UNIVERSITÀ DEGLI STUDI DI TORINO

Dipartimento di Scienze Veterinarie

DOTTORATO DI RICERCA IN NEUROSCIENZE
CICLO XXXIV

TITOLO DELLA TESI:

γ phosphorylation of the H2AX histone in the old mouse brain and its correlation with BrdU incorporation and cell death

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ANNI ACCADEMICI:
1 ottobre 2018-30 settembre 2022

SETTORE SCIENTIFICO-DISCIPLINARE DI AFFERENZA: VET/01

ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere gratitude to my supervisors Professors Laura Lossi and Adalberto Merighi who made this work possible. Their help and guidance carried me through my PhD study and research.

I wish also to thank Professor Alberto Granato for his precious contribution in reviewing my thesis.

I am thankful to all my colleagues for their friendship, support, and advice: Patrizia Aimar, Susanna Romboli, Fulvia Cerruti, Graziana Gambino, Chiara Salio, Francesco Ferrini and Emma Merlin. Thank you for all the laughter we shared together in the last four years.

Last but not least, I would like to thank my parents for their love, support and encouragement.

LIST OF ABBREVIATIONS

^{3}H-thymidine	tritiated thymidine
53BP1	p53-binding protein 1
BrdU	5-bromo-2-deoxyuridine
BW	body weight
CA	hippocampal cornu Ammonis
CASP3	caspase 3
cCASP3	cleaved (active) caspase 3
ChP	choroid plexus of the lateral ventricle
CI	confidence interval
CldU	5-Chloro-2'-deoxyuridine
CNS	central nervous system
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
DDR	DNA damage response
DG	dentate gyrus
DLC	double-labeled cells
DSBs	double-stranded DNA breaks
EdU	5-ethynyl-2'-deoxyuridine
GF	growth fraction
IdU	5-Iodo-2'-deoxyuridine
i.p.	intraperitoneal
IR	ionizing radiation
LI	labeling index
NGS	normal goat serum
NSCs	neural stem cells
OB	olfactory bulb
OCP	observed co-labeling percentage
PB	phosphate buffer
PBS	phosphate-buffer saline
PCP	predicted co-labeling percentage
pHH3	phosphorylated form of histone H3
PLL	poly-L-lysine
PTM	post-translational modification

RMS	rostral migratory stream
ROS	reactive oxygen species
SD	standard deviation
SGZ	subgranular zone of the hippocampus
SSBs	single-stranded DNA breaks
SVZ	subventricular zone of the lateral ventricle
γH2AX	γ phosphorylated form of histone H2

1 INTRODUCTION

1.1 HISTONES

Nucleic acids are a long chain or polymer of repeating subunits, called nucleotides. Each nucleotide subunit is composed of three parts: a five-carbon sugar, a phosphate group, and a base (Dickerson, 1983). The sugar contained in the nucleotide subunits of DNA is deoxyribose. The bases are nitrogen-containing molecules. Two of them, adenine (A) and guanine (G) have a double carbon-nitrogen ring structure, while the other two bases, thymine (T) and cytosine (C), have a single ring structure (Chargaff, 1951). The phosphate functional group (PO_4) gives DNA the property of acid at physiological pH. The linking bonds that are formed by phosphates are esters that have the additional property of being stable, yet easily broken by enzymatic hydrolysis.

DNA has a double-helical structure with sugar-phosphate backbones on the outside and base pairs on the inside. However, the eukaryotic cell usually stores its genetic information in DNA molecules that can be over 1 m in length. Given its length, the DNA is hierarchically packed in the nucleus with the aid of proteins to form a complex called chromatin. The nucleosome core particle represents the first level of chromatin organization (Fig. 1) and was characterized for the first time by Kornberg in 1974 through X-ray diffraction patterns from cell nuclei. It is composed of 147 base pairs of DNA wrapped around a protein core formed by an octamer of two copies of each of the four histones proteins H2A, H2B, H3, and H4 (Kornberg, 1974). By this point, the linear DNA molecule has been compacted by a factor of 30-40.

A specific region of DNA, the linker region, separates two adjacent nucleosomes, forming a 10-nm beads-on-a-string array (Fig. 1). Histone H1 binds the linker DNA close to the sites of DNA entry and exit to the nucleosome core. Histone H1 association has a role in the establishment and maintenance of the assembly of three-dimensional chromatin structures, leading to an increased degree of chromatin compaction (Luger, 2003). The distance in terms of the number of nucleotides between two adjacent nucleosomes is variable according to the

organism of interest and is known as the nucleosome repeat length (Ausió, 2015). In the eukaryotic genome, nucleosomes occur every 200 ± 40 bp.

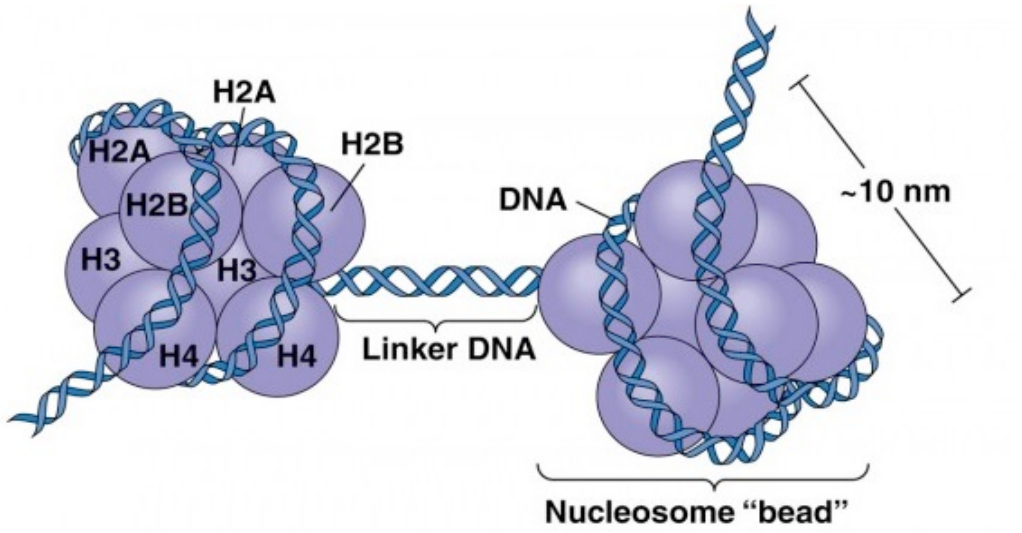


Figure 1. A schematic representation of the nucleosome structure. Note the linker DNA connecting two adjacent nucleosomes.

The interactions between each of the chains of the histone were clarified by the 2.8 Å resolution structure of the nucleosome core particle (Luger *et al.*, 1997). Each histone protein (H3, H4, H2A, and H2B) shares a common structural domain that consists of 3 α-helices (α1, α2, and α3) separated by two loops (L1 and L2), called the histone fold, which facilitates heterodimerization of H2A with H2B and H3 with H4. These interactions result in the basic dimeric structural unit of the nucleosome. Each pair forms a 'handshake' motif that depends on a head-to-tail arrangement of the two histone-fold domains. This leads to the apposition of the L1 and L2 loops on both ends of the dimer. These loops are essential for nucleosome-DNA interaction.

The next level of the structural unit is the four-helix bundle that brings the histone pairs together. Interaction between α-helices of H3 brings the two H3–H4 pairs together to form the central tetrameric axis of the nucleosome. A similar four-helix bundle forms between H2B and H4 on each side of the tetramer to assemble an octamer in an ordered manner (Smith and Stillman, 1991). As a result of this structural organization, the H3–H4 tetramer forms a stable core, whereas the two H2A–H2B dimers can be removed more easily (Kulaeva, Hsieh and Studitsky,

2010). Consistent with this observation, H2A–H2B dimers in vivo have a higher rate of exchange than the H3–H4 tetramer (Jamai, Imoberdorf and Strubin, 2007). Furthermore, the replacement of the H3–H4 tetramer requires the prior removal of the H2A–H2B dimers (English *et al.*, 2006; Henikoff, 2008; Luger, Dechassa and Tremethick, 2012), thereby setting a sequence of events for the process of H3 and H4 exchange.

Furthermore, each core histone contains also a tail (Fig. 2).

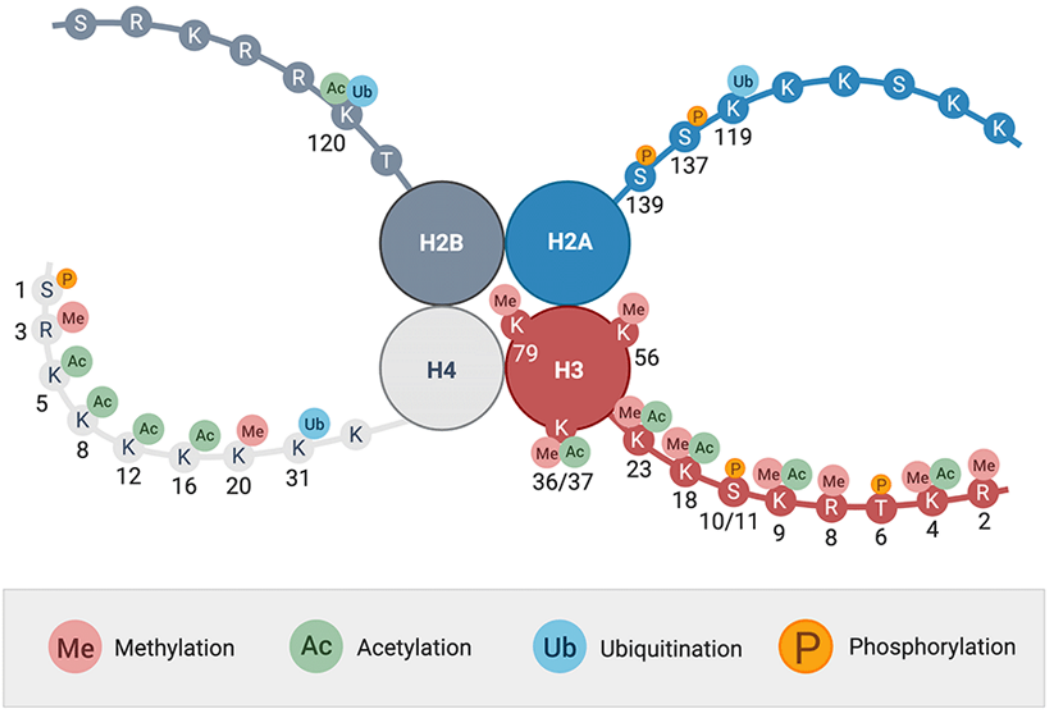


Figure 2. A schematic representation of the histone tails. The different types of post-translational modifications are indicated using color coding.

These tails are regions prevalently amino-terminal to the histone fold domain except for H2A, whose tails are extended to both sides of its fold region. These regions are highly basic and contribute to the formation of the chromatin fiber through interactions with neighboring nucleosomes, in addition, they have a role in supporting the establishment of higher-order structures by facilitating nucleosome assembly or disassembly (Zheng and Hayes, 2003). The N-tails protrude as disordered structures from the center of the nucleosome and play important roles in nucleosome stability (Brower-Toland *et al.*, 2005).

The core histones make contact with the DNA primarily through the phosphodiester backbone. The lack of specific contacts between the core histones and the DNA bases explains how nucleosomes can pack DNA in a sequence-independent fashion. A higher resolution structure of the nucleosome core revealed that DNA binding to the octameric core is accomplished by mainly three to six hydrogen bonds between the protein main chain amides and the DNA phosphate backbone (Davey *et al.*, 2002).

Importantly, nucleosomes are the main characters in determining DNA accessibility. The characteristics of each nucleosome are related to the DNA sequence incorporated, and this can have functional significance for specific gene promoters (Wallrath *et al.*, 1994). Nucleosomes can be more or less compacted, thus determining the folding of chromatin and consequently influencing the rate of gene transcription. Indeed, it is possible to define two configurations of chromatin according to their compaction status and transcriptional activity: euchromatin and heterochromatin. Euchromatin is characterized by a more relaxed compaction rate, thus being considered more accessible to transcription factors, while heterochromatin is tightly compacted and is related to stable transcriptional repression.

However, this organization is reversible and dynamic. One mechanism used by the cell to keep chromatin fluid is histone exchange, which involves the removal of parts of the nucleosome or the entire nucleosome, followed by replacement with either newly synthesized histones or different components. This swapping mechanism, which is also known as histone turnover, has many implications for the composition, structure, and function of different genomic regions. For example, increased histone exchange could increase the accessibility of a particular genomic region to cellular components such as Pol II (polymerase II), thereby facilitating transcription. However, if nucleosome components are replaced with alternatives that block subsequent rounds of exchange, this could limit or prevent DNA availability, thus restricting transcription. Moreover, canonical histones can be replaced with histone variants that alter the chemical nature and physical properties of the nucleosome, thereby affecting distinct cellular processes (Talbert and Henikoff, 2010).

Numerous factors regulate histone exchange during transcription; these include enzymes that add histone post-translational modifications (PTMs), energy-dependent chromatin remodelers, and histone chaperones that function either individually or in combination with one another.

1.1.1 HISTONE MODIFICATIONS

PTMs are a component of the epigenome that elicit changes to DNA and its connected proteins. These changes do not result in modifications to the original DNA sequence. Remarkably, PTMs can be added and removed enzymatically in a reversible way, acting like switches for the regulation of gene expression.

The first experiment that demonstrated that histone proteins are subject to PTMs at their N- terminal tails date back to the early sixties (Allfrey, Faulkner and Mirsky, 1964).

Collectively, all PTMs are today thought to form a so-called histone code, whereby combinations of histone modifications have specific meanings (Turner, 1993; Jenuwein and Allis, 2001). Although it is not clear whether all histone PTMs encode functions as initially suggested by Allis and Jenuwein in the histone code hypothesis (Jenuwein and Allis, 2001), it is clear that many of them affect the processing of the underlying DNA template (Kouzarides, 2007).

The majority of histone PTMs occur on the unstructured tails that emerge from the compact structure of the nucleosome (Fig. 2) but also PTMs within the histone fold have been identified (Cosgrove, Boeke and Wolberger, 2004; Freitas, Sklenar and Parthun, 2004). Nowadays, about 100 different modifications have been recognized and described, decorating over 60 different amino acid residues and including methylation, acetylation, propionylation, butyrylation, formylation, phosphorylation, ubiquitylation, sumoylation, citrullination, glycosylation, proline isomerization and ADP ribosylation.

