric trials. Although valid concerns have been raised regarding lithium protecting not only neurons and neural stem cells but also the remaining tumour cells, there are studies demonstrating that lithium does not promote tumour growth ([92,94]) or abet the onset of relapse ([94,124]). Furthermore, an increasing number of clinical trials are investigating the effects of lithium in the treatment of malignant diseases, including acute myeloid leukaemia ([125]), neuroendocrine tumours ([126]) thyroid cancer ([127]), and glioblastoma ([128]).

1.5 Neural stem cells in the post-natal brain

The discovery of neural stem cells in the postnatal mammalian brain dates back to the seminal work of Altman, who used ^{3}H-thymidine to label and visualise

actively proliferating cells ([129,130]). This pioneering work paved the way for the development of lineage tracing tools, such as the thymidine analogue bromodeoxyuridine (BrdU) ([131]) and more specific transgenic tools ([132,133]), to track newly-born neurons throughout their maturation and integration in a process widely known as neurogenesis **Fig. 2**. Neurogenesis peaks during prenatal development ([134]), and in the postnatal brain it is restricted to two areas, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) ([135]). Neurogenesis decreases with age ([131]), and occurs in animals and humans ([130,136-138]). NSPCs can be isolated from these two regions ([139]) and maintained *in vitro* as sphere or adherent cultures ([140]) under the constant proliferative drive of epidermal growth factor and fibroblast growth factor 2 ([141]). The neurosphere culture assay represents a heterogeneous system composed of dying, differentiated cells, quiescent neural stem cells (qNSCs) and amplifying neural progenitors (ANPs); therefore, this assay system is believed to better represent the composite *in vivo* scenario than adherent culture assays do ([142]). Indeed, when a qNSC is recruited into asymmetric division, which occurs rarely *in vivo*, it gives rise to a replica of itself and an ANP ([143]). The ANPs divide more rapidly and account for a larger proportion of the proliferating pool ([144]), but are subjected to a wave of apoptosis within the first 4 days of their birth ([145]). This considerable loss of neuronal precursors is believed to be responsible for the homeostatic regulation of the neurogenic process and the *in vivo* cellular turnover ([146,147]). Throughout life, the qNSCs are thought to divide a restricted number of times and subsequently give rise to astrocytes ([144]), whereas the surviving ANPs proceed a few steps toward maturation ([148]) and by fine regulation of crucial transcription factors, such as Pax6 and Olig2, become committed to being either neurons or oligodendrocytes, respectively ([149-151]). Importantly, Wnt and bone morphogenetic protein are pivotal players in dictating the time of entering and exiting the cell cycle in ANPs and qNSC, respectively ([152,153]). Upon injury, ANPs and qNSCs have the ability to increase

their rates of proliferation and differentiation and to migrate to the damaged areas ([154,155]), suggesting their latent role in regenerating and healing areas affected by a lesion ([156,157]). However, recent studies have reported that seizure ([158]) and stab-wound injury ([159]) recruit qNSCs that start dividing through the uncommon symmetric division, leading to an initial increase in ANPs and a consequent depletion of qNSCs, thus permanently impairing neurogenesis. Moreover, the integration of newly generated neurons after an injury is highly dependent on features of the compromised microenvironment, and this dependency often results in an aberrant neuronal network ([160,161]). This process is particularly crucial for neurogenesis because the accurate integration of newly-born neurons into the hippocampus is pivotal for performing several cognitive tasks, including pattern separation ([162]) and memory processes ([163]). Hippocampal qNSCs and ANPs are highly responsive to many environmental factors, such as enriched environment, voluntary running ([164]), stress ([165]), antidepressant therapy ([166]), and injury ([167]). Several studies have shown that these cells also react positively to lithium through increased proliferation *in vitro* ([71,168-170]) and *in vivo* ([109,171,172]), decreased apoptosis, increased survival, and higher neuronal than astrocytic differentiation. Further *in vivo* studies generated compelling evidence that lithium also enhances *in vivo* neuronal functional integration and synaptic plasticity of the newly generated cells born during the drug treatment ([111]), resulting in better cognitive performances ([173]). Therefore, given the overt effects of ionizing radiation on proliferation and neuronal integration of hippocampal NSPCs ([21]), scientists have recently started investigating whether lithium may restore the loss of functions in a similar model. Thus far, the data indicate that this is indeed the case in animal models of irradiation ([77,92,93,174]).

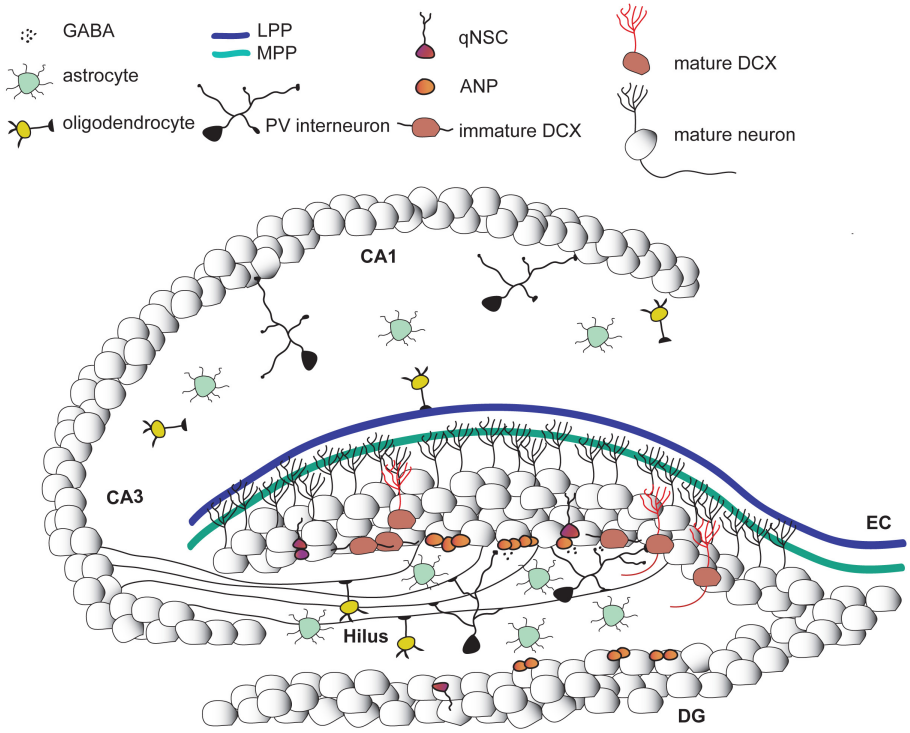

Figure 2. Representative figure of the hippocampal network. Input signals from the entorhinal cortex (EC) are carried through two connectional routes made of the axons of the medial (light green) and lateral (blue) perforant pathways. These axons establish stable synapses with the dendrites of the mature granule cells neurons and weak ones with the immature DCX cells in the dentate gyrus (DG). At the boundary of the DG and the hilus is the subgranular zone (SGZ), where quiescent neural stem cells (qNSC) give rise to amplifying neural progenitors (ANP) allowing the continuous neuronal re-population of the DG. The qNSC and ANPs are multipotent stem cells, capable of giving rise to astrocytes, oligodendrocytes and neurons. The input signal from the DG is relayed to the proximal Cornu Ammonis region (CA3) through the axons of the mature granule cells that form the mossy fibre projection. The signal transduction continues to the CA1 region through the Schaffer collaterals fibres and ultimately to further cortical areas. Parvalbumin (PV) interneurons in the hilus are important in modulating, through feed-back and feed-forward inhibition, the input signals and NSC proliferation through the release of the neurotransmitter GABA. Author: Giulia Zanni.