These modifications act to influence the chromatin architecture, as demonstrated by the X-ray structure of the nucleosome (Luger *et al.*, 1997). Besides governing nucleosome interactions, histone PTMs were shown to be fundamental in regulating nearby transcription by acting as scaffolds to which several effector proteins bind (Lee *et al.*, 2007). PTMs may also prevent proteins from binding to

chromatin (Venkatesh *et al.*, 2012). Depending on their effect on transcription, most of these chemical marks are classified as activating or repressing (Smolle and Workman, 2013).

The histone PTMs can function both synergically and antagonistically and can be deposited asymmetrically within the nucleosome adding further layers of complexity and plasticity to their functions (Voigt *et al.*, 2012).

Most histone PTMs are reversible, as the cell contains separate enzymes that add and remove these marks. This separation not only regulates the genomic localization of the marks, which ensures the removal of aberrantly added marks but also controls how long a PTM remains at a particular genomic location (Papamichos-Chronakis *et al.*, 2011).

The most functionally characterized histone PTMs are acetylation and methylation of lysine residues, or the phosphorylation of threonine, tyrosine, and serine.

Histone acetylation was the first described (Phillips, 1963). It causes the neutralization of the positive charge of lysines, weakening the interactions between histone and DNA and thus leading to enhanced accessibility of DNA to the transcription machinery. Indeed, acetylated histones are found to be enriched in actively transcribed regions. Interestingly, several studies have demonstrated that it is the charge neutralization, rather than the acetylation of specific lysines, to influence the transcriptional status of modified gene loci (Zentner and Henikoff, 2013). It has been shown that histone acetylation is also important in the DNA replication process and is required for replication origin firing (Unnikrishnan, Gafken and Tsukiyama, 2010). Lysine acetylation is the enzymatic product of histone acetyltransferases (HATs). These enzymes show low substrate specificity and can acetylate both cytoplasmic-free and nucleosomal histones. HATs are present at sites of active transcription to facilitate polymerase transit. Their action is exerted by weakening DNA–histones interactions (Allis *et al.*, 2007). Acetylation can be removed by the action of histone deacetylases (HDACs), which restore the positive charge of lysines leading to transcriptional repression.

A further layer of regulation is constituted by the fact that acetylated lysines can be recognized by specific domains defined as bromodomains that are present in

several proteins with chromatin remodeling activity, thus influencing the chromatin structure (Zeng and Zhou, 2002).

Methylation of histones can instead occur on both arginine and lysine residues and, contrarily to acetylation, it does not lead to charge changes. It can be catalyzed in different manners, thus providing several combinations and functional outcomes: arginines can be monomethylated or symmetrically and asymmetrically demethylated (Bedford and Clarke, 2009) while lysines can be mono-, di-, or trimethylated. It has been demonstrated that the effects on transcriptional regulation and nucleosome dynamics seem to be exerted almost completely by lysine methylation rather than methylated arginines (Zentner and Henikoff, 2013). Methylation of histones can either increase or decrease the transcription of genes, depending on which amino acids in the histones are methylated, and how many methyl groups are attached. This modification is indeed associated with transcriptional repression if deposited on H3K9 or H3K27, while it determines transcriptional activation when involves H3K36 or H3K4. The enzymes responsible for these PTMs are the histone lysine methyltransferases (HKMTs).

Although acetylation and methylation of histones have been discovered since the mid-1960s, progress in the last decade or so has greatly expanded our understanding of the diversity and complexity of covalent histone modifications (for review, see (Felsenfeld and Groudine, 2003; Berger, 2007; Kouzarides, 2007)). All four nucleosomal histone tails contain acceptor sites that can be phosphorylated by several protein kinases and dephosphorylated by phosphatases. Histone phosphorylation can occur on serine, threonine, and tyrosine residues and constitutes an essential part of the histone code. Histone phosphorylation is often a direct outcome of activated intracellular signaling pathways, and functions to translate extracellular signals into appropriate nuclear biological outputs. Some proteins containing phospho-binding modules such as 14-3-3 and BRCT domains that can recognize phosphorylated histones have been identified and characterized as downstream effectors (for review, see (Taverna *et al.*, 2007; Yun *et al.*, 2011)).

Histone phosphorylation has been linked to multiple cellular functions, such as transcriptional activation, DNA damage response (see chapter 1.2), mitosis, as well as apoptosis.

A substantial number of phosphorylated histone residues are associated with gene expression. Interestingly, these are often related to the regulation of proliferative genes. For example, phosphorylation of serines 10 and 28 of H3 and serine 32 of H2B has been associated with the regulation of epidermal growth factor (EGF)-responsive gene transcription. H3S10ph and H2BS32ph have also been linked to the expression of proto-oncogenes such as c-fos, c-jun, and c-myc (Chadee *et al.*, 1999; Choi *et al.*, 2005; Lau *et al.*, 2011).

While H3 phosphorylation is involved in chromatin relaxation and regulation of gene expression, this modification was originally identified to be associated with chromosome compaction during mitosis and meiosis. In total, four phosphorylated residues within the N-terminal tail of H3 were discovered to be associated with chromosome condensation and segregation: T3, S10, T11, and S28 (Sauvé *et al.*, 1999; Wei *et al.*, 1999; De La Barre *et al.*, 2000).

Furthermore, several intriguing connections exist between histone phosphorylation and apoptosis. Early work demonstrated that phosphorylation of the N-terminal tail of histone H2B was essential for apoptosis-induced chromatin condensation (Ajiro, 2000; De La Barre *et al.*, 2001). Subsequently, serine 14 was identified as the phosphorylated residue in apoptotic mammalian cells (Cheung *et al.*, 2003).

1.1.2 HISTONE VARIANTS

Chromatin fiber can be modified by the incorporation of histone variants (Malik and Henikoff, 2003; Henikoff, Furuyama and Ahmad, 2004; Kamakaka and Biggins, 2005). This would change local chromatin structure by promoting nucleosome subunit exchange to facilitate processes such as DNA transcription (Park *et al.*, 2005) or more in general cell development (Saeki *et al.*, 2005).

Most histones present in chromatin are replication coupled: they are synthesized only during the S-phase of the cell cycle and are deposited onto newly replicated DNA by dedicated histone chaperones that work in concert with the DNA

polymerases (Panne *et al.*, 2018). Their genes are present as multiple, intronless copies in histone clusters within the genome, and their expression is tightly regulated at both the transcription and translation levels. Histones are now divided into “canonical” histones that today are considered to have the basic packaging DNA function, as opposed to the “non-canonical” ones, i.e. the histone variants.

Histone variants are non-allelic isoforms of canonical histones that are adopted throughout the evolution of discriminating functions in gene expression, replication, recombination, and chromosome segregation (Malik and Henikoff, 2003). They are expressed independently of the S-phase from single or lower copy genes than the canonical histones and also have dedicated chaperones for their incorporation (Skene and Henikoff, 2013). Histone variants unlike canonical histones have generally polyadenylated mRNAs, though exceptions exist (Marzluff *et al.*, 2002; Bönisch and Hake, 2012).

Some variants are highly conserved and appear to have arisen only once in evolution, while other variants seem to have diverged repeatedly in different lineages (Talbert and Henikoff, 2010). Histone variants also have highly similar isoforms called ‘subvariants’ (encoded mainly by pseudogenes), which are not conserved among species and might play a role in regulating tissue-specific gene expression (Maehara *et al.*, 2015).

The incorporation of histone variants into chromatin contributes to the diversity of nucleosome structure and function (Venkatesh and Workman, 2015; Buschbeck and Hake, 2017). The changes to chromatin associated with histone variant incorporation can be profound, resulting in nucleosomes that contain vastly different histone sequences and domains compared with their replicative counterparts, or relatively modest, with variant-containing nucleosomes differing by just a few amino acids yet displaying distinct genomic enrichment profiles and properties.

These histone variants are subject to dynamic exchange, and their deposition, selective eviction, and/or recycling are regulated by specific chaperones or chromatin remodeling complexes that recognize amino acid differences between these variants and their replication-coupled counterparts (De Koning *et al.*, 2007). One of the initial events that facilitate histone exchange is the disruption of

histone-DNA contacts, which results in the removal of the histones. Several factors facilitate the process of histone exchange through a weakening of both the histone octamer-DNA interactions and the histone-histone interactions by the addition of PTMs on histones, by the use of ATP-dependent chromatin remodelers, or by altering nucleosomal composition with the help of histone chaperones.

Histone chaperones can be classified based on the histone substrates to which they bind. Interestingly, most histone chaperones bind either to H3-H4 or to H2A-H2B oligomers (English *et al.*, 2006; Bowman *et al.*, 2011; Hondele *et al.*, 2013), although some bind to both hetero-oligomers using different domains. A few histone chaperones can only bind to specific histones (canonical or variant) alone (Tagami *et al.*, 2004; Luk *et al.*, 2007; Obri *et al.*, 2014), and this often contributes to their location and/or their function. In addition to acting as histone sinks, histone chaperones have a crucial role in regulating transcription-specific histone PTMs. Histone chaperones are required to promote specific histone PTMs for sites that are normally inaccessible, such as the globular domain of histones. Interestingly, such chaperone-assisted, exchange-dependent marks may activate or repress transcription (Tsubota *et al.*, 2007; Du, Fingerman and Briggs, 2008; Du and Briggs, 2010; Kolonko *et al.*, 2010).

While the variants of H2B, H3, and H4 histone represent smaller groups of variants with sometimes only a single amino acid change, the group of H2A is the most diverse having 19 variants found in humans so far which also represents the most extensive structural changes for nucleosome upon their incorporation (Bönisch and Hake, 2012; Vardabasso *et al.*, 2014). The reason for this most likely is the marginal position of H2A in the nucleosome (Bönisch and Hake, 2012). Most of the modifications of H2A variants are located on the C-terminus which is situated in the entry/exit region of the nucleosome. Modifications at this location could provoke actual structural changes, altering the stability and dynamics of the nucleosome and adjacent or associated factors (Tropberger and Schneider, 2013).

1.2 H2AX

The histone family H2A consists of the members, H2A.1, H2A.2, H2AX, and H2AZ (Redon *et al.*, 2002). The main part of the family is represented by H2A.1 and

H2A.2. The two isoforms differ in only a few amino acids and, thus far, they appear not to have different functions. In eukaryotes, H2AZ comprises about 10% of the H2A histones, with H2AX comprising even up to 25%. Specifically in mammals, however, H2AX is much less expressed and represents only up to 10% of the H2A histones (Kuo and Yang, 2008).

H2AX is 143 amino acids in length and differs from the other members of the H2A family by a highly conserved 22 amino acid domain at the C-terminus. The C-terminal motif Ser139-Gln140-Glu141-Tyr142 is used for post-translational modification of the histone, where the protein can be phosphorylated at Ser139 (γH2AX) (Kuo and Yang, 2008; Sharma, Singh and Almasan, 2012). H2AX, as with all other histones, serves to structure and stabilize the DNA (Varvara *et al.*, 2019). It also has a very specific function in the complex DNA damage detection and repair machinery of higher eukaryotes. Ser139 is highly conserved from plants to humans, suggesting a crucial role throughout evolution (Siddiqui *et al.*, 2013).

DNA double-strand breaks (DSBs) are the most lethal type of DNA damage, and their inefficient or inaccurate repair can create mutations and chromosomal translocations that induce genomic instability and, ultimately, cancer development (Jeggo and Löbrich, 2007; McKinnon and Caldecott, 2007). Many events are responsible for generating DSBs. Among them, one can recall here ionizing radiations (IR) or treatment with radiomimetic drugs (Rogakou *et al.*, 1998; Ismail, Wadhra and Hammarsten, 2007), reactive oxygen species, drugs, and DNA modifications that induce replication or transcription stress (Takahashi and Ohnishi, 2005; Pommier, 2006; Guirouilh-Barbat, Redon and Pommier, 2008; Jingsong, Ghosal and Junjie, 2009), and ultraviolet radiation in S-phase cells (Ward and Chen, 2001; Marti *et al.*, 2006). However, DSBs can also arise during normal physiological processes, such as V(D)J recombination, class-switch recombination, and meiosis (Chicheportiche *et al.*, 2007; Edry and Melamed, 2007; Soulas-Sprauel *et al.*, 2007).

To deal with DNA DSBs, cells are equipped with two major repair pathways, the non-homologous end-joining (NHEJ) pathway, and the homologous recombination (HR) pathway. In the NHEJ pathway, DSB ends are simply joined directly or joined after limited processing. Therefore, NHEJ occurs rapidly and is

used throughout the cell cycle. On the other hand, HR mainly takes place during the late S and G2 phases since it needs a sister homolog as a template for repair (Moynahan and Jasin, 2010).

In addition to these repair pathways, cells also possess evolutionarily conserved pathways that are collectively known as the DNA damage response (DDR). DDR enables the cell to sense DNA damage, propagate DNA damage signals, and activate signaling cascades that subsequently evoke a large number of cellular responses aiming to fix or eliminate the damaged DNA (Fig. 3).

The formation of the gamma phosphorylated form of H2AX (γH2AX) was first discovered in yeast where NHEJ was impaired with the loss of the C-terminus of H2A, which contained Ser-129, the yeast homolog of the mammalian Ser-139 (Downs, Lowndes and Jackson, 2000).

H2AX null mice were then demonstrated to be extremely radiosensitive and have aberrant genomes, require longer DNA damage repair times, and have a high incidence of aberrant metaphases as well as defective G2/M cell cycle kinetics (Celeste *et al.*, 2003; Sedelnikova *et al.*, 2003). This showed that although γH2AX may not be a critical component of either the mammalian HR or NHEJ repair pathways, it has a role in the repair of a subset of DSBs, increases the efficiency of DNA repair, and reduces radiosensitivity (Sedelnikova *et al.*, 2003; Karagiannis and El-Osta, 2004; Soutoglou *et al.*, 2007; Kinner *et al.*, 2008).

In the DDR, the phosphorylation of Ser139 is one of the first signals for the detection of DSBs and an essential step for the initialization of DNA repair (Stiff *et al.*, 2004; Johnson *et al.*, 2021).

DSBs cause the spread of γH2AX foci, which extend for up to 50 kb on each side of the DSBs in *Saccharomyces cerevisiae* (Shroff *et al.*, 2004) and for up to several Mb in mammals (Rogakou *et al.*, 1999).

In response to DSBs, the conserved C-terminal tail of H2AX becomes rapidly phosphorylated at Ser139 by members of the PI3 kinase family, including ataxia telangiectasia mutated (ATM), ATR (AT and Rad3-related protein), and DNA-dependent protein kinase (DNA-PK) (Fernandez-Capetillo *et al.*, 2004; Stiff *et al.*, 2004, 2006).

MDC1 (Mediator of DNA damage checkpoint protein 1), a regulator of the S and G2/M-phase checkpoints, works very closely with γH2AX in DDR binding directly to it through its BRCT domains, consisting of repeats containing approximately ~90-100 amino acids (Stucki *et al.*, 2005; Lou *et al.*, 2006). This binding between MDC1 and γH2AX regulates the intra-S-phase checkpoint in response to DNA damage (Huen *et al.*, 2007; Chapman and Jackson, 2008; Melander *et al.*, 2008; Spycher *et al.*, 2008; Wu *et al.*, 2008), and leads to the spreading of H2AX phosphorylation up to megabase regions surrounding DSBs (Stucki and Jackson, 2006), serving as a landing site for the accumulation of other DDR proteins.

In the last decade, many works highlighted that H2AX and its phosphorylation on Ser 139 could not be simply considered as a specific DSB marker with a role restricted to the DNA damage response. Many reports presented H2AX as a 'protagonist' in other scenarios, as listed below.

H2AX, Cell Cycle Checkpoints, and Genomic Instability

Genomic instability is a general term used to describe a genetic propensity for an increase in chromosomal pathology secondary to inaccurate repair or deficiency in cell cycle checkpoints.

H2AX-deficient mouse embryo fibroblasts and T cells contain chromosomal breaks and translocations. However, $H2AX^{-/-}$ B-cells do not show such aberrations, presumably because the apoptotic machinery eliminates B cells that carry the chromosomal pathology (Celeste *et al.*, 2002; Limoli *et al.*, 2002). Consistent with this idea, loss of checkpoint regulation by p53 deletion produces chromosomal aberrations that can be seen in all $H2AX^{-/-}$ and $p53^{-/-}$ cell types (Celeste *et al.*, 2003).

Moreover, $H2AX^{-/-}$ cells exposed to low doses of irradiation that generate few DSBs fail to properly arrest at the G2 phase of the cell cycle and progress to mitosis (Fernandez-Capetillo *et al.*, 2002). This defective G2/M checkpoint response seems to be linked to the impaired accumulation of factors such as 53BP1 at DSBs, which may be an essential amplification step at threshold levels of DNA damage (DiTullio *et al.*, 2002; Fernandez-Capetillo *et al.*, 2002; Wang *et al.*, 2002).

H2AX and Mitosis

Chromatin alterations occurring during mitosis not only are required for the condensation of chromosomes into discrete units but also have functional roles, such as transcription factor displacement and maintenance of memory of transcriptional programs after cell division. It has become clear in the past few years that PTMs of histones play critical roles in both structural and functional chromatin regulation during mitosis (Wang and Higgins, 2013).

Changes in the γH2AX foci pattern during the cell cycle in mammalian cells were documented for the first time in 2005 (McManus and Hendzel, 2005). The authors demonstrated that, without exogenous or artificial sources of DNA damage, hundreds of H2AX phosphorylated sites exist throughout the genome of normally growing mammalian cell lines. The existence of two distinct yet highly discernible γH2AX focal populations was documented: a first small population of large amorphous foci, morphologically similar to the ionizing irradiation-induced DSB foci, colocalizing with numerous DSB repair proteins and likely representing naturally occurring DSBs; and a second previously undescribed but much more abundant population of small foci, that do not associate with proteins involved in DNA DSB repair. Cell cycle analyses revealed that γH2AX signal intensities increased as cells progressed from G1 into S, G2, and M phases, reaching maximal levels at metaphase.

Subsequently, other studies contributed to describing the γH2AX dynamics during the cell cycle, highlighting its specific presence during mitosis (Ichijima *et al.*, 2005; An *et al.*, 2010; Tu *et al.*, 2013).

It was then demonstrated a mitotic role for MDC1 and ATM in regulating SAC activation and it was shown that H2AX is phosphorylated at mitotic kinetochores by ATM. This phosphorylation is needed for MDC1 and, subsequently, MAD2 and CDC20 localization at kinetochores and the formation of an intact mitotic checkpoint complex (Eliezer *et al.*, 2014).

H2AX and Apoptosis

Soon after the discovery of γH2AX, it was shown to be the earliest epigenetic modification during apoptosis (Rogakou *et al.*, 2000). Then, in 2009, a careful examination of single cells by confocal immunofluorescence microscopy revealed

a previously unnoticed staining pattern for γH2AX during apoptosis induced by the TNF-related apoptosis-inducing ligand (TRAIL) (Solier and Pommier, 2009; Solier *et al.*, 2009). TRAIL is a pro-apoptotic agonist that activates death receptors at the cell membrane, leading to caspase-8 activation and mitochondrial apoptosome formation with downstream activation of initiator caspase-9 and effector caspase-3 (Yagita *et al.*, 2004; Taieb *et al.*, 2006; Wang, 2008). The γH2AX staining produced by TRAIL could be divided into three successive patterns: first, a ring staining, which is best detected in early apoptotic cells without massive alteration of nuclear size, then a pan-staining of the nucleus, which retains its overall morphology and size, and finally a pan-staining that persists within apoptotic bodies (Solier and Pommier, 2009; Solier *et al.*, 2009). The ring constitutes an epigenetic landmark of early apoptosis and differs from the focal patterns of DNA damage foci produced by DNA-damaging agents.

Although total H2AX is diffuse in the nucleus due to the random incorporation of H2AX in nucleosomes, γH2AX is initially limited to the outer portion of the nucleus, thereby forming the annular staining ("the ring") in confocal immunofluorescence microscopy (Solier and Pommier, 2009; Solier *et al.*, 2009). The ring is located inside the nuclear envelope and its peripheral distribution coincides with the peripheral heterochromatic nuclear region (Solier *et al.*, 2009). Importantly, the apoptotic γH2AX ring colocalizes with broken DNA ends, indicating it is initiated by the early wave of DNA breaks at the nuclear periphery (Solier *et al.*, 2012).

Interestingly, 53BP1, which is a landmark of γH2AX in DDR foci is not present in the apoptotic ring (Solier *et al.*, 2009; Solier and Pommier, 2011), indicating that the DDR proteins act differently during apoptosis and DNA damage/repair.

An important clue for the apoptotic γH2AX response is that it does not include MDC1, which normally binds to γH2AX and amplifies the DDR (Stewart *et al.*, 2003; Stucki *et al.*, 2005; Eliezer *et al.*, 2009) because MDC1 is cleaved by activated caspase-3 (Solier and Pommier, 2011). MDC1 cleavage by caspase-3, at the early phase of apoptosis, separates the MDC1 fork-head-associated (FHA) domain from its BRCT domains and therefore abrogates the interactions between the different DDR factors, consequently aborting DDR activation and DNA repair (Xie *et al.*, 2007).

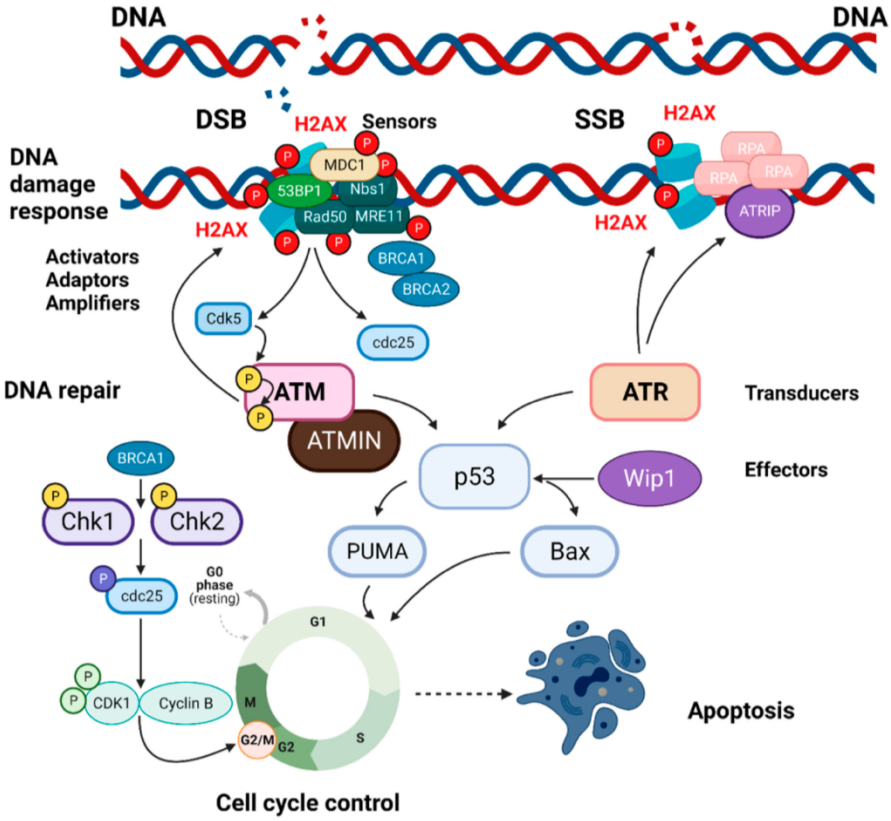


Figure 3. Simplified graphical summary of the pathways of the DDR and cellular responses that require the participation of γH2AX. H2AX becomes phosphorylated during DDR and has a primary role in the repair of DSBs (left), but it also participates in the repair of SSBs (right). γH2AX is also involved in the G2/M checkpoint of the cell cycle (from Merighi *et al.*, 2021).

1.3 CELLULAR RESPONSE TO IONISING RADIATION

Ionizing radiation is a term used for any radiation that is capable of ionizing atoms or molecules of the medium being traversed.

All types of ionizing radiation induce a wide range of damage and effects, including DNA damage, chromosomal aberrations, mutations, cell transformation, and cell killing (NRC, 1999, 2005; UNSCEAR, 2000; Valentin, Cox and Kellerer, 2003; ICRP, 2007).

In fact, it has been shown that exposure to high doses of radiation leads to apoptosis or dysfunction in differentiating cells amalgamated into the hippocampal network and also leads to changes in synaptic protein levels, dendritic complexity, morphology and spine density alterations. (Parihar and Limoli, 2013).

Further, it has also been established that radiation inhibits neurogenesis in a dose dependent manner (Low to High; >2 Gy to 45 Gy) through radiosensitive populations of neural stem and progenitor cells housing in the subgranular zone of the dentate gyrus of the brain (Acharya *et al.*, 2015). This can block the generation of new cells in the brain and cause neuroinflammation (Belarbi *et al.*, 2013; Greene-Schloesser, Moore and Robbins, 2013).

The cel- lular response to radiation-induced damage is influenced by the cell type, timing (e.g. cell cycle phase in which the damage occurs), the extent of damage and the integrity of the pathways that govern these responses.

1.3.1 RADIATION-INDUCED DNA DAMAGE

There are different types of DNA damage such as base lesions, DNA crosslinking, single- (SSBs), and double-strand breaks (Lindahl, 1993; Mehta and Haber, 2014). SSBs are discontinuities in one strand of the DNA double helix and are often associated with damaged or mismatched 5'- and/or 3'-termini at the sites of SSBs (Caldecott, 2008). SSBs can arise from oxidized nucleotides/bases during oxidative stress, intermediate products of DNA repair pathways, such as base excision repair (BER), and aborted activity of cellular enzymes, e.g. DNA topoisomerase 1 (Caldecott, 2008; Yan, Sorrell and Berman, 2014). Unrepaired SSBs compromise DNA replication and transcription programs, leading to genome instability, and are associated with diseases such as cancer and neurodegenerative disorders (Caldecott, 2008). Moreover, unrepaired SSBs during DNA replication can be converted to more deleterious DSBs (Kuzminov, 2001). DSBs can also be caused by mechanical stress on chromosomes and apoptotic-associated DNA fragmentation, and are the main cytotoxic lesion for IR (Huang *et al.*, 2004). DSBs are relatively rare but have a strong impact on neurodevelopment (Rulten and Caldecott, 2013). On the other hand, SSBs occur more than 10,000 times per mammalian cell each day, representing the most common type of DNA damage. They are however less severe as they undergo repair very quickly and are unlikely to cause developmental defects. Another difference between SSBs and DSBs is their propensity to provoke apoptosis. Namely, p53, one of the main molecules

involved in the DNA damage repair machinery, accumulates faster after SSBs compared to DSBs, leading to apoptosis more easily (Ma and Dai, 2018).

The major exogenous cause of DNA double-strand breaks is IR. The DNA molecule can be damaged by IR through indirect or direct ionization. Indirect ionization is caused by IR rays passing through the cell, producing free oxygen radicals (ROS) via water radiolysis and inducing the generation of reactive nitrogen species by stimulating inducible nitric oxide synthase (Azzam, Jay-Gerin and Pain, 2012). IR rays can also hit directly the DNA structure, causing oxidized/reduced base damage and base loss.

The simplest classes of radiation-induced DNA damage include simple, isolated SSBs and excised bases, which are produced in abundance in healthy cells but repaired efficiently. What makes ionizing radiation so lethal to cells is its ability to produce DNA damage in clusters: even if the total number of lesions to DNA induced by a dose of 1 Gray is significantly lower than the number of lesions caused by endogenous processes per day, they are considerably more effective in causing severe biological consequences (Burkart, Jung and Frasch, 1999). IR usually leads to two or more ionizations within a radius of 1–4 nm. Multiple ionizations result in multiply damaged sites which consist of two or more SSBs, abasic sites, oxidized purine or pyrimidine bases, and most importantly DSBs, formed within one or two helix turns from the same energy deposition event (Lomax, Folkes and O'Neill, 2013; Vignard, Mirey and Salles, 2013).