1.6 Neuronal maturation and plasticity

The generation of new neurons in the DG can be divided into four processes: cell proliferation, migration, cell survival, and neuronal differentiation **Fig. 2** ([157]). The maturation process requires at least 2 months, and it progresses over at least two stages of ANP lineage-determined progenitor cells (type-2 and type-3 cells) to early post-mitotic and to mature neurons. Throughout these stages, transition markers, such as doublecortin (DCX), the polysialylated form of neural cell adhesion molecule, calbindin, calretinin, and neuronal nuclei (NeuN), are expressed. Both the selection process of functionally mature neurons and other governing stimuli differentially affect the various stages of development ([175]), and these processes are crucial for neuronal integration into the pre-existing adult network, ensuring a correct synaptic transmission. Newborn neurons display a high input resistance ([176]), receive less inhibition ([177]), and exhibit considerably greater synaptic plasticity than mature granular neurons ([178,179]). Immature cells rely on tonic γ–aminobutyric acid (GABA) activation, which leads to depolarisation due to the high concentration of intracellular chloride ([176,178]). In the maturation stage, newborn neurons start receiving synaptic GABAergic input, mostly from parvalbumin-expressing interneurons ([180]), and expressing glutamatergic receptors so that the direction of the chloride gradient eventually switches, and GABAergic input then elicits hyperpolarisation ([176-178]). After 4 weeks, new neurons receive weak synaptic glutamatergic input from layer II of the entorhinal cortex through the medial perforant pathway (MPP), similar to mature cells ([181,182]). However, these new neurons are weakly coupled to GABA inputs and inhibitory postsynaptic currents as well as to glutamatergic input compared with mature granule neurons. This coupling occurs with enough delay to ensure a high plasticity range ([183]). Once fully mature, newborn neurons are indistinguishable physiologically from developmentally born granule neurons. It is now widely accepted that in light of these unique properties, young neurons are likely to be more excitable than

mature neurons ([176]); thus in response to presynaptic inputs, the synapses formed by newborn neurons may be more dynamic than the existing synapses, contributing to the unique function of adult neurogenesis. It is now established that the DG represents the first relay station for information processing in the hippocampus, and neurogenesis in particular is necessary in pattern separation, a mechanism necessary to disambiguate distinct inputs and facilitate memory formation ([162,184,185]). Using radiation, as well as selective forms of neural stem cell ablation, it was shown that LTP at MPP synapses in young adult-born granule cells of the rat DG accounts for approximately 10% of the total dentate gyrus LTP ([186,187]). Because newborn, in contrast to mature, granule cells exhibit depolarising GABAA-mediated responses, this LTP in synapses onto adult-born granule cells can be isolated using high-frequency stimulation in the presence of intact GABAA-mediated signalling ([187,188]). Impairment of this process has been further correlated with the cognitive decline observed in young patients who undergo radiotherapy ([186,189,190]). Lithium showed striking effects in rescuing this form of LTP in a Down syndrome model ([111]), and hope currently rests in achieving the same effect in cranial irradiation animal models.

1.7 Radiotherapy targets

Radiotherapy uses ionizing radiation (IR), high-energy photons, to displace electrons from atoms and molecules. In living tissues, IR will generate severe damage *directly* through breakage of chemical bonds and *indirectly* by ionization of H_2O and O_2 molecules, which generate free radicals ([191]). All of the resulting products are highly reactive with the surrounding environment and may ultimately lead to apoptosis or cell death. One of the many targets is DNA, and owing to its role in encoding information, DNA is the most vulnerable target, with major damage recapitulating in single- and double-stranded breaks of the double helix ([192]). Upon DNA damage, the interplay between multiple protein modifications, including phosphorylation, ubiquitylation, acetylation, and

sumoylation that combine to propagate the DNA damage signal eventually elicits cell cycle arrest, DNA repair, apoptosis, and senescence ([193]). Mammalian cells have evolved ways to offset these insults through numerous well-conserved cellular enzymatic mechanisms that can directly repair damaged DNA or allow tolerance of DNA lesions, guaranteeing faithful transmission of the genome. These processes include base excision repair, nucleotide excision repair, nonhomologous end joining, homologous recombination, and mismatch repair ([194]). Interestingly, cancer cells are more sensitive than noncancerous cells to IR due to their dysfunctional repair mechanisms and apoptotic signalling as well as derangement in their growth regulation ([195]). Nevertheless, tumours are often in a hypo-oxygenated state, decreasing the probability of generating free radicals and overall conferring a level of protection on cancer cells ([196]). In addition, during cell division, the cell cycle stages present different sensitivities toward IR, with the G_2-M phase more sensitive than the S phase ([197]). The aforementioned factors have been pivotal in evolving the conventional fractionated radiation therapy, aimed at delivering lower radiation doses over a longer period of time to spare normal tissues at the expense of tumours while avoiding loss of tumour control ([198]). However, the stem cell population residing in the postnatal brain has dividing rates comparable to cancer cells, and the response to IR manifests in a dramatic drop in their cell number immediately after the procedure that may persist ([199]). Monje *et al.* elegantly showed that SGZ neural precursors derived from irradiated rats failed to expand *in vitro* in a dose-dependent manner, and, more interestingly, naïve neural precursors transplanted into an irradiated brain were more likely to become astrocytes rather than neurons. These seminal findings suggest that the proliferative capacity of neural precursors is lost after irradiation, and, more importantly, that the compromised microenvironment imposes a coercive control on neural precursors. A recent study similarly showed that irradiation, prompting a DNA damage response (DDR) via activation of the kinase ataxia telangiectasia-mutated protein (ATM) and the