Studies in vitro assessing the DNA damaging effects of IR have been carried out on several different types of neuronal primary cells or cell lines. Among these studies, experiments carried out on rat cortical neurons and astrocytes showed that the percentage of neurons undergoing apoptosis appeared to be dose-dependent up to 16 Gray. The fraction of nonapoptotic cells decreased at a relatively rapid rate from 0 to 16 Gray. However, the decrease in viability as the dose increased from 16 to 64 Gray was comparatively small. They also demonstrated that when cells were irradiated with a dose of 32 Gray, virtually all cells in the forebrain were killed (Gobbel *et al.*, 1998). More recently, a study carried out on a neuroblastoma cell line demonstrated that cells treated with a 10 Gray dosage of X-rays started expressing γH2AX (Mergui *et al.*, 2008). The same results were shown in a study

performed on cultured normal human astrocytes also irradiated with a 10 Gray dose (Bylicky, Mueller and Day, 2019).

1.3.2 CELL CYCLE AND RADIATION

A common cellular response to DNA-damaging agents is cell cycle arrest due to activation of cell cycle checkpoints. These checkpoints act as barriers to prevent propagation of cells with a damaged genome. Especially the G2/M checkpoint is important in the response to ionising radiation, since cell cycle arrest in the G2 phase allows time for repair of DNA damages in the replicated genome before cell division.

The ataxia-telangiectasia mutated (ATM) kinase is the most proximal initiator of signal transduction to the cell cycle machinery after IR-induced DNA damage. When a cell is exposed to IR, ATM is rapidly activated and phosphorylates numerous downstream targets, including p53, MDM2, CHK2, NBS1 and BRCA1 (Canman *et al.*, 1998; Cortez *et al.*, 1999; Pawlik and Keyomarsi, 2004). Several of these targets function to block progression through the cell cycle.

The tumour suppressor p53 has a central role in the cellular response to DNA-damaging agents, since it coordinates DNA repair with cell cycle progression and apoptosis (Fei and El-Deiry, 2003). Damage recognition and checkpoint activation leads to p53 phosphorylation, which alters its conformation and greatly increases its stability. This is a rapid response and p53 levels increase within minutes after DNA damage. p53 has the ability to bind to DNA and functions as a transcription factor. It induces gene transcription of several cell cycle regulators, including p21, GADD45 and members of the 14-3-3 family (Gudkov and Komarova, 2003). p53 also induces BAX and other proteins involved in apoptosis, but suppresses DNA repair through down regulation of RAD51 (Gudkov and Komarova, 2003; Arias-Lopez *et al.*, 2006).

Therefore, exposure of normal cells to radiation results in interruption of the G1/S transition, thereby halting further advancement into the S-phase progression, which allows more time to repair DNA damage prior to DNA replication. The arrest is often transient but can become permanent following exposure to IR. When

double-stranded DNA breaks occur, a G2/M arrest occurs, which prevents cells from entering the M (mitosis) phase, and this cell cycle arrest in G2 allows the coordinated repair of the damage (Wang, 2014). In severe cases of radiation, the recovery process can be delayed, and sometimes, irreparable DNA damage can lead to mitotic catastrophes that cause cell death (Huang and Zhou, 2020).

1.3.3 RADIATION-INDUCED CELL DEATH

Ionising radiation induces two different modes of cell death: apoptosis and mitotic cell death (also mitotic catastrophe). Mitotic cell death is thought to be the major mechanism by which solid tumours are killed by radiotherapy (Ross, 1999). It occurs during mitosis, or after several rounds of cell divisions (Castedo *et al.*, 2004). This mode of cell death is coupled to failure to completely or accurately repair DNA damage. Also, attempts to enter mitosis before the completion of DNA replication in S phase can result in mitotic catastrophe (Castedo *et al.*, 2004). In addition, deficient cell cycle checkpoints increase the probability of mitotic cell death. Cells that are unable to halt in G2/M after DNA damage might enter repeated rounds of failed cell division that result in aneuploid cells and eventually mitotic catastrophe. It seems that the apoptotic machinery is activated to execute the mitotic cell death, and that it involves activation of caspase 2, 3 and 9 (Castedo *et al.*, 2004).

Radiation also induces apoptosis. This is attributed to the damage of intracellular molecules and the activation of nuclease and proteolytic enzymes after high doses of radiation, which leads to degradation of chromatins as well as spillover of histone. This is followed by shrinkage and lysis of cell nuclei, the rapid destruction of normal nuclear morphous, and ultimately cell death. Factors such as disruption of membrane structure and disorder of cell energy metabolism after irradiation are also important contributors to apoptotic death (Yang *et al.*, 2017; Kang *et al.*, 2019).

1.4 THE CELL CYCLE

The highly regulated series of events that leads to eukaryotic cell generation is called the cell cycle.

The cell cycle consists of two consecutive processes, mainly characterized by DNA replication and the segregation of replicated chromosomes into two separate daughter cells (Murray and Hunt, 1993). Originally, the cell cycle was divided into two stages: mitosis (M), the process of nuclear division, also referred to as a part of the M-phase, and interphase, the interlude between two M-phases. This second part of the cycle is devoted to the duplication of all the cell components. Most cell components (cytoplasmic organelles, membranes, structural proteins, and RNAs) are replicated continuously throughout the cell cycle, resulting in the gradual doubling of cell size by the end of the cycle. The chromosomes, however, are present in single copies only and must therefore be duplicated once per cycle. Under the microscope, interphase cells simply grow in size, but different techniques revealed that the interphase includes G1, S, and G2 phases (Norbury and Nurse, 1992). The G1 and G2 phases are thus named as they correspond to an interval (gap) preceding and following the phase of DNA synthesis (S-phase).

Early along the cell cycle, the DNA is replicated and chromosomes are duplicated in S-phase. This process begins at specific DNA sites called replication origins, which are scattered in large numbers along the chromosomes. At these sites, proteins open the DNA double helix, exposing it to the enzymes that carry out DNA synthesis, which move outward in both directions from the origins to copy the two DNA strands.

The M-phase is typically composed of two major events: nuclear division (mitosis) and cell division (cytokinesis). Mitosis is the process that distributes the duplicated chromosomes equally into the two daughter nuclei. The pairs of sister chromatids are attached in early mitosis to the mitotic spindle, a bipolar array of protein polymers called microtubules. By the midpoint of mitosis (metaphase), sister chromatids in each pair are attached to the microtubules fixed to the opposite poles of the spindle. At the next stage (anaphase), sister-chromatid cohesion is destroyed, resulting in their separation. The microtubules of the spindle pull the

separated sisters to opposite ends of the cell (sister-chromatid segregation) and the two sets of chromosomes are each packaged into the new daughter nuclei. Following mitosis, the cytoplasm itself divides by cytokinesis. During this process new plasma membrane is deposited at a position that bisects the long axis of the mitotic spindle, ensuring that the newly separated chromosome sets are distributed into individual cells. In many organisms, the deposition of new membrane components is guided by a contractile ring of actin filaments and myosin motor proteins that forms beneath the cell membrane at the site of cell division. The cell cycle is completed when the contraction of this ring pinches the cell in two.

The two gap phases provide additional time for cell growth. In particular, the G1 phase corresponds to the interval between mitosis and initiation of DNA replication. During the G1 phase, the cell is metabolically active and continuously grows but does not replicate its DNA. On the other hand, during the G2 phase, proteins are synthesized in preparation for mitosis while cell growth continues. The two gap phases serve as more than simple time delays to allow cell growth. They also provide time for the cell to monitor the internal and external environment to ensure that conditions are suitable and preparations are complete before the cell commits itself to division. The G1 phase is especially important in this respect. Its length can vary greatly depending on external conditions and extracellular signals from other cells. If extracellular conditions are unfavorable, cells delay progress through G1 and may even enter a specialized resting state known as G0, in which they can remain for days, weeks, or even years before resuming proliferation (Molinari, 2000; Malumbres and Barbacid, 2001). If extracellular conditions are favorable and growth and division signals are present, cells in early G1 or G0 progress through a commitment point near the end of G1 known in yeast as the Start or the restriction point in mammalian cells. After passing this point, cells are committed to DNA replication, even if the extracellular signals that stimulate cell growth and division are removed.

1.4.1 CELL CYCLE REGULATION

The cell cycle involves numerous regulatory proteins that direct the cell through a specific sequence of events culminating in mitosis and the production of two daughter cells. Central to this process are the cyclin-dependent kinases (CDKs) and the cyclin proteins that regulate the cell progression through the stages of the cell cycle. CDKs are a family of serine/threonine protein kinases whose members are small proteins (34–40 kDa) that are activated at specific points of the cell cycle (Morgan, 1997; Malumbres and Barbacid, 2001; Vermeulen, Van Bockstaele and Berneman, 2003).

Until now, nine CDKs have been identified and, of these, five are active during the cell cycle (CDK1, 2, 4, 6, and 7). CDK1 and CDK2 operate primarily in the M-phase and S-phase respectively, while CDK4 and CDK6 are important in regulating entry into the cell cycle in response to extracellular factors, which is why they are expressed during the G1-phase (Morgan, 1995; Pines, 1995). Another family member, CDK7, contributes indirectly by acting as a CDK-activating kinase (CAK) that phosphorylates other CDKs (Fisher and Morgan, 1994).

CDKs catalyze the covalent attachment of phosphate groups derived from ATP to protein substrates. This phosphorylation results in changes in the substrate's enzymatic activity or its interaction with other proteins. As the cell progresses through the cycle, abrupt changes in the enzymatic activities of these kinases lead to changes in the phosphorylation state, and thus the state of activation of proteins that control cell-cycle processes. These CDK substrates are phosphorylated at serine (S) or threonine (T) residues in a specific sequence context that is recognized by the active site of the CDK protein. In most cases, the target S or T residue is followed by a proline (P); it is also highly favorable for the target residue to have a basic amino acid two positions after the target residue. The typical phosphorylation sequence for CDKs is [S/T*]PX[K/R], where S/T* indicates the phosphorylated serine or threonine, X represents any amino acid and K/R represents the basic amino acid lysine (K) or arginine (R) (Ubersax *et al.*, 2003). The CDKs phosphorylate a variety of substrates. In G2 and M, CDK substrates include nuclear lamins and microtubules that form the nuclear cytoskeleton (Kill and Hutchison, 1995; McNally, 1996). In G1, an important target of the CDKs is the retinoblastoma protein (Rb), which is phosphorylated on multiple residues (Kato

et al., 1993). Hypophosphorylated pRb binds the E2F transcription factor, making it unavailable for transcription. Once the cyclin-CDK phosphorylates pRb, pRb releases E2F, freeing it to participate in the transcription of proteins necessary for the progression of the cell cycle (Arroyo and Raychaudhuri, 1992; Mudrak *et al.*, 1994).

CDKs are regulated by several methods, one of which is the binding of a regulatory cyclin subunit.

Cyclins are a diverse family of proteins whose defining feature is that they bind and activate members of the CDK family. Most cyclins display dramatic changes in concentration during the cell cycle, which help to generate the oscillations in CDK activity that form the foundation of the cell-cycle control system (Evans *et al.*, 1983; Pines, 1995).

Different cyclins are required at different phases of the cell cycle. The three D-type cyclins (cyclin D1, cyclin D2, and cyclin D3) bind to CDK4 and CDK6, and CDK-cyclin D complexes are essential for entry into G1 (Sherr, 1994). Unlike the other cyclins, cyclin D is not expressed periodically but is synthesized as long as growth factor stimulation persists (Assoian and Zhu, 1997). Another G1 cyclin is cyclin E which associates with CDK2 to regulate the progression from G1 into the S phase (Ohtsubo *et al.*, 1995). Cyclin A binds with CDK2 and this complex is required during S-phase (Girard *et al.*, 1991; Walker and Maller, 1991). In late G2 and early M, cyclin A complexes with CDK1 to promote entry into the M-phase. Mitosis is further regulated by cyclin B in complex with CDK1 (King, Jackson and Kirschner, 1994; Arellano and Moreno, 1997).

Sixteen cyclins have been identified so far but, like CDK, not all of them are cell cycle-related (Okamoto and Beach, 1994; Rickert *et al.*, 1996; Peng, Marshall and Price, 1998). Different types of cyclins are produced at different cell cycle phases, resulting in the periodic formation of distinct cyclin–CDK complexes that trigger different cell cycle events. Moreover, part of their cyclic expression is also due to regulated degradation. In particular, cyclins A and B contain a destruction box, while cyclins D and E contain protein motifs rich in proline (P), glutamate (E), serine (S), and threonine (T) residues (PEST) sequences. These protein sequences are

required for efficient ubiquitin-mediated cyclin proteolysis at the end of a cell cycle phase (Glotzer, Murray and Kirschner, 1991; Rechsteiner and Rogers, 1996). Some cyclins contain sequence information that targets them and their CDK partners to specific subcellular locations, providing another mechanism by which a cyclin can direct its catalytic partner to the right substrates. This mechanism is used, for example, to regulate the function of vertebrate cyclin B (Miller and Cross, 2001). There are two forms of this cyclin, cyclins B1 and B2. One of the main targets of cyclin B1–CDK1 is the nuclear lamina, the cytoskeletal network that lies under the nuclear envelope. Before mitosis, the cyclin B1–CDK1 complex is held in the cytoplasm, preventing its access to targets inside the nucleus. In the late prophase, however, cyclin B1–CDK1 is rapidly translocated into the nucleus and immediately phosphorylates the proteins of the nuclear lamina, triggering the breakdown of the nuclear envelope. The second type of cyclin B, cyclin B2, associates with the Golgi apparatus and stimulates the phosphorylation of proteins that cause fragmentation of this organelle during mitosis.

Cyclin binding alone is not enough to fully activate CDKs involved in cell-cycle control. Complete activation of a CDK also requires phosphorylation of a threonine residue adjacent to the kinase active site (threonine 161 in CDK1, threonine172 in CDK4, and threonine 160 in CDK2). Phosphorylation at this site is catalyzed by CAK. These phosphorylations induce conformational changes and enhance the binding of cyclins (Jeffrey *et al.*, 1995; Paulovich and Hartwell, 1995).

CAK is a trimeric complex containing a CDK-related protein kinase known as CDK7, along with its activating partner, cyclin H, and a third subunit, Mat1 (Morgan, 1997). Its activity is maintained at a constant high level throughout the cell cycle and is not regulated by any known cell-cycle control pathway.