JAK/STAT3 pathways, fostered astrocytic differentiation of neural precursors *in vitro* and *in vivo*, shifting the cellular homeostasis ([200]). Radiotherapy, in addition to affecting actively proliferating precursors, also has pernicious consequences on the function of terminally differentiated cells, such as neurons, astrocytes, microglia, oligodendrocytes, and endothelial cells. Astrocytes have evolved a unique mechanism that confers radioresistance through the lack of activating the ATM-mediated DDR signalling while preserving the DNA repair capacity ([201]). This is in contrast to neurons, which due to their high metabolic state are markedly more affected by IR and are thereby more likely to perish or derange their neuronal function consequent to alterations of dendritic complexity, spine density and morphology, increased postsynaptic density protein 95 (PSD-95), and impaired synaptic plasticity ([189,190,202]). Sustained inflammation is also an important adverse effect of IR-induced damage, and this inflammation is mediated by activation of astrocytes and microglia ([199]), the resident immune-competent cells in the brain, that upon injury secrete pro-inflammatory cytokines to negatively impact other cell types ([203]). Importantly, oligodendrocytes, responsible for the myelination process, are more susceptible than astrocytes and microglia to IR-induced oxidative stress, apparently due to their relatively low antioxidant capacity and high iron content ([204]). Lastly, the vasculature has been identified as an active participant in the IR-induced stimulation of NSPC apoptosis *in vivo* ([205]). Despite the efficiency of IR in targeting and halting the growth spurts of tumours, the various effects of IR on multiple biological systems and its causal relationship with the neurocognitive sequelae observed in cancer patients treated with radiotherapy represent its main drawbacks ([22]).

1.8 Radiosensitivity of the developing brain

The first postnatal years are critical given that the brain growth spurt is maximal and changes have a great impact on brain function during this period ([134]). The accurate development of neurogenic niches during the postnatal period represents the hub for life-long neuronal production in the SVZ and DG. In addition, other cell types, such as endothelial cells, microglia, and oligodendrocytes, undergo significant maturation during the first postnatal weeks. Radiotherapy is known to affect all of these processes and to a larger extent in a young than an adult brain ([206,207]). Therefore, it is noteworthy that disturbances in brain homeostasis during this critical period lead to long-lasting functional and structural changes. Current therapies and rehabilitation strategies ([22]) that aim to improve neurogenic signals and recover lost functions of neuronal networks may, therefore, provide the right approach for ensuring the accurate functional maturation of the brain into adulthood.

2 AIM

The aim of the work presented in this thesis was to examine the effects of lithium on the developing brain after radiotherapy, focusing on neurogenesis in the female rodent hippocampal DG. Lithium is the most potent mood stabiliser used in the treatment of BPAD, and its treatment outcomes have been extensively investigated in the adult brain. Although lithium was shown to be effective in restoring neurogenesis and ameliorating synaptic plasticity in several pre-clinical studies, little is known about its effects in the juvenile brain. Therefore, we examined the early as well as the late responses of the developing brain and neural stem/progenitor cells to lithium administered before, during, or after radiotherapy. Specifically we investigated the following aspects:

I. Effects of lithium treatment on synaptic transmission in the dentate gyrus of the irradiated developing rat brain.

II. Effects of lithium pre-treatment on growth dynamics and cell cycle progression of irradiated young neural stem/progenitor cells.

III. Spatial regionalisation of lithium following long-term administration and lithium-associated lipid changes in the juvenile mouse brain.

IV. Immediate and late effects of delayed chronic lithium treatment on neurogenesis after irradiation of the developing mouse brain.

3 MATERIAL AND METHODS

Refer to papers I, II, III and IV for the details of the methodological approaches used in this thesis.

4 RESULTS AND DISCUSSION

Irradiation of the juvenile brain permanently alters synaptic plasticity

A previous study by our group was pivotal in defining the higher sensitivity of the developing brain compared with the adult brain to radiotherapy ([207]), providing important correlative evidence for the cognitive impairments induced by this treatment in children. In **Paper I**, to extend this line of evidence, we examined the electrophysiological properties of the DG in 4-month-old rats after administering radiation at a dose of 6 Gy to rats on postnatal day (PND) 11 that received 14 days of LiCl intraperitoneal (i.p.) injections starting on PND 7. First, we evaluated the efficacy of the basal excitatory transmission in the MPP **Fig. 3**. To this end, we conducted input/output measurements and plotted the magnitude of the fibre volley (reflecting the number of activated axons) versus the field excitatory postsynaptic potential (reflecting the activated population of synapses) evoked at increasing stimulation intensities. We observed that irradiation consistently resulted in a long-lasting enhancement of basal synaptic transmission at MPP granule cell synapses. These synapses encompass the developmentally generated as well as adult-born granule cells that are found at different maturation stages at any given time. Notably, the integration of the young adult-born neurons in the DG is progressively impaired following irradiation ([207-209]). For this reason, we quantified the young granule cell population, and as previously reported ([208]), we confirmed that irradiation of the juvenile brain markedly reduced the population of immature neurons expressing DCX. Lithium administration that began prior to irradiation and continued after irradiation was insufficient to rescue this loss, which persisted into adulthood. Our electrophysiological assessment was consistent with a previous study showing that, compared with control mice, irradiated adult mice presented reduced DCX expression as well as stronger activation of the DG in response to perforant path activation, which the authors attributed to a reduced

inhibitory tone ([210]). To address the contribution of the inhibitory network, we quantified the number of parvalbumin-expressing inhibitory interneurons in the hilus, but found no evidence indicating a reduced number of these cells in either the irradiated or lithium-treated groups. However, because we neither investigated other types of GABAergic interneurons nor specifically examined inhibitory synaptic transmission, we cannot rule out that long-term effects of irradiation in the DG involve alterations in inhibition. Nevertheless, we propose that this increased synaptic strength is due to the depletion of adult-born granule cells, which generally exhibit weaker coupling to the MPP input and silent glutamatergic synapses compared with mature granule cells ([181,182,211,212]).

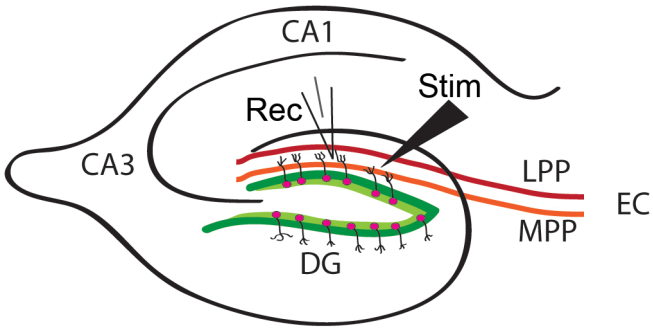

Figure 3. Representative figure of the site of the electrophysiological recordings. A bipolar tungsten electrode (Stim) was placed in the medial perforant path (MPP) in the middle of the molecular layer. The evoked excitatory response was recorded 300 µm away of the stimulation electrode at the same distance from the granule cell layer of the dentate gyrus (DG) using a glass capillary micropipette (Rec) filled with 1 M NaCl. LPP= lateral perforant pathway, EC=entorhinal cortex, CA= cornus ammonis. Author: Giulia Zanni.