Two inhibitory phosphorylations do have important functions in the regulation of CDK activity. One is at a conserved tyrosine residue (Tyr 15) that is found in all major CDKs. Additional phosphorylation of an adjacent threonine residue (Thr 14) further blocks CDK activity (Lew and Kornbluth, 1996). Thr 14 and Tyr 15 are located on the roof of the kinase ATP-binding site and their phosphorylation probably inhibits activity by interfering with the orientation of ATP phosphates. Changes in the phosphorylation of these sites are particularly important in the

activation of M–CDKs at the onset of mitosis, and they are also thought to influence the timing of G1/S- and S-phase CDK activation.

The phosphorylation state of Tyr 15 and Thr 14 is controlled by the balance of opposing kinase and phosphatase activities acting at these sites. One enzyme responsible for Tyr 15 phosphorylation is Wee1 (McGowan and Russell, 1993). On the other hand, dephosphorylation of inhibitory sites is carried out by phosphatases of the Cdc25 family (Sebastian, Kakizuka and Hunter, 1993). Vertebrates also contain a second protein kinase, Myt1, related to Wee1, which catalyzes the phosphorylation of both Thr 14 and Tyr 15 (Lew and Kornbluth, 1996).

Wee1 and Cdc25 provide the basis for the switch-like features of M-phase CDK activation, which allows abrupt and irreversible entry into mitosis. Both enzymes are regulated by their mitotic substrate, the M-phase cyclin–CDK complex. Phosphorylation by M-phase–CDK inhibits Wee1 and activates Cdc25. Thus, M-phase–CDK activates its activator and inhibits its inhibitor, and the resulting feedback loops are thought to generate switch-like CDK activation at the beginning of mitosis (Hoffmann *et al.*, 1993; Izumi and Maller, 1993).

CDK activity can be counteracted by cell cycle inhibitory proteins, called CDK inhibitors (CKI) which bind to CDK alone or the CDK-cyclin complex and regulate CDK activity. Two distinct families of CKI have been discovered, the INK4 family and Cip/Kip family (Sherr and Roberts, 1995). The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), and p19 (INK4d), which specifically inactivate G1-phase CDKs (CDK4 and CDK6). These CKI form stable complexes with the CDK enzyme before cyclin binding, preventing association with cyclin D (Carnero and Hannon, 1998). The second family of inhibitors, the Cip/Kip family, includes p21 (Waf1, Cip1), p27 (Cip2), and p57 (Kip2). These inhibitors inactivate CDK-cyclin complexes (Polyak *et al.*, 1994; Harper *et al.*, 1995; Lee, Reynisdottir and Massague, 1995). They inhibit the G1 CDK- cyclin complexes, and to a lesser extent, CDK1-cyclin B complexes (Hengst and Reed, 1998). p21 also inhibits DNA synthesis by binding to and inhibiting the proliferating cell nuclear antigen (PCNA) (Pan *et al.*, 1995; Waga, Li and Stillman, 1997). CKI are regulated both by internal and external signals: the expression of p21 is under transcriptional control of the

p53 tumor suppressor gene. The p21 gene promotor contains a p53-binding site, that allows p53 to transcriptionally activate the p21 gene (El-Deiry *et al.*, 1993). The expression and activation of, respectively, p15 and p27, increase in response to transforming growth factor β (TGF-β), contributing to growth arrest (Hannon and Beach, 1994; Reynisdóttir *et al.*, 1995).

Transitions from one cell-cycle stage to the next should ideally be unidirectional and irreversible. This is achieved in part through mechanisms that provide all-or-none, irreversible CDK activation. Irreversibility is also achieved by the proteolytic destruction of regulatory proteins (Hershko and Ciechanover, 1998; Pickart, 2001). Proteolysis is particularly critical at the metaphase-to-anaphase transition, where sister-chromatid separation and mitotic exit are triggered by the irreversible destruction of mitotic cyclins and proteins that control sister-chromatid cohesion (Scholey, Brust-Mascher and Mogilner, 2003). Destruction of cyclins also helps establish the state of low CDK activity in G1. In addition, the destruction of CDK inhibitor proteins at the end of G1 helps drive the irreversible activation of S–CDKs. Cyclins, CDK inhibitor proteins, and other cell-cycle regulators are targeted for degradation by the attachment of multiple copies of the small protein ubiquitin, in a process known as ubiquitination. Ubiquitinated proteins are recognized and destroyed by giant protease complexes called proteasomes.

Two large, multisubunit ubiquitin-protein ligases are crucial for the G1/S and metaphase-to-anaphase transitions. For the ubiquitination and proteolysis of targets such as CKI at the G1/S transition, the key ubiquitin-protein ligase is an enzyme called SCF. The metaphase-to-anaphase transition is promoted by an even larger and more complex ubiquitin-protein ligase known as the anaphase-promoting complex (APC) or cyclosome (Scholey, Brust-Mascher and Mogilner, 2003).

The intracellular localization of different cell cycle-regulating proteins also contributes to a correct cell cycle progression. The CDK-inactivating kinases Wee1 and Myt1 are located, respectively, in the nucleus and Golgi complex and protect the cell from premature mitosis (Heald, McLoughlin and McKeon, 1993; Liu *et al.*, 1997). The 14-3-3 group of proteins regulate the intracellular trafficking of different proteins. During interphase, the CDK activating kinase, Cdc25, is kept in

the cytoplasm through interaction with 14-3-3 proteins. Sequestration of the CDK1-cyclin B complex in the cytoplasm following DNA damage is also mediated by 14-3-3 proteins (Peng *et al.*, 1997; Yang *et al.*, 1999).

The cell-cycle control system drives progression through the cell cycle at regulatory transition points called checkpoints (Hartwell and Weinert, 1989; Elledge, 1996).

The first occurs late in G1 and controls progression from G1 to S. This regulatory point was first defined by studies of budding yeast (*Saccharomyces cerevisiae*). In the presence of the appropriate growth factors, cells pass the restriction point and enter the S phase. On the other hand, if appropriate growth factors are not available in G1, progression through the cell cycle stops at the restriction point. Such arrested cells then enter the G0 phase, in which they can remain for long periods without proliferating (Pardee, 1974). G0 cells are metabolically active, although they cease growth and have reduced rates of protein synthesis.

The second major checkpoint group consists of the DNA damage checkpoints (Nyberg *et al.*, 2002). In response to DNA damage, checkpoints arrest the cell cycle to provide time for DNA repair. DNA damage checkpoints are positioned before the cell enters the S phase (G1-S checkpoint) or after DNA replication (G2-M checkpoint) and there appear to exist DNA damage checkpoints also during S and M phases.

At the G1/S checkpoint, cell cycle arrest induced by DNA damage is p53-dependent. Usually, the cellular level of p53 is low but DNA damage can lead to rapid induction of p53 activity (Levine, 1997). p53 stimulates the transcription of different genes including p21, Mdm2, and Bax (Agarwal *et al.*, 1998). The induction of p21 results in CDK inhibition and cell cycle arrest, preventing the replication of damaged DNA (Ko and Prives, 1996). Mdm2 plays an important role in the regulation of p53: it binds to and inhibits p53 transcriptional activity and contributes to the proteolytic degradation of p53 by facilitating its ubiquitination, thereby providing a negative feedback loop (Oren and Rotter, 1999). The binding of regulatory proteins can also modulate p53 ubiquitination. The p19 protein binds to Mdm2 and this prevents the Mdm2-mediated p53 proteolysis (Zhang, Xiong and Yarbrough, 1998). In the case of severely damaged cells, p53 induces cell

death by activating genes (like Bax, Fas, and genes involved in oxidative stress pathways) that are involved in apoptotic signaling (Owen-Schaub *et al.*, 1995; Polyak *et al.*, 1997; Gottlieb and Oren, 1998).

Different protein kinases recognize DNA damage, like ataxia-telangiectasia-mutated (ATM), ataxia, and rad3-related (ATR). These kinases phosphorylate p53 in response to DNA damage, resulting in p21 blocking the cell cycle, at least at the G1/S checkpoint (Siliciano *et al.*, 1997).

When DNA damage occurs during G2, cells can initiate a cell cycle arrest in the presence or absence of p53. The entry into mitosis is prevented by maintaining CDK1 in its inhibited form through inhibitory phosphorylation or by sequestration of components of the CDK1-cyclin B complex outside the nucleus. This is achieved by the protein kinases Chk1 and Chk2, which are activated during DNA damage in an ATM-dependent manner and phosphorylate Cdc25. Phosphorylation of Cdc25 inhibits its activity and promotes its binding to 14-3-3 proteins, sequestering it outside the nucleus and preventing it from activating CDK1-cyclin B and mitotic entry (Sanchez *et al.*, 1997; Zeng *et al.*, 1998). Besides the induction of inhibitory phosphorylations on CDK, p53 may also play a role in the regulation of the G2/M checkpoint. DNA damage-dependent increase of p53 results, as during the G1/S checkpoint, in increased transcription of p21 and of 14-3-3 σ. Increased binding of cyclin B to 14-3-3 σ actively excludes it from the nucleus. p53 also mediates the dissociation of CDK1-cyclin B1 complexes by induction of the growth arrest and DNA damage-inducible gene (Gadd45) (Hermeking *et al.*, 1997; Taylor and Stark, 2001).

The third major checkpoint, called the spindle checkpoint, is the metaphase-to-anaphase transition, which leads to sister-chromatid segregation, completion of mitosis, and cytokinesis (Scholey, Brust-Mascher and Mogilner, 2003). Progression through this checkpoint occurs when M-phase cyclin–CDK complexes stimulate APC, which causes the proteolytic destruction of cyclins and of proteins that hold the sister chromatids together. Activation of this enzyme, therefore, triggers sister-chromatid separation and segregation. Destruction of cyclins leads to the inactivation of all CDKs in the cell, which allows phosphatases to dephosphorylate CDK substrates. Dephosphorylation of these substrates is required for spindle disassembly and the completion of mitosis, and cytokinesis. On the other hand,

mitotic arrest deficient (Mad) and budding uninhibited by benomyl (Bub) proteins are activated when defects in microtubule attachment occur and inhibit the Cdc20 subunit of APC, resulting in the prevention of metaphase-anaphase transition (Fang, Yu and Kirschner, 1998; Amon, 1999).

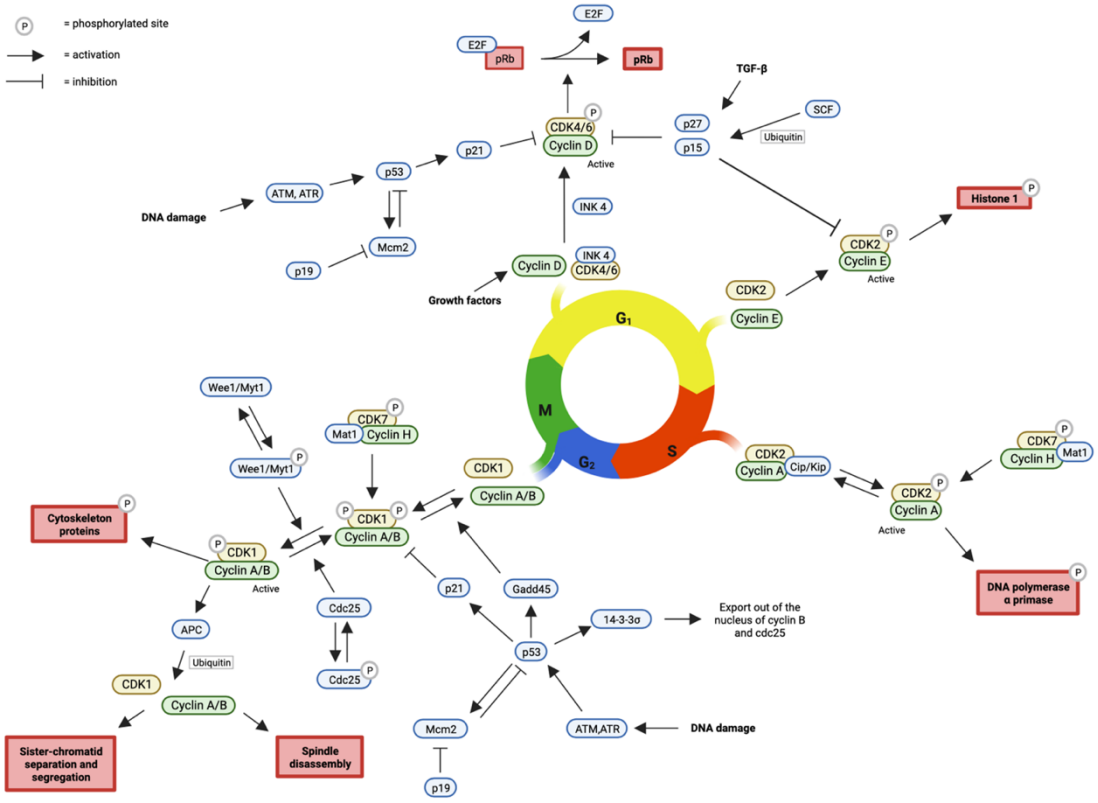


Figure 4. A schematic overview showing the canonical cell cycle and its main regulatory mechanisms. The cell cycle consists of four distinct cellular phases: G1, a stage of cell growth, S phase, during which DNA synthesis and replication take place, G2, the second phase of cell growth and active synthesis of factors required for cell division or mitosis (M). Cell cycle progression is driven by the cyclin-dependent kinases (CDKs), heterodimeric complexes consisting of a catalytic CDK subunit associated with a regulatory cyclin subunit. Sequential assembly and activation of CDK–cyclin complexes are regulated by a complex network of activators and inhibitors.

1.4.2 MARKERS OF THE CELL CYCLE

The first studies on cell division provided very little insight into the cell cycle status per se because they were essentially limited to the morphological description of cellular features, such as cell rounding and changes in chromatin condensation upon entry into mitosis. Since these first schematic illustrations of dividing cells,

we have come a long way in characterizing the cell cycle status of eukaryotic cells thanks to some different approaches.

Cells actively engaged in DNA replication can be identified using thymidine analogs, which are incorporated into nascent DNA during the labeling period (Darzynkiewicz, Bedner and Smolewski, 2001). This family of probes shares a similar mechanism of action, but their labeling/detection protocols carry distinct advantages and disadvantages (Cavanagh *et al.*, 2011). Halogenated thymidine nucleosides like 5-Bromo-2'-deoxyuridine (BrdU), 5-Iodo-2'-deoxyuridine (IdU), and 5-Chloro-2'-deoxyuridine (CldU) are detected by immunostaining with specific antibodies, whereas cell nuclei that have incorporated ^{3}H-thymidine are visualized by autoradiography. First, BrdU is incorporated into newly synthesized DNA in cells. The incorporated BrdU is then stained with specific (fluorescently labeled) anti-BrdU antibodies, and the levels of cell-associated BrdU are measured using either microscopy or flow cytometry. Using the latter approach, a dye that binds to total DNA, such as 4',6'-di-amidino-2-phenylindole (DAPI) or 7-aminoactinomycin D (7-AAD), is often used in conjunction with immunofluorescent BrdU staining. This allows the identification of the individual cell cycle phases in a given population into G0/G1, S, and G2/M phases. The detection of BrdU following its incorporation into DNA requires the denaturation of DNA to allow targeting by antibodies. These protocols result in cell and tissue disruption, together with the degradation of proteins and nucleic acids, limiting the utility of BrdU as a probe where concurrent measurement of protein content or molecular analysis is required. Its use as a quantitative tool for measuring rates of cell genesis and as a labeling tool for measuring cell fate is therefore limited (Cameron and Mckay, 2001; Kee *et al.*, 2002; Taupin, 2007).

A new technique for detecting DNA synthesis using nucleoside analogs in proliferating cells in vivo, and in vitro, has been developed in recent years (Salic and Mitchison, 2008). An alkylated thymidine nucleoside, 5-ethynyl-2'-deoxyuridine (EdU), can be coupled via a covalent bond using "click" chemistry (Buck *et al.*, 2008; Salic and Mitchison, 2008), namely, a copper [Cu(I)]-catalyzed [3+2] cycloaddition reaction, to a fluorescent dye-conjugated azide, which has the advantages of superior labeling sensitivity and minimal disruption to the DNA

staining signal as no DNA denaturation is required (Salic and Mitchison, 2008; Cavanagh *et al.*, 2011). “Click” chemistry is a rapid chemical reaction that occurs readily at room temperature and is catalyzed by copper Cu(I), resulting in the formation of a covalent bond between an azide and an alkyne group. The small-sized dye-azide allows for efficient EdU detection.

Detection of thymidine analogs after their incorporation into replicating DNA is also used to study the progression through the cell cycle and the cell proliferation kinetics. An approach allowing us to trace the progression of cells through the cell cycle is based on the use of two labels delivered with a time interval (Hayes and Nowakowski, 2000; Vega and Peterson, 2005; Encinas *et al.*, 2011). With the lengthening of the interval between delivery of the labels, the proportion of cells that have incorporated both labels declines progressively. This is because cells marked with the first label leave the S-phase, while unlabeled cells enter the S-phase and become marked only with the second label. The intercept of the declination line with the time axis reveals the time point when all cells marked with the first label exit the S-phase, giving an estimation of the S-phase duration. Lengthening the time interval between the two labels allows for an increase in the proportion of double-labeled cells, which reflects the entry of cells that have incorporated the first label into the next S-phase. Further increasing the length of the time interval causes the proportion to decline again. The point when the peak is reached corresponds to the cell cycle duration.

Further details on the cell cycle status can be obtained by indirect immunofluorescence thanks to specific antibodies that recognize cell-cycle regulators with well-defined spatial and temporal patterns of expression and subcellular localization, such as cyclins. For example evaluation of levels and subcellular localization of cyclins A, B and E allow the S-phase, G2, and M-phases to be distinguished, because cyclin E appears at nuclear foci at the onset of replication, closely followed by nuclear accumulation of cyclin A during the S phase, whereas cyclin B resides mainly in the cytoplasm and at centrosomes, and accumulates in the nucleus during the late G2 (Pines and Hunter, 1994; Ohtsubo *et al.*, 1995).

Expression of the Mcm2–7 proteins allows cells engaged in the cell division cycle to be clearly distinguished from cells residing in out-of-cycle states (Blow and Hodgson, 2002), similar to PCNA and Ki-67 (Cuylen *et al.*, 2016). PCNA, a cofactor of DNA polymerase, is expressed during the S-phase (Kurki *et al.*, 1986), but also in cells undergoing DNA repair and in some non-proliferating neurons (Takahashi and Caviness, 1993; Ino and Chiba, 2000). Ki67, the mitotic chromosome-dispersing protein, is expressed during all phases of the cell cycle except for G0, and its expression reaches a peak during DNA synthesis and mitosis (Lopez *et al.*, 1991; Endl and Gerdes, 2000; Zacchetti *et al.*, 2003). Ki67 has a very short half-life and it cannot be detected during DNA repair mechanisms (Takahashi and Caviness, 1993; Scholzen and Gerdes, 2000; Zacchetti *et al.*, 2003).

Geminin, which prevents relicensing of replication origins after the initiation of DNA synthesis, is only present in cells progressing through S, G2, and M phases, as are the mitotic engine kinases Plk1, Aurora A, and Aurora B (Crosio *et al.*, 2002; Hook, Lin and Dutta, 2007; Kulkarni *et al.*, 2007). These three kinases control most mitotic events, including centrosome maturation and separation, chromosome orientation, and segregation (Nigg, 2001).

Other markers of the cell cycle, like phosphohistone H3 and RNR, are useful during in situ studies of cell proliferation (Engström *et al.*, 1985; Hendzel *et al.*, 1997; Zhu, Wang and Hansson, 2003; Zhu, Dahlström and Hansson, 2005). Notably, histone H3 is a substrate for the Aurora kinases and is phosphorylated at serine 10 only during the initial stages of chromatin condensation in late G2, up to the anaphase (Hendzel *et al.*, 1997; Wei *et al.*, 1999; Crosio *et al.*, 2002). Phosphohistone H3 is therefore an M-phase marker. RNR, the ribonucleotide reductase, has its peak of expression during S-phase, and its M1 subunit is expressed by proliferating cells but not by quiescent ones (Engström *et al.*, 1985).

Hence, multiparameter analysis of these G1–S and G2–M regulators, using immunodetection methods, provides a detailed readout of the cell cycle state.

1.5 CELL DEATH

In multi-cellular organisms, there is a constant effort to maintain a homoeostatic balance between the number of new cells that are generated through mitosis and

the number of damaged or unrequired cells that are removed from the body. Cell death plays a pivotal role in embryonic development, maintaining the homeostasis of the organism and eliminating damaged cells, but it is also important for tissue remodeling during adult life and intervenes in several forms of pathology.

Cell death causes macroscopic and microscopic alterations. Together with the mechanisms whereby dead cells and their fragments are disposed of, such morphotypes have historically been employed to classify cell death into three different forms (Green and Llambi, 2015): type I cell death (apoptosis), type II cell death (autophagy) and type III cell death (necrosis).

Dead cells are eventually removed from the tissue in either a controlled (programmed) manner involving a series of biochemical and molecular events or in a poorly controlled manner, resulting in the spilling of the cellular contents into surrounding tissues (Kerr, Wyllie and Currie, 1972).

The most well-characterized and prevalent form of controlled cell death is apoptosis. Classic apoptosis is characterized by the compartmentalization of intracellular components of the cell and the removal of cellular debris without any collateral damage occurring to surrounding tissues. An alternative form of apoptosis has been identified (Boise and Collins, 2001) that although following a programmed series of caspase-dependent events, is pro-inflammatory. This form of cell death is termed 'pyroptosis' (Bergsbaken, Fink and Cookson, 2009).

Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001).

Autophagic cell death is characterized by the appearance of large intracellular vesicles, plasma membrane blebbing, enlarged organelles, and the depletion of cytoplasmic organelles in the absence of chromatin condensation (Liu and Levine, 2015). During autophagy, cellular components such as macro proteins or even whole organelles are sequestered into lysosomes for degradation (Shintani and Klionsky, 2004; Mizushima *et al.*, 2008). The lysosomes are then able to digest these substrates, the components of which can either be recycled to create new

cellular structures and/or organelles or can be further processed and used as a source of energy.

Autophagy can be initiated by a variety of stressors, most notably by nutrient deprivation, or can result from signals present during cellular differentiation and embryogenesis and on the surface of damaged organelles (Mizushima *et al.*, 2008). Autophagy has also been shown to be involved in both the adaptive and the innate immune system where it may degrade intracellular pathogens and deliver antigens to MHC class II holding compartments (Levine and Deretic, 2007).

Necrosis is a form of cell death that is induced by external factors, such as hypoxia, inflammation, infections, toxins, and physical injuries (Elmore, 2007). This leads to morphological alterations, such as cytoplasmic swelling, plasma membrane rupture, and the subsequent loss of intracellular organelles without severe chromatin condensation, but randomly degraded DNA, resulting in a cascade of inflammation and tissue damage (Weerasinghe and Buja, 2012).

In contrast to apoptosis, necrosis is an energy-independent form of cell death, where the cell is damaged so severely by a sudden shock that it is unable to function (Syntichaki and Tavernarakis, 2002).

As early as 2005, a novel form of cell death, that showed characteristics of necrosis, but unlike previous observations appeared to be tightly regulated, was identified. This form of cell death was termed necroptosis (Degterev *et al.*, 2005). Necroptosis is a form of regulated cell death initiated by perturbations of the extracellular or intracellular microenvironment detected by specific death receptors (Vercammen *et al.*, 1997, 1998; Degterev *et al.*, 2005, 2008; Galluzzi *et al.*, 2014), or pathogen recognition receptors (Upton, Kaiser and Mocarski, 2010, 2012; Kaiser *et al.*, 2013).

It is now clear that necroptosis not only mediates adaptative functions upon failing responses to stress but also participates in developmental safeguard programs, as well as in the maintenance of adult T-cell homeostasis (Kaczmarek, Vandenabeele and Krysko, 2013; Dara, Liu and Kaplowitz, 2016; Weinlich *et al.*, 2017).

1.5.1 APOPTOSIS

The word apoptosis was first used in a 1972 paper by Kerr, Wyllie, and Currie to describe a morphologically distinct type of cell death (Kerr, Wyllie and Currie, 1972). Our understanding of the mechanisms involved in the process of apoptosis in mammalian cells transpired from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (Horvitz, 1999). In this organism, 1090 somatic cells are generated in the formation of the adult worm, of which 131 undergo apoptosis. These 131 cells die at particular points during development, which is essentially invariant between worms, demonstrating the remarkable accuracy and control in this process. Apoptosis has since been recognized and accepted as a distinctive and important mode of programmed cell death, which involves the genetically determined elimination of cells.

Light and electron microscopy have identified the various morphological changes that occur during apoptosis (Häcker, 2000). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (Kerr, Wyllie and Currie, 1972). With cell shrinkage, the cells are smaller in size, the cytoplasm is denser and the organelles are more tightly packed. Pyknosis is the result of chromatin condensation and this is the most characteristic feature of apoptosis. On histologic examination with hematoxylin and eosin stain, the apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments.

Electron microscopy can better define the subcellular changes. Early during the chromatin condensation phase, the electron-dense nuclear material characteristically aggregates peripherally under the nuclear membrane although there can also be uniformly dense nuclei.

Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called “budding”. These apoptotic bodies can be phagocytosed by the surrounding cells, although this behavior is observed primarily in cell culture (Elmore, 2007). In vivo, cells such as macrophages often remove apoptotic cells before they fragment. This

results in the containment of the injured tissue, and consequently, reduces the risk of collateral damage to surrounding cells.

The process of apoptosis is highly conserved within multi-cellular organisms and is genetically controlled (Lockshin and Zakeri, 2004). Apoptosis can be initiated by the cell itself when it detects damage via several intracellular sensors; a mechanism known as the intrinsic pathway. Alternatively, it can result from the interaction between a cell of the immune system and a damaged cell, which is known as the extrinsic pathway of apoptosis (Sica *et al.*, 1990; Oppenheim *et al.*, 2001).

The initiation of apoptosis is dependent on the activation of a series of cysteine-aspartic proteases known as caspases. Once caspases are initially activated, there seems to be an irreversible commitment toward cell death. To date, ten major caspases have been identified and broadly categorized into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7), and inflammatory caspases (caspase 1, 4, 5) (Cohen, 1997; Rai *et al.*, 2005).

The intrinsic pathway, also known as the mitochondrial pathway of apoptosis (Igney and Krammer, 2002) involves a variety of stimuli that act on multiple targets within the cell, see (Lossi, 2022) for a recent review. This form of apoptosis is dependent on factors released from the mitochondria and is initiated either from a positive or negative pathway. Negative signals arise from the absence of cytokines, hormones, and growth factors in the immediate environment of the cell. Without these pro-survival signals, pro-apoptotic molecules within the cell, such as Puma, Noxa, and Bax, that are normally inhibited, become active and initiate apoptosis. Other factors that initiate apoptosis include exposure to hypoxia, toxins, radiation, reactive oxygen species, viruses, and a variety of toxic agents (Brenner and Mak, 2009).

The initiator caspase that controls the intrinsic pathway of apoptosis is caspase 9, which can bind to the adapter protein apoptotic protease activating factor 1 (APAF1).

The binding of caspase 9 to APAF1 causes drastic changes in the inner mitochondrial membrane that results in an opening of the mitochondrial

permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential, and release of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (Saelens *et al.*, 2004), such as cytochrome c, Smac/DIABLO and HtrA2/Omi (Du *et al.*, 2000; Cain, Bratton and Cohen, 2002; Van Loo *et al.*, 2002; Garrido *et al.*, 2006). In particular, cytochrome c induces apoptosis by binding to APAF1 monomers, thus allowing several APAF1s to assemble into a complex known as an apoptosome (Acehan *et al.*, 2002). The apoptosome recruits and activates several procaspase 9 proteins. These activated caspase 9 enzymes can activate the executioner procaspase 3 (Cain, Bratton and Cohen, 2002).

The extrinsic pathway, also known as the death receptor pathway of apoptosis (Igney and Krammer, 2002) is initiated by perturbations of the extracellular microenvironment (Ashkenazi and Dixit, 1998; Strasser, Jost and Nagata, 2009; Flusberg and Sorger, 2015; Gibert and Mehlen, 2015), and in particular by patrolling natural killer cells or macrophages.

Extrinsic apoptosis is driven by transmembrane receptor-mediated interactions that involve the so-called death receptors (see (Lossi, 2022)). Depending on different downstream cascade reactions, extrinsic apoptosis can be divided into two categories. One type of apoptosis cascade is mediated by the death receptor Fas, while the other involves the tumor necrosis factor receptor 1 (TNFR1) (Chicheportiche *et al.*, 1997; Ashkenazi and Dixit, 1998; Peter and Krammer, 1998). Members of these receptor families have a cytoplasmic domain of about 80 amino acids called the “death domain” (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways.