During the first 3 weeks after their birth, young granule neurons undergo extensive morphological and synaptic changes encompassing formation of glutamatergic and GABAergic synapses, both exerting an excitatory function ([176,213,214]). Thus, young granule cells are thought to display enhanced synaptic plasticity with lower induction thresholds. Additional previous studies examining adult neurogenesis identified young granule neurons as responsible for a unique form of long-term plasticity that can be elicited in the presence of GABAergic inhibition and is ablated by irradiation ([178,186,187,215]). Hence, to examine the effect of early-age irradiation on LTP in adulthood, we applied a protocol of four trains of high-frequency stimulation (HFS; 100 Hz for 1 second with a 15 second inter-train interval). We found that irradiation of the young developing brain resulted in ablation of LTP in slices obtained from adult mice. LTP was not only ablated, but the response to HFS resulted in long-term depression (LTD). By contrast, in the sham group, a small but consistent LTP was elicited, as previously described ([187]). It is well known that it is difficult to induce LTP in adult granule cells using HFS in the slice preparation when GABAergic inhibition is intact ([187,215,216]). Indeed, in contrast to newborn granule cells, mature granule cells are under strong inhibitory control, which likely explains the difficulty of inducing LTP in general and in particular when the DG is depleted of newborn granule cells ([187,215]). However, to our knowledge, HFS has not previously been shown to elicit LTD in the DG. It has been reported that HFS results in LTD, instead of LTP, in CA1 pyramidal neurons when NMDA receptor channels are blocked with an open channel blocker ([217]). This shift to LTD does not occur when the NMDA receptor is blocked by an antagonist obstructing the glutamate binding site, suggesting that the induction of NMDA receptor-dependent LTD relies on metabotropic NMDA receptor function, rather than on ionotropic NMDA receptor function with calcium influx through the channel ([217,218]). We propose that when the inhibitory control of the ionotropic NMDA receptor function is strong and

newborn granule cells are depleted, the metabotropic NMDA receptor-dependent LTD in mature dentate granule cells may be unmasked.

LTP and LTD are experimental tools that can be used to demonstrate the long-lasting modifications of individual synapses. However, it is a difficult to prove that these activity-dependent modifications support functional roles. Yet, these two opposing phenomena are still considered the principal candidates for mediating learning and memory, as well as other types of experience-dependent plasticity ([219]).

The integration of young adult-born granule cells in a pre-existing network has proved to be important for processing information during discriminatory tasks, such as pattern separation ([220,221]). Conversely, impairment of neurogenesis showed to correlate with increase generalisation and negatively affect declarative memory, thereby proving a link to cognitive functions ([186,222,223]). Therefore, the absence of newborn neural cells and the impaired synaptic transmission observed in the present study are likely to correlate with the cognitive decline observed in both rodents and humans after irradiation of the young brain.

Previous studies demonstrated that lithium administered chronically to adult mice for 4 weeks had beneficial effects in replenishing adult-born granule cells after radiation-induced loss of neurogenesis and in rescuing LTP in a Down syndrome model ([77,111]). However, despite encouraging results in a mouse model ([174]), in the current study, we observed no clear effect of lithium treatment on any of the parameters described. One plausible explanation is that we limited the lithium treatment to 2 weeks. In addition, we evaluated the results after the lithium effect had already washed out, possibly resulting in a lack of measurable effects. Evaluation at an earlier time point or chronic treatment would be needed to rule out these possibilities.

Lithium enhances growth dynamics and accelerates cell cycle progression in young NSPCs

In **paper II**, we first sought to address the dose-response effect of lithium on young hippocampal NSPCs isolated from mouse brain and grown in culture under the proliferative drive of epidermal growth factor and fibroblast growth factor 2. We selected the neurosphere *in vitro* model due to its putative resemblance to the *in vivo* scenario in which, in addition to NSCs, neuronal and glial progenitors at various stages of differentiation are preserved, most closely representing *in vivo* heterogeneity ([224]) and making this model a suitable tool for investigating how extrinsic stimuli affect various growth parameters ([225]). The rationale for using 1 and 3 mM LiCl in **paper II** stems from previous observations supporting the notion that the dynamic lithium distribution in the brain may not reflect plasma levels ([43,44]). As also reported in **paper III**, lithium likely regionalises in areas with high cell density, particularly in neurogenic regions, suggesting that these local lithium concentrations may be higher than those considered in the therapeutic range (0.6–1.2 mmol/L).

Wexler at al. ([71]) showed that the proliferation of adult rat hippocampal neural progenitors exposed to lithium dose-dependently increases through activation of Wnt signalling and that within the therapeutically relevant lithium concentrations (1–3 mM) neurogenesis is favoured without significantly altering gliogenesis. A similar scenario may be predicted for young NSPCs; however, the scarcity of reports about this and the known differences in dynamic cell-autonomous regulation ([226]) prompted our generation of supporting evidence. Our results examining the multipotency of young NSPCs showed that lineage commitment was not affected by lithium treatment, strengthening previous *in vitro* and *in vivo* findings ([71,109]). We then tested the proliferative potential of NSPCs by measuring BrdU incorporation as well as sphere volume at various times. We found that lithium increased both the proportion of dividing cells and

the volume of the clusters of dividing cells formed into neurospheres in concentration- and time-dependent manners. Next, we hypothesised that this increase in proliferative capacity is likely to involve cell cycle entry and progress ([227-230]) because higher proliferative potentials are often correlated with the shortening of the cell cycle ([231-233]). Indeed, our cell cycle analysis demonstrated that the percentage of cells in G_1 phase was reduced in favour of a marked increase in S and G_2/M phases. Therefore, in **paper II**, we provided additional evidence that the lithium concentration-dependent proliferative effect reflected in a redistribution of NSPCs across the cell cycle specifically shortened the G_1/S phase transition or the time spent in G_1, ultimately resulting in an acceleration of the cell cycle.