As a general rule, upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of the Fas ligand to the Fas receptor results in the binding of the adapter protein FADD and the binding of the TNF ligand to the TNF receptor then results in the binding of the adapter protein TRADD with the recruitment of FADD and RIP (Hsu, Xiong and Goeddel, 1995; Wajant, 2002). FADD is then associated with procaspase-8 via dimerization of the death effector domain. At this point, a death-

inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (Kischkel *et al.*, 1995). The recruitment of several procaspase 8 monomers to the DISC results in their dimerization and activation, with the resultant caspase 8 able to induce apoptosis via one or the other of two distinct sub-pathways. The particular sub-pathway that is induced depends on whether the cells are categorized as type I or type II cells (Samraj *et al.*, 2006). In type I cells, caspase 8 directly cleaves executioner caspases and therefore immediately initiates apoptosis. In type II cells, inhibitors of apoptosis proteins (IAPs) inhibit direct caspase 8 activations of the executioner caspases, unless the IAPs are inhibited by proteins released from the mitochondria (Spencer *et al.*, 2009).

The extrinsic and intrinsic pathways both end at the point of the execution phase, which is considered the final pathway of apoptosis. It is the activation of the execution caspases that triggers this phase of apoptosis (Lossi, 2022). Execution caspases activate cytoplasmic endonuclease, which degrades the nuclear material, leading to DNA fragmentation (Enari *et al.*, 1998), and proteases that degrade the nuclear and cytoskeletal proteins (Arur *et al.*, 2003; Lossi *et al.*, 2009). Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (Slee, Adrain and Martin, 2001).

1.5.2 MARKERS OF CELL DEATH

Since apoptosis occurs via a complex signaling cascade that is tightly regulated at multiple points, there are many opportunities to evaluate the activity of the proteins involved. As the activators, effectors, and regulators of this cascade continue to be elucidated, a large number of apoptosis assays have been devised to detect and count apoptotic cells. However, many features of apoptosis, necrosis, and other aforementioned forms of cell death can overlap, and it is, therefore, crucial to employ at least two or more distinct assays to confirm that

cell death is occurring via one specific mechanism. In the case of apoptosis, one assay may detect early apoptotic events and a different assay may target a later event. The second assay, used to confirm apoptosis, is generally based on a different principle.

The experimental procedures to identify dead and dying cells differ from one another in regard: to specificity, sensitivity, detection range, precision, throughput, cell death stage, cell death parameters, or readout.

There is a large variety of assays available, but each assay has advantages and disadvantages which may make it acceptable to use for one application but inappropriate for another (Watanabe *et al.*, 2002; Otsuki, Li and Shibata, 2003). Assays, based on methodology, can be classified into six major groups (see e.g. Lossi, Mioletti and Merighi, 2002).

Cytomorphological alterations

The evaluation of hematoxylin and eosin-stained tissue sections with light microscopy does allow the visualization of apoptotic cells.

Because the morphological events of apoptosis are rapid and the fragments are quickly phagocytized, considerable apoptosis may occur in some tissues before it is histologically apparent. Additionally, this method detects the later events of apoptosis, so cells in the early phase of apoptosis will not be detected.

This methodology depends on the nuclear and cytoplasmic condensation that occurs during apoptosis. The tissue and cellular details are preserved with this technique and surveys of large tissue regions are distinct advantages. However, smaller apoptotic bodies will not be detected and healthy cells with large dense intracellular granules can be mistaken for apoptotic cells or debris. Additionally, there is a loss of antigenicity during processing so immunohistological or enzyme assays cannot always be performed on the same tissue.

Transmission electron microscopy (TEM) was considered, until recently, the gold standard to confirm apoptosis. This is because the categorization of an apoptotic cell is irrefutable if the cell contains certain ultrastructural morphological characteristics (White and Cinti, 2004). These characteristics are an electron-dense nucleus, nuclear fragmentation, an intact cell membrane even late in the cell disintegration phase, disorganized cytoplasmic organelles, large clear

vacuoles, and blebs at the cell surface. More recent investigations, however, have shown that the different types of cell death may share some ultrastructural features and that there is the possibility to observe forms of death with intermediate characteristics between one or the other (see e.g. Lossi, Castagna and Merighi, 2015; Lossi, 2022).

DNA fragmentation

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis (Wyllie, 1980). This assay involves the extraction of DNA from a lysed cell homogenate followed by agarose gel electrophoresis. After the run, a characteristic "DNA ladder" can be seen with each band in the ladder separated in size by approximately 180 base pairs.

This methodology is useful for tissues and cell cultures with high numbers of apoptotic cells per tissue mass or volume, respectively. On the other hand, it is not recommended in cases with low numbers of apoptotic cells. Moreover, since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis. Additionally, DNA fragmentation can occur during the preparation making it difficult to produce a nucleosome ladder and necrotic cells can also generate DNA fragments.

Membrane alterations

Externalization of phosphatidylserine residues on the outer plasma membrane of apoptotic cells allows detection via Annexin V in tissues, embryos, or cultured cells (Bossy-Wetzel and Green, 2000). Once the apoptotic cells are bound with FITC-labeled Annexin V, they can be visualized with fluorescent microscopy. The advantages are sensitivity (can detect a single apoptotic cell) and the ability to confirm the activity of initiator caspases. On the other hand, the membranes of necrotic cells are labeled as well.

Detection of apoptosis in whole mounts

Apoptosis can also be visualized in whole mounts of embryos or tissues using dyes such as acridine orange (AO), Nile blue sulfate (NBS), and neutral red (NR) (Zucker,

Hunter and Rogers, 2000). Since these dyes are acidophilic, they are concentrated in areas of high lysosomal and phagocytotic activity.

However, these dyes cannot distinguish between lysosomes degrading apoptotic debris from the degradation of other debris such as microorganisms. Although all of these dyes are fast and inexpensive, they have certain disadvantages. AO is toxic and mutagenic and quenches rapidly under standard conditions whereas NBS and NR do not penetrate thick tissues and can be lost during preparation for sectioning.

Mitochondrial assays

Mitochondrial assays and cytochrome c release allow the detection of changes in the early phase of the intrinsic pathway. Laser scanning confocal microscopy (LSCM) creates submicron-thin optical slices through living cells that can be used to monitor several mitochondrial events in intact single cells over time (Bedner *et al.*, 1999; Darzynkiewicz *et al.*, 1999). Mitochondrial permeability transition (MPT), depolarization of the inner mitochondrial membrane, Ca^{2+} fluxes, mitochondrial redox status, and reactive oxygen species can all be monitored with this methodology. The main disadvantage is that the mitochondrial parameters that this methodology monitors can also occur during necrosis. The electrochemical gradient across the mitochondrial outer membrane (MOM) collapses during apoptosis, allowing detection with a fluorescent cationic dye (Poot and Pierce, 1999). In healthy cells this lipophilic dye accumulates in the mitochondria, forming aggregates that emit a specific fluorescence. In apoptotic cells, the MOM does not maintain the electrochemical gradient and the cationic dye diffuses into the cytoplasm where it emits a fluorescence that is different from that of the aggregated form.

Other mitochondrial dyes can be used that measure the redox potential or metabolic activity of the mitochondria in cells. However, these dyes do not address the mechanism of cell death.

Cytochrome c release from the mitochondria can also be assayed using fluorescence and electron microscopy in living or fixed cells respectively (Scorrano *et al.*, 2002). However, cytochrome c becomes unstable once it is released into the cytoplasm (Goldstein *et al.*, 2000).

Apoptotic or anti-apoptotic regulator proteins such as Bax, Bid, Bcl-2, and several others can also be detected using fluorescence and confocal microscopy (Tsien, 1998; Zhang *et al.*, 2002).

Detection of caspases, cleaved substrates, regulators, and inhibitors

There are more than 13 known caspases or procaspases that can be detected using various types of caspase activity assays (Gurtu, Kain and Zhang, 1997). There are also immunohistochemistry assays that can detect cleaved substrates such as PARP and known cell modifications such as phosphorylated histones (Love, Barber and Wilcock, 1999; Talasz *et al.*, 2002). Fluorescently conjugated caspase inhibitors can also be used to label active caspases within cells (Grabarek, Amstad and Darzynkiewicz, 2002). Caspase activation can be detected in a variety of ways including western blot, immunoprecipitation, and immunohistochemistry. Both polyclonal and monoclonal antibodies are available to both procaspases and active caspases.

A major disadvantage of caspase localization to detect apoptosis is that caspase activation does not necessarily indicate that apoptosis will occur. Moreover, there is a tremendous overlap in the substrate preferences of the members of the caspase family, affecting the specificity of the assay.

1.6 NEUROGENESIS IN THE ADULT CNS

Neurogenesis is the process by which new neurons (and glia in a broader sense) are generated, starting from the proliferation of multipotent cells commonly referred to as neural stem cells (NSCs). NSCs are defined as cells that can self-renew and give rise to the three major cell types of the central nervous system (CNS): neurons, astrocytes, and oligodendrocytes (Gage, 2000). During the development of the CNS, neurons, and glia arise from NSCs following a stereotyped sequence in which neurons are generated first, mostly during the embryonic period, while the majority of glial cells differentiate after most neurons are born (McKay, 2000; Temple, 2001).

In the adult CNS, the continued generation of glia has been observed in many different regions, and these cells are thought to derive from NSCs (Kuhn *et al.*, 1997; Horner *et al.*, 2000; Lie *et al.*, 2002).

On the other hand, neurogenesis in its narrower definition, i.e. the process of producing functional neurons from precursor cells, was conventionally perceived to occur only during the embryonic, pre-natal, and early post-natal stages in mammals (Ming and Song, 2005). In fact, since the early 1900s, it was generally believed that the adult CNS of mammals had a very limited regenerative capacity (Cajal, 1928). Therefore, the CNS was considered incapable to recover from injury and predominant repair mechanisms were thought to be postmitotic, such as changes in neurotransmitter-receptor expression, synaptic reorganization, and sprouting of axon terminals.

The fixed brain model was first undermined by Altman in the 1960s. He used tritiated thymidine (^{3}H-thymidine) labeling and light microscopy to show that new cells with neuronal morphologies could be found in the olfactory bulb (OB), hippocampus, and neocortex of adult rats and cats (Altman, 1962). In the following year, Altman published another study that corroborated his initial findings and revealed labeled neurons in the dentate gyrus (DG) of the hippocampus and the cerebral cortex, strengthening his claim of possible neurogenesis in the adult brain (Altman, 1963). Then, in 1965, Altman and Das presented the first histological evidence of newly formed DG granule cells in the hippocampus of the adult rat (Altman and Das, 1965), and then identified the source of new postnatal granule cell neurons in the OB (Altman, 1969).

In the 1980s, Nottebohm and his colleagues published a series of studies showing that a substantial number of new neurons are produced in the song system of adult birds. The new cells were generated from the lining of the cerebral ventricles and migrated through the mature nervous tissue to reach their final destinations (Goldman and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988), where they attained the typical morphological and ultrastructural characteristics of neurons (Burd and Nottebohm, 1985; Alvarez-Buylla, Theelen and Nottebohm, 1988).

Moreover, in 1982 Bayer presented novel evidence that the granule cell population in the DG increased with age. His findings demonstrated a linear

increase of 35–43% in the granule cell population between 1 month and 1 year of age, suggesting that in the mammalian brain neurons increase in number in adulthood (Bayer, 1982; Bayer, Yackel and Puri, 1982).

During the same years, Kaplan and Hinds performed autoradiographic studies on tissue slices from normal adult rat brains 30 days after an intraperitoneal injection of ^{3}H-thymidine (Kaplan and Hinds, 1977). Their research revealed labeled cells in the OB and the granular layers of the hippocampal DG. Furthermore, using electron microscopy, they were able to show that the labeled cells were indeed neurons with distinguishable dendrites, axons, and synapses. Shortly thereafter, Kaplan released another study in which he described neuronal proliferation in the hippocampus of adult rats injected with ^{3}H-thymidine (Kaplan and Bell, 1983).

On the other hand, one of the fiercest opponents of adult neurogenesis at this time was Pasko Rakic. In 1985, Rakic analyzed 12 rhesus monkeys ranging from 6 months to 11 years of age by injecting them with radiolabeled thymidine. He planned to trace neural division at various time intervals in different regions of the brain, including the hippocampus, OB, and the visual, motor, and association neocortex (Rakic, 1985). However, he couldn't find any new neurons and proposed that primates had traded the ability to generate new neurons for the ability to retain plasticity in the old neurons as a requisite for their social and cognitive lives. Three years later, Rakic published a similar study on the cells in the hippocampal DG, providing further opposition to adult neurogenesis (Eckenhoff and Rakic, 1988).

A few years later, Gould demonstrated neurogenesis first in the hippocampal DG of adult monkeys (Gould, Reeves, Fallah, *et al.*, 1999) and later in three areas of the neocortex linked to cognitive function (Gould, Reeves, Graziano, *et al.*, 1999). The experimental design consisted of injecting 12 macaque monkeys with bromodeoxyuridine (BrdU) and examining labeled cells immunohistochemically for specific markers, finding new neurons arising in the subventricular zone (SVZ) of the lateral ventricles.

At about the same time, Rakic and his group also published a study confirming neurogenesis in the hippocampus of the adult macaque monkey (Kornack and Rakic, 1999).

The concept of adult neurogenesis in humans was first proposed in 1998 when Eriksson and colleagues applied the current ‘‘gold standard’’ adult hippocampal neurogenesis method, which was previously established in animal studies, on the human hippocampus (Eriksson *et al.*, 1998). They identified patients who had received infusions of the thymidine analog BrdU for tumor-staging purposes but did not receive any treatment that was thought to affect cell generation and analyzed the brains postmortem. Their conclusion from five brains was that adult neurogenesis could be detected in the human hippocampus in the same location and numbers as expected based on work in rats.

Another study reported observing neurogenesis in postmortem samples of the human striatum, including migration of newly formed interneurons to the neocortex measuring the concentration of the nuclear bomb test-derived radioactive carbon (C^{14}) in genomic DNA (Ernst *et al.*, 2014).