Lithium rescues young NSPCs from radiation-induced cell cycle arrest

Considering the overt effects of IR on cell cycle progression ([193]), we examined whether this lithium-dependent proliferative gain led to a rescue of young NSPCs after irradiation *in vitro*, or rather promoted radiosensitisation and apoptosis, as previously observed in cancer cell lines ([95]). For this purpose, in **paper II**, we exposed young NSPCs derived from mouse hippocampus to a radiation dose of 3.5 Gy, resulting in a 5.3- and 7.5-fold decrease in neurosphere volumes compared with sham cells 24 and 48 hours after irradiation, respectively. Surprisingly, the NSPCs receiving lithium pre-treatment with 3 mM, but not 1 mM, for 12 hours displayed a 2-fold increase in neurosphere volume 24 and 48 hours after irradiation as well as a 16-fold increase in BrdU incorporation at 48 hours. These data argue against the possibility that lithium treatment sensitises NSPCs in the developing brain to irradiation as previously observed for cancerous cells ([169,234]). Our results may be due to lithium acting on genes with differential roles in distinct DNA repair pathways, which are frequently aberrant in cancer ([95,235]), strongly encouraging the concurrent use of lithium treatment with radiotherapy ([93,95,235]). In addition, an analysis of the cell

cycle after irradiation revealed that the significant reduction of NSPCs in S phase was fully rescued by 3 mM LiCl as early as 6 hours after irradiation, and this protection was maintained for at least 72 hours. More interestingly, our results showed that lithium concentration-dependently ameliorated G_1 arrest after irradiation for at least 72 hours. However, the accumulation in G_2 phase after irradiation was more prominent in irradiated NSPCs treated with lithium concentrations at both 1 and 3 mM. Irradiation is known to activate the DDR pathway, resulting in a cascade of events that ultimately promotes post-translational modification of proteins involved in DNA damage repair, modulation of apoptosis and/or cell cycle progression ([236,237]). In particular, actively proliferating cells use cell cycle checkpoints to ensure that there is enough time for repair to occur, guaranteeing faithful transmission of the genome to the daughter cells even after genotoxic stress ([236]). The arrest in G_1 phase following irradiation has been previously related to activation of p53, a tumour suppressor gene that in turn upregulates $p21^{Cip1}$ and $p16^{Ink4a}$, inhibitors of cyclin-dependent kinases, leading to cell cycle arrest ([238-240]). We surmised that at the time of irradiation there is a heterogeneous pool of NSPCs, with cells at various cell cycle stages, which activate different DDR signalling pathways. We speculated that cells that have recently entered interphase are more likely to activate a p21-dependent G_1 arrest, whereas cells that are recruited into proliferation by lithium treatment and have initiated the elongation process are prone to arrest in G_2 phase ([236,241]).

We further speculated that a higher proliferative capacity may be concurrent with a higher apoptotic rate as a homeostatic mechanism of self-renewal, which has been observed *in vivo* in the DG of the mouse ([146,147]). Thus, we investigated two parameters indicative of apoptosis/cell death: annexin V, which binds to the phosphatidylserine expressed in early apoptotic cells; and the sub-G_1 cell cycle fraction, which reflects the population of dying cells with fragmented DNA ([242,243]). We found that irradiated NSPCs displayed, beginning 24 hours

after irradiation, a higher and sustained apoptotic rate, as determined by the results of both annexin V and sub-G_1 analyses at all time-points examined. This increase in apoptosis was not reversed by lithium, neither in the irradiated nor in the sham NSPCs. This evidence led us to postulate that, despite the presence of lithium, DNA-damaged young NSPCs, which are generally driven into apoptosis ([208,244]), remain committed to programmed cell death to the same extent as their untreated counterparts. Therefore, lithium may forestall the potentially carcinogenic transmission of damaged NSPCs bearing accumulated genotoxic stress through cell division.

Another important observation made in **paper II** was the significant, though modest, reduction in radiation-induced γH2AX activation in 3 mM LiCl-treated NSPCs, providing supporting evidence that the lithium-mediated rescue of proliferation in NSPCs was accompanied by a less genotoxic stress response and possibly a higher degree of protection. The mechanisms formerly proposed included activation of DNA-dependent protein kinase, which in turn modulates the pro-survival PI3K/Akt pathway, causing a decrease in γH2AX foci and an increase the nonhomologous end joining repair pathway, supporting the beneficial effects of lithium on the DDR ([92,93,245,246]).

In vivo response to long-term lithium treatment

In **paper III**, we investigated the *in vivo* response to long-term lithium treatment during development. Serum lithium levels were analysed after administering a loading dose of LiCl (4 mmol/kg) followed by chow supplemented with 0.24% Li_2CO_3 to PND 21 female mice for 28 days. The initial bolus injection caused a peak serum lithium level, and 5 hours after the onset of treatment, lithium reached a steady state level of approximately 1.2 mmol/L, which is within the therapeutic range for bipolar disorder in humans. This stabilisation within the therapeutic range suggests that this mode of delivery may be appropriate for

causing an effect in young animals. However, we also noticed that, compared with controls, mice administered long-term lithium displayed lower body weight gain 2 days after the onset of lithium treatment, and this lack of growth persisted at 7 days, 14 days, and 28 days. This lower growth rate was initially concomitant with a lower food intake, but intake reached normal levels at 14 and 28 days. These data indicated that although the food intake was restored to basal levels after 14 days, body weight did not reach that of control mice, even when a partial recovery was observed. Additionally, we observed that lithium-treated animals suffered from excessive urination, suggesting that this dose and mode of lithium administration predisposed juvenile mice to nephrogenic diabetes insipidus. This has not been reported previously using this type of chow, although lithium-induced nephrogenic diabetes insipidus is a well-known phenomenon that is under investigation to determine amelioration for this condition ([247]). In our study, 0.24% Li_2CO_3-supplemented chow was provided on PND 21 to female mice. However, previous studies used adult male animals. Thus, it is plausible that young female mice are less tolerant than older male mice of this lithium dose ([111]). Interestingly, rats and mice treated with daily intraperitoneal (i.p.) injections of lithium did not display this slower weight gain or signs of polyuria ([86,107,108,174]), suggesting that fluctuating serum concentrations after i.p. administration may be, as previously observed, preferable to avoid negative effects on distal renal filtration ([248]). For future studies, it will be important to investigate the pharmacokinetics of the two modes of administration and to establish the appropriate duration of lithium treatment to avoid adverse effects and to optimise the therapeutic window in young patients. It is still a matter of debate whether the doses required to control bipolar disorder are the same as those required for neuroprotection in the developing brain.

Lithium displays a region-selective uptake and boosts proliferation in the juvenile mouse brain

As previously described ([249]), in **paper III**, we identified in the developing brain the spatial distribution of the most prominent chemical species in the brain, including cholesterol and choline, as well as lithium, using time-of-flight secondary ion mass spectrometry (ToF-SIMS) **Fig. 4**. With this approach, the specific chemical profile of each of these species can be used to delineate an anatomical region of interest (ROI). Cholesterol localises in the white matter region, whereas lithium and choline are prevalent in the grey matter.

We conducted a principal component analysis and found that lithium followed a spatial dependent pattern of distribution in the following regions: olfactory bulb (OB), SVZ, rostral migratory stream, hippocampus, DG, cerebral cortex, cerebellum, and basal ganglia. Lithium has a tendency to accumulate in areas of the brain with higher cell density, and the ToF-SIMS analysis conducted here confirmed this ([42,43,44]).

The lithium signal in the brain was high on day 2 but then declined in non-neurogenic regions (i.e., cerebellum and basal ganglia), while in neurogenic regions (i.e., DG, OB, and SVZ), the signal was maintained at a relatively constant level. This strongly argues in favour of lithium distributing in regions with higher cellular densities, indicating that lithium uptake occurs chiefly in the cell body rather than in axons, as previous studies also hypothesised ([43,44]).

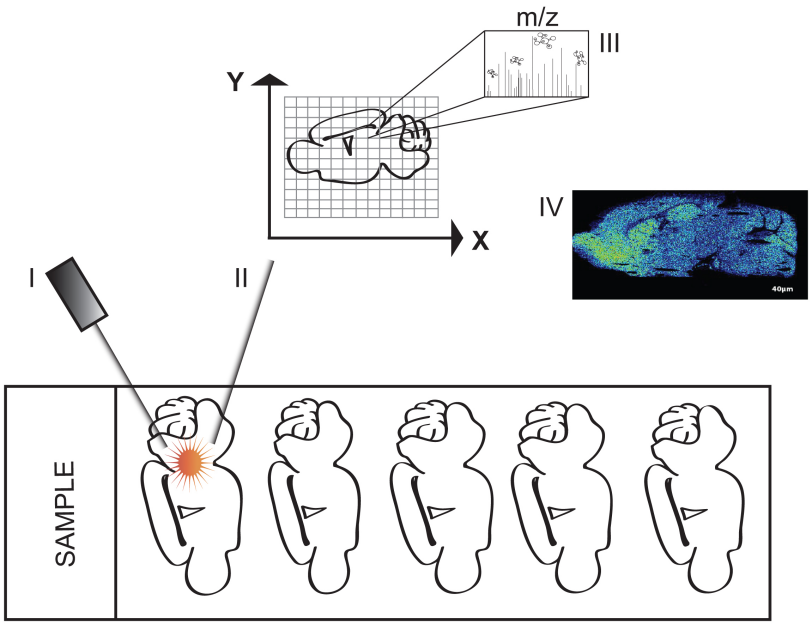

Figure 4. Principle of imaging mass spectrometry. (I) Sagittal frozen tissue sections are mounted on a glass and probed with an ionbeam, generating low molecular weight secondary ions (m/z > 1000 Da) (II). One mass spectrum is acquired for every XY coordinate of the scanned tissue (III) and a single ion image (IV) can be generated by mapping the intensity of an individual ion signal (m/z; rel. Int) over the whole tissue slice. Author: Giulia Zanni.

This regionalisation may be related to the positive effects previously observed on neurogenesis and other molecular processes relying on the generation and integration of new neurons in a pre-existing network ([71,109,111,174]). Indeed, **in paper III**, we investigated the effect of lithium on neurogenesis immediately after discontinuing lithium-supplemented chow and found that long-term treatment led to a significant 1.34-fold increase in cellular proliferation, as determined by quantifying the proliferation marker Ki67 in the SGZ of the DG, thus corroborating the *in vitro* findings discussed in **paper II**. However, no direct effect of lithium was observed on the integration of newly-born neurons into the DG network, as determined by quantifying DCX. In agreement with a

previous study using the same dosage regimen as that used in **paper III**, lithium was found to predominantly target the initial stage of progenitors, enhancing the turnover of NSPCs but failing to increase numbers of immature neurons ([171]). As will be discussed later in **paper IV**, our data suggest that discontinuation of lithium treatment is necessary to allow the proliferating cells to differentiate and integrate.

Lithium regional uptake is associated with lipid changes

In **paper III**, we further examined the lipid distribution, analysing the association of any lipid changes in the various ROIs with the distribution of lithium treatment. The key finding was that all the lipids and lipid-associated species examined, including phosphatidylcholine, choline, vitamin E, sphingomyelin and cholesterol, were differentially changed in the ROIs analysed. Particularly in the cortex the levels of choline and phosphatidylcholine showed to be significantly elevated on day 28 and similarly the levels of cholesterol were increased at both day 14 and 28. Elevated levels of the lipid fragment m/z 246.10 were also observed on day 28 in the cortex, hippocampus and DG. On the contrary sphingomyelin was found to be decreased in virtually all ROIs in the lithium treated groups as compared to their age-matched controls particularly in the cortex. Lipids in the brain are relevant components in cell signalling functions and in neural stem cell differentiation in particular ([250]). Both cholesterol and sphingomyelin are the main components of lipid rafts, which function in membrane signalling and trafficking ([251]). The exact mechanisms by which lipid rafts act are poorly understood; however, they are implicated in mediating the cell signalling triggered by growth factors and cytokine receptors, and ultimately in modulating the maintenance, polarisation, and differentiation of NSPCs ([250]). Additionally, lipogenesis is pivotal for ensuring life-long neurogenesis. Thus, targeting this metabolic pathway may reveal new therapeutic approaches for treating neurogenesis-related cognitive

decline ([252]). Although the broader implications of our findings need further elucidation, the evidence that we provided for lithium associating with overt lipid changes may deepen knowledge on the effects of lithium in the developing brain.

We also found that levels of vitamin E in the cerebellum, hippocampus and DG displayed an initial increase at day 2 followed by stabilisation to control levels at later times. Vitamin E is a lipid with a strongly electrophilic group capable of efficiently quenching carbon radicals, making it a strong antioxidant and an efficient neutraliser of unstable lipid peroxy-radicals generated from polyunsaturated fatty acids ([253,254]). Previous findings showed that vitamin E is highly expressed in cerebellar Purkinje cells ([255]), and its antioxidant role in neurodegenerative diseases has been extensively investigated ([256]). These findings together with our data showing a strong effect of lithium on vitamin E brain distribution provide supporting evidence for a likely beneficial effect of lithium in the developing cerebellum. These data also demonstrated that the resolving power of ToF-SIMS is reliable for determining the spatial distribution of small molecules and that this method has the potential to correlate locally confined changes in various biochemical species with underlying cellular processes.

Delayed lithium treatment rescues NSPC proliferation after irradiation of the juvenile mouse brain

In **paper IV**, as shown in the study design in **Fig. 5**, we sought to develop a novel lithium administration paradigm following whole-brain irradiation. A single dose of 4 Gy was delivered to each animal on PND 21, which is comparable to a therapeutically relevant but rather low dose ([196,209]) of radiation administrated to the brain of a 2- to 3-year-old child ([134]). Female mice received chow supplemented with 0.24% Li_2CO_3 starting 4 weeks after irradiation on

PND 49, an age comparable to an 18-year-old human ([134]). The lithium-supplement chow was maintained for 4 weeks, from PND 49 to PND 77.

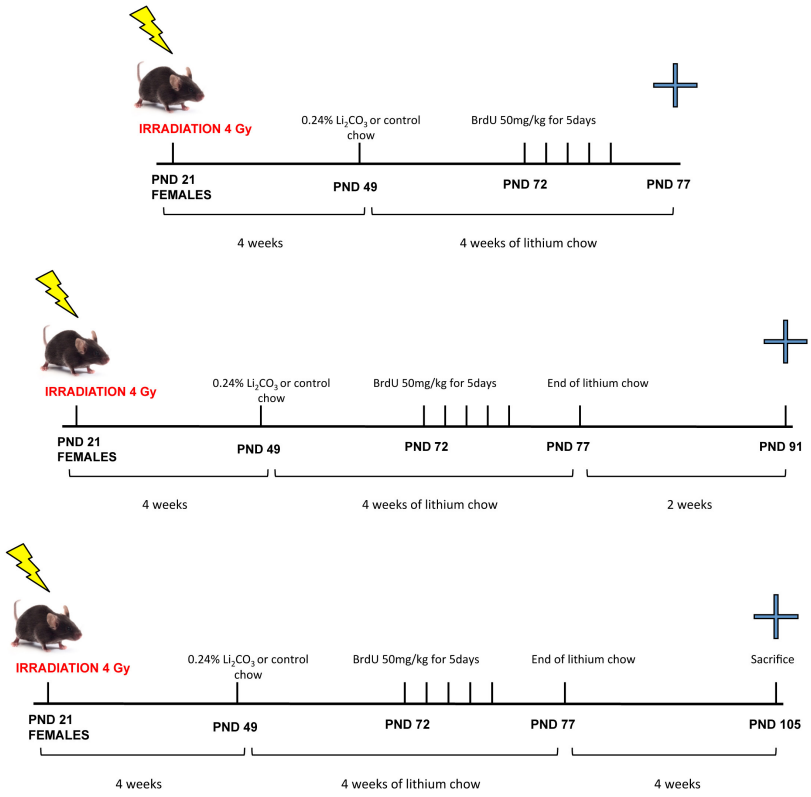

Figure 5. Delayed lithium treatment study design. Postnatal day 21 (PND21) female mice were irradiated with a 4 Gy dose. They were randomly assigned to either lithium or control chow four weeks after irradiation, from PND49 to PND77. All animals received 50 mg/kg 5-Bromo-2´-Deoxyuridine (BrdU) dose the last five days of the lithium chow and sacrifice at different time points: PND77, PND91 and PND105 and the assessments of proliferation, integration and survival were conducted at each time point, respectively. Author: Giulia Zanni.

Although several studies showed the neuroprotective role of lithium in rodent models of brain injury ([107,110,174]), delayed administration of lithium to rescue neurogenesis following cranial irradiation has never been demonstrated.

Radiation-induced hippocampal injury affects the neurogenesis cascade within the SGZ on multiple levels, producing an increase in NSPC apoptosis, a decrease in the number of surviving NSPCs, a decreased tendency of those NSPCs to differentiate, and sustained inflammation ([174,209,257]). In **paper IV**, sagittal sections obtained on PND 77 from irradiated mice immediately following discontinuation of lithium chow revealed that late onset of lithium administration increased the proliferation of NSPCs as indicated by the increased density of $BrdU^+$ cells in the SGZ, strengthening our findings in **papers II** and **III**. Lithium was recently shown to promote the proliferation of qNSCs ([171]). However, in response to pro-neurogenic stimuli, such as physical exercise, enriched environment, and antidepressants, most proliferating cells in the DG are ANPs ([166,258-260]). Hence, it is plausible that lithium targets the symmetric divisions of the ANP population, which would be therapeutically beneficial because those cells represent a renewable source of neuronal precursors. By contrast, enhancement of qNSC proliferation would be detrimental for neurogenesis because it would deplete this non-renewable source of neuronal precursor cells.

Additionally, as previously observed in **paper III**, we found no difference in the number of immature DCX^+ granule cells upon lithium treatment both in sham and irradiated animals. It may be argued that lithium induced apoptosis in the immature DCX-expressing cells, however due to the pro-proliferative and anti-apoptotic properties of lithium *in vivo* this is an unlikely scenario. In further support of this hypothesis, previous studies also showed that lithium *in vivo* decreases apoptosis through inhibition of glycogen synthesis kinase-3β activity ([53,261]), which translates to an upregulation of the pro-apoptotic molecules B-cell

lymphoma protein-2, brain-derived neurotrophic factor, and β-catenin ([262-264]). Instead, we proposed that lithium may induce neural progenitor cells to adopt the immature DCX phenotype only transiently. We further speculated that their DCX-expressing stage of the neuronal differentiation cascade might have been accelerated before they became mature granule cells.

Lithium discontinuation is necessary to allow the integration of immature neurons after irradiation

During neuronal differentiation, approximately 4 to 6 weeks are required for a progenitor cell to become a mature neuron. DCX expression occurs during the first 3 weeks, peaking between the fourth and seventh day ([265,150]). In this regard, the neurogenic cascade is a structured one ([144]) in which neuronal migration is an unceasing progression of neurons differentiating from the ANP pool into neuroblasts and then into immature neurons, gradually changing the orientation of the leading neurite relative to the granule cell layer during each of these phases **Fig. 2** ([149,266-268]). An important parameter that determines the functional integration of DCX cells into the granule cell layer is the orientation of the leading process. Immature neuroblasts display an elongated cell body flanked by dendritic processes that lie parallel to the SGZ, contrary to DCX cells at late stages of maturation that exhibit instead a radial process ([149,269]). Thus, in **paper IV**, to assess whether the cells that were observed proliferating on PND 77 survived and differentiated into immature neurons, we analysed the density of the DCX cells as well the orientation of dendritic processes in cells double-labelled with BrdU and DCX in the DG on PND 91, 2 weeks after lithium discontinuation. Our results showed that when sham and irradiated mice were no longer exposed to lithium, the density of DCX cells significantly increased in both those groups compared with saline-treated sham and irradiated-only mice, respectively. Interestingly, when we analysed the orientation of the leading neurite, we observed that irradiation produced a significant increase in the

percentage of parallel processes, whereas the number of radial processes was significantly decreased. These data are in line with previous studies showing that irradiation perturbed the structural integration of immature neurons ([208,270,271]). More importantly, we found that whereas lithium had no effect on radial processes, in irradiated lithium-treated mice, the orientation of the parallel processes reverted to the sham control level. These data suggest that it is unlikely that lithium-induced immature cells to switch their orientation, avoiding the DCX stage, but the permanence at this stage might have been reduced. Lithium may therefore protect against radiation damage by limiting DCX cells to stay an immature phenotype for an undetermined period of time. It could be argued that the lack of effect of lithium on radial process orientation is because the cellular fates of DCX immature neurons have already been determined, such that those neurons are already oriented along their correct routes. However, the percentages of radial cells co-labelled for BrdU and DCX in both the sham-irradiated and irradiated lithium-treated groups remained unaltered after lithium treatment. To preserve or increase neurogenesis, lithium should preferably target the late critical period of newborn cell survival as well as their structural and synaptic integration. The role of lithium may involve not only guaranteeing that dendritic process orientation is correctly maintained, but also stimulating dendritic maturation. Indeed, irradiation permanently affects dendritic complexity as well as spine density ([270]) and previous studies attributed these changes to the radiation-induced increase in the expression of the synaptic plasticity-regulating protein PSD-95 ([272,273]). This seems to have an important role in controlling dendritic morphology, and when overexpressed, it adversely affects dendritic complexity ([272]). In our study, however, the capacity of lithium to stimulate dendritic sprouting in DCX immature neurons will require further validation.

Lithium discontinuation partly reversed the radiation-induced switch in lineage commitment

To investigate the effect of lithium on neuronal maturity, we assessed, in **paper IV**, the percentage of $BrdU^+/NeuN^+$ cells in the DG obtained from mice sacrificed on PND 105, 4 weeks after lithium discontinuation. Our results showed that compared with irradiated-only mice, the number of $BrdU^+/NeuN^+$ neurons in the irradiated plus lithium-treated group was significantly increased reaching sham-control levels. We did not find any difference in the number of $BrdU^+/NeuN^+$ neurons between sham mice administered saline and those treated with lithium. In addition, lithium had no effect on the density of DCX cells measured on PND 105 in the sham or the irradiated groups. Next, we examined the effect of lithium on astrocytic maturation by quantifying the percentage of co-labelled cells positive for $BrdU^+$ and the glial-specific marker $S\text{-}100\beta^+$ in the DG and found a trend suggestive of lithium's ability to reduce the number of astrocytes generated after cranial irradiation. This radiation-induced alteration in lineage commitment has been previously observed in adult as well as young mice ([199,274,275]). In addition, compelling evidence suggests that chronic inflammation secondary to increased apoptosis and sustained production of reactive oxygen species cause the cells to adopt a senescent phenotype, and concomitant elevation of cytokine secretion levels promotes increased glial differentiation ([200,276-279]). This radiation-induced perturbation of the DG homeostasis was positively modulated by lithium, such that neuronal maturation of the surviving neurons was restored, and the neurogenic lineage in the DG was preserved.

5 CONCLUSION

Despite the improved survival rates of paediatric patients treated for brain tumours, cranial irradiation remains responsible for numerous adverse effects, including cognitive impairments, growth retardation, and social inadaptability, in the surviving patients (280,281). The results of the work presented in this thesis better define the mechanisms underlying the extreme sensitivity of the developing brain to irradiation, which manifest overtly in structural changes and synaptic transmission re-arrangements chiefly affecting neurogenic regions (189,207) that likely correlate with the neurocognitive sequelae of radiotherapy. It is tempting to speculate that preserving or promoting neurogenesis may help mitigate the lasting, progressive cognitive deficits observed in radiotherapy-treated survivors of brain tumours (271). Lithium, the most potent mood stabiliser established for treatment of bipolar disorders, has proven efficacious in the treatment of several other diseases. Pre-clinical studies have demonstrated that short- and long-term lithium administration protect against the neurodegenerative effects of cranial radiotherapy through their pro-proliferative, anti-inflammatory, and anti-apoptotic effects in both young and adult animals [92,174]. Striking positive effects on rescued neurogenesis and synaptic plasticity have also been corroborated [111], and other pre-clinical studies have validated the efficacy of lithium in preventing neural degeneration and restoring synaptic networks in models of Parkinson disease, Alzheimer disease, and fragile X syndrome [112,113,282]. There are, however, limited published pre-clinical and clinical data in support of a neuroprotective or neuroregenerative role of lithium in children treated with radiotherapy, and the contribution of the work in this thesis will impact this endeavour.

Our results showed that caution should be used when translating a well-established treatment for the planning and safety assessment of paediatric trials, especially to avoid the known adverse effects of lithium treatment (247). The

results in this thesis showed that delayed lithium treatment at a therapeutically relevant dose was able to rescue neurogenesis even long after irradiation of the juvenile brain. Studies herein examining lithium discontinuation suggest that a sequential therapeutic scheme for lithium treatment after irradiation may be preferable to a chronic or life-long protracted regimen. Valid concerns have been expressed about lithium protecting not only healthy cells but also the remaining tumour cells and thereby encouraging relapse ([94,124]), despite numerous studies demonstrating that lithium does not appear to foster tumour growth ([92,94]). Although there is strong evidence of lithium acting as an anti-tumour treatment in medulloblastoma ([95,283]), glioblastoma ([70]) or gliomas ([284]), its actions on different tumour types and stages remain indeterminate. It is therefore important to generate evidence supporting the most appropriate use of lithium in children undergoing radiotherapy.

We speculated that lithium regionalisation in brain structures with higher neurogenic potential is associated with the positive effects on NSPCs and neurogenesis observed in both our *in vitro* and *in vivo* models, although we did not exclude that other reasons for the regionalisation not related to the observed effects are possible. We demonstrated that lithium is important for both regenerative and anti-tumourigenic purposes.

The ability of lithium to modulate the cell cycle in proliferating cells and protect against DNA damage offers a promising approach to therapy, and this is particularly relevant after irradiation for restoring the depleted pool of proliferating NSPCs ([93,232,279]).

Additionally, our novel findings associating brain levels of lithium with those of lipids and vitamin E support further investigation of latent antioxidant effects targeting extra-neurogenic regions.

Ultimately, the results of the studies comprising this thesis demonstrate that the administration of lithium to children with cancer who were treated with radiotherapy is warranted because lithium has the potential to improve the quality of life for those children who survive their cancer.

6 FUTURE PERSPECTIVES

Encouraging results strongly support the use of lithium in combination with radiotherapy to enhance the protective effects ([95,283]). However, because the effects of lithium on different cancer types remain uncertain, post-radiotherapy lithium treatment may represent a better initial approach to safely exclude the prospect of lithium increasing the risk of relapse. The results of the studies comprising this thesis will serve as background for the design and safety assessment of a clinical trial that will soon be conducted involving children of Nordic countries and France treated for medulloblastoma, including radiotherapy.

Our short-term, pre-clinical plan is to further investigate at the DNA level whether irradiation acts on the functional specificity of transcriptional repressor checkpoints, controlling the premature growth arrest, and whether the lithium effect is associated with the prevention or reversibility of this downstream mechanism.

Additionally, we plan to conduct electrophysiological assessments in mice and determine whether neurons born during the lithium treatment are functionally integrated and display the expected intrinsic properties during their different maturation stages. We also plan to define which stages of NSPCs lithium acts on, with the hope of excluding that the quiescent renewable source of neurons is negatively affected by lithium. It will be equally important to conduct behavioural experiments to see if the effects of lithium also are reflected in a functional read-out.

Overall, we believe that our work strongly encourages future clinical trials aimed at treating young patients with lithium after the curative phase of radiotherapy.

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