The most robust study to date disputing the concept of adult human hippocampal neurogenesis was done by Sorrells (Sorrells *et al.*, 2018). They collected postmortem and postoperative hippocampal tissue from humans. The researchers stained the samples with the fluorescent marker antibodies Ki-67+SOX1+SOX2 to identify neural progenitor cells, and DCX+PSA-NCAM to identify young neurons. Their results showed that young neurons decreased in density from roughly 1,618 cells/mm^2 at birth to 12 cells/mm^2 at age 7 to 2 cells/mm^2 by age 13, and almost no young neurons in the samples in individuals over the age of 18.

On the other hand, the most robust study that supports the validity of the concept of adult human hippocampal neurogenesis was done by Boldrini (Boldrini *et al.*, 2018) who used immunohistological methods similar to the Sorrells study. Here, investigators autopsied hippocampal DG from humans aged 14 to 79 years and found that the total numbers of intermediate neural progenitors and immature neurons, mature granule neurons, and glia didn’t show any significant differences between the age groups.

More recently, other evidence supporting hippocampal neurogenesis in adult humans has emerged. Moreno-Jiménez (Moreno-Jiménez *et al.*, 2019), identified thousands of immature neurons exhibiting variable degrees of maturation along

the differentiation stages of the DG in neurologically healthy humans up to the ninth decade of life.

Tobin also reported observing persistent hippocampal neurogenesis in adult humans through the 10th decade of life, including patients with mild cognitive impairments and Alzheimer's disease, though not as significant as those with no cognitive impairments (Tobin *et al.*, 2019).

Lastly, Kumar recently published research (Kumar *et al.*, 2019) using bioinformatics methods to analyze the differential expression of neurogenesis signature markers as per maturation stages in the developmental transcriptome in human hippocampi. They found persistent but minimal hippocampal neurogenesis in adult humans.

1.6.1 NEUROGENESIS IN THE SVZ AND OLFACTORY BULB

The SVZ forms during embryonic development between the striatum and the lateral ventricle. At the late stages of embryonic life, the SVZ generates cells destined not only for the adjacent basal ganglia but also for other structures in the brain, including the diencephalon (Rakić and Sidman, 1969) and the cerebral cortex (De Carlos, López-Mascaraque and Valverde, 1996; Anderson *et al.*, 1997).

Three types of precursor cells exist in the adult SVZ: type B progenitor cells, type C transit amplifying cells, and type A migrating neuroblasts (Fig. 5). Type B cells, which resemble astrocytes, express GFAP, glutamate aspartate transporter, and brain lipid-binding protein (Doetsch, García-Verdugo and Alvarez-Buylla, 1997; Codega *et al.*, 2014; Mich *et al.*, 2014). Activated type B cells express nestin and divide asymmetrically (Doetsch, García-Verdugo and Alvarez-Buylla, 1997; Ponti, Obernier and Alvarez-Buylla, 2013). Type C cells express achaete-scute homolog 1 and distal-less homeobox 2 and divide asymmetrically two to three times, subsequently differentiating into type A cells (Ponti, Obernier and Alvarez-Buylla, 2013). Neuroblasts represent the final stage of differentiation within the SVZ, dividing one to two times and migrating through the rostral migratory stream (RMS) towards the OB (Doetsch and Alvarez-Buylla, 1996; Lois, García-Verdugo and Alvarez-Buylla, 1996; Wichterle, García-Verdugo and Alvarez-Buylla, 1997;

Ponti, Obernier and Alvarez-Buylla, 2013). These cells express the microtubule-associated protein DCX as well as collapsing-response mediator protein-4, which is involved in axonal guidance. These proteins together allow the newly generated neuroblasts to interact with microtubules and myosin II, allowing them to travel toward the OB (Wichterle, García-Verdugo and Alvarez-Buylla, 1997; Francis *et al.*, 1999; Nacher, Rosell and McEwen, 2000).

In the SVZ, progenitor cells are adjacent to the ependymal cell layer of the lateral ventricle, which expresses the protein Noggin that may promote SVZ neurogenesis by blocking the glycogenic effects of the bone morphogenetic proteins (Lim *et al.*, 2000). However, Nogging alone is insufficient to induce the neuronal differentiation of adult NSCs. Wnt signaling is another pathway that could instruct NSCs to adopt a neuronal fate (Lee and Jessell, 1999). Ependymal cells may also promote the self-renewal of adult NSCs through pigment epithelium-derived factors (Ramírez-Castillejo *et al.*, 2006). In addition, dopaminergic fibers are found near SVZ precursor cells. Dopaminergic signaling may promote SVZ proliferation through the D2-like dopamine receptors (Höglinger *et al.*, 2004).

In rodents, neuroblasts from SVZ migrate tangentially up to a distance of 5mm before reaching the OB (Lois and Alvarez-Buylla, 1994). This migration is not guided by radial glia or existing axon fibers but through “chain migration”, by forming elongated cell aggregates that are enclosed in tubular structures formed by specialized astrocytes (Lois, García-Verdugo and Alvarez-Buylla, 1996; Wichterle, García-Verdugo and Alvarez-Buylla, 1997). Although astrocytes do not appear to play an essential role in this chain migration, they may be involved in modulating the level of GABA, which reduces the speed of neuroblast migration. Migration velocities (ranging from 40 µm/h to 80 µm/h) are not constant but rather saltatory, with periods of inactivity interspersed between migratory spurts. Furthermore, cells move bidirectionally, occasionally migrating back in the direction of the SVZ (Davenne *et al.*, 2005).

Tangential migration requires the presence of polysialated neural cell adhesion molecule (PSA-NCAM) that allows cell-cell and cell-extracellular matrix interactions (Bonfanti and Theodosis, 1994; Cremer *et al.*, 1994; Hu *et al.*, 1996;

Chazal *et al.*, 2000), while the directionality of this migration is regulated by a series of factors, including the Slit-Robo signaling (Hu, 1999; Wu *et al.*, 1999; Nguyen-Ba-Charvet *et al.*, 2004). A gradient of Slit1 and Slit2 is established by the cilia, with the highest concentration being in the SVZ, driving type A cells away (Sawamoto *et al.*, 2006).

After reaching the core of the OB, neuroblasts detach from their chains and turn radially towards the granule cell layer and to the periglomerular cell layer through a process regulated by factors such as tenascin-R and Reelin. First Reelin, a secreted glycoprotein synthesized in the mitral cell layer, induces type A cells detachment from chains (Hack *et al.*, 2002). Second, tenascin-R, found in the granule cell layer, not only induces detachment but also triggers the radial migration of the neuroblasts (Saghatelyan *et al.*, 2004).

Subsequently, type A cells differentiate mainly into neurons that grow in dendritic trees. Most of the neuroblasts (about 95%) differentiate into GABA-containing granule cells, whereas the rest differentiate into periglomerular cells expressing GABA and/or the dopamine-synthesizing enzyme tyrosine hydroxylase (Betarbet *et al.*, 1996; Luskin, 1998; Kato *et al.*, 2001; Petreanu and Alvarez-Buylla, 2002; Winner *et al.*, 2002; Belluzzi *et al.*, 2003; Carleton *et al.*, 2003).

Only a small portion of these cells survive to become mature granule cells. Quantitative studies have demonstrated that nearly half of newborn interneurons are eliminated between two and eight weeks after their birth (Doetsch *et al.*, 2002; Winner *et al.*, 2002).

The survival of newborn granule cells is subject to regulation by diverse mechanisms, based on the olfactory experience of animals (Corotto, Henegar and Maruniak, 1994; Biebl *et al.*, 2000; Petreanu and Alvarez-Buylla, 2002; Rochefort *et al.*, 2002). Further studies revealed that it is olfactory learning, not simple exposure to odors, that enhances SVZ neurogenesis (Alonso *et al.*, 2006).

However, once a cell is fated to survive, it does so for a long period: 50% of newly generated neurons that survive the initial period of cell elimination survive for at least 19 months (Winner *et al.*, 2002). These cells tend to establish connections to mitral or tufted cells (Petreanu and Alvarez-Buylla, 2002).

Neurogenesis in the SVZ is regulated by several intrinsic and extrinsic factors, such as growth factors, signaling pathways, neurotransmitters, and genes. Furthermore, neurogenesis in the SVZ can occur as a result of neurological diseases, such as stroke or seizures (Fares *et al.*, 2019), or physical exercise (Lee *et al.*, 2016; Mastrorilli *et al.*, 2017; Nicolis di Robilant *et al.*, 2019); moreover, an enriched environment can modulate neurogenesis (Zhang *et al.*, 2018; Tang *et al.*, 2019; Zhan *et al.*, 2020). The main growth factors involved include the mitogens fibroblast growth factor 2 (FGF-2) and the epidermal growth factor 2 (EGF-2), both of which are expressed by astrocytes and provide proliferative signals to the NSCs in the SVZ (Morita *et al.*, 2005). Indeed, at least a subpopulation of proliferating cells in the SVZ express the EGF-receptor (Morshead *et al.*, 1994; Doetsch *et al.*, 2002), and a null mutation for the EGF-receptor ligand TGFα leads to a significantly decreased stem/progenitor cell proliferation within the SVZ (Tropepe *et al.*, 1997). Moreover, delivery of either EGF or FGF-2 to the adult rodent CNS by different routes has been demonstrated to increase the proliferation of progenitor cells in the SVZ (Craig *et al.*, 1996; Wagner, Black and DiCicco-Bloom, 1999). Other growth factors involved include ciliary neurotrophic factor (CNTF), which is involved in NSC self-renewal; vascular endothelial growth factor (VEGF), which is important for angiogenesis; betacellulin, which increases proliferation; and BDNF, which acts as the main trophic factor required for survival and maturation into granule cells (Kirschenbaum and Goldman, 1995; Palmer, Willhoite and Gage, 2000; Pencea *et al.*, 2001; Jin *et al.*, 2002; Emsley and Hagg, 2003; Greenberg and Jin, 2005; Ramírez-Castillejo *et al.*, 2006; Gómez-Gaviro *et al.*, 2012).

Regarding neurotransmitters, serotonin (5HT) and serotoninergic transmission play a critical role in the initial stages of SVZ cell proliferation, increasing proliferation, and neurogenesis. In particular, 5HT increases the proliferation of B1 cells through the activation of 5HT2C receptors (Banasr *et al.*, 2004; Tong *et al.*, 2014). GABA can inhibit both cell proliferation and neuronal differentiation in this neurogenic region. However, type B and type C cells express the diazepam-binding inhibitor protein, which prevents GABAergic neurotransmission and promotes cell proliferation (Liu *et al.*, 2005; Fernando *et al.*, 2011; Alfonso *et al.*, 2012). Lastly, the SVZ population of neurons expressing choline acetyltransferase also regulates

neuroblast proliferation through the activation of fibroblast growth factor receptor (FGFR)-mediated signaling (Paez-Gonzalez *et al.*, 2014).

Some additional genes influence neurogenesis in the SVZ. For example, the KAT6B (Lysine Acetyltransferase 6B) gene is expressed at high levels in this neurogenic niche and plays an important role in adult neurogenesis (Sheikh *et al.*, 2012). In keeping with this observation, KAT6B-deficient mice have reduced numbers of NSCs and migrating neuroblasts in the RMS (Niklison-Chirou *et al.*, 2020). Additionally, the Btg1 gene is known to control the proliferation of stem/progenitor cells in the SVZ and is associated with the maintenance and self-renewal of the stem cells in this niche (Farioli-Vecchioli *et al.*, 2012; Micheli *et al.*, 2015).

1.6.2 NEUROGENESIS IN THE HIPPOCAMPUS

The hippocampus is a part of the temporal lobe and belongs to the limbic system, being responsible for memory, learning, and emotions. Several distinct regions characterize the mammalian hippocampus each with a different function, including a macroscopically well-identifiable part referred to as the Hammon's horn (cornu Ammonis). The Hammon's horn is subdivided histologically into four fields named cornu Ammonis (CA)1, CA2, CA3, CA4, and the DG (Giap *et al.*, 2000). The DG neurons project to the CA2 region (Llorens-Martín *et al.*, 2015), which plays a role in social memory and contextual discrimination (Hitti and Siegelbaum, 2014; Wintzer *et al.*, 2014). The adult-born DG neurons promote excitation of the CA3 pyramidal neurons, mossy cells, and hilar interneurons (Gu, Janoschka and Ge, 2013), which is essential for memory recovery and for delivering feedback inhibition into the mature DG neurons (Temprana *et al.*, 2015).

Evidence that adult hippocampal neurogenesis exists for a large number of species, ranging from rodents (Snyder *et al.*, 2009; Jessberger and Gage, 2014) to monkeys (Gould *et al.*, 1998; Gould, Reeves, Fallah, *et al.*, 1999), strongly suggests that this phenomenon is common to most mammalian species. In the rodent brain, the number of new neurons is substantial, with some studies reporting as many as 9,000 new granule cells being added to the young adult rat DG daily (Cameron and Mckay, 2001). Relatively high numbers of new neurons have also

been reported in the adult human hippocampus (Eriksson *et al.*, 1998; Knoth *et al.*, 2010; Spalding *et al.*, 2013; Boldrini *et al.*, 2018). Even though a few studies contradict these findings (Cipriani *et al.*, 2018; Sorrells *et al.*, 2018), the majority of reports provide positive evidence for adult neurogenesis in the hippocampus of humans.

In the hippocampus, adult neurogenesis produces one type of neuron, the DG granule cell (Fig. 5). The process begins with the asymmetric division of radial glial stem cells residing in the subgranular zone (SGZ), a region between the granule cell layer and the hilus (Seri *et al.*, 2001). These precursor cells, called type-1 cells, have triangular somas and branches projecting into the inner molecular layer. Type-1 cells express the undifferentiated neural progenitor cell marker nestin, the glial fibrillary acidic protein (GFAP), and the Sry-related HMG box transcription factor Sox2 (Seri *et al.*, 2001; Filippov *et al.*, 2003; Fukuda *et al.*, 2003; Garcia *et al.*, 2004; Suh *et al.*, 2007).

The daughter cells are highly proliferative amplifying stem cells, called type-2 cells. They have a dense, irregular-shaped nucleus, only short processes, and do not express GFAP. These cells are capable of tangential migration from the SGZ into the granule cell layer (Gonçalves *et al.*, 2016). Type-2 cells can be further characterized based on the expression of the immature neuronal marker doublecortin (DCX) into either DCX-negative type-2A cells, which are less differentiated, or DCX-positive type-2B cells, which are more differentiated and committed to the neuronal lineage (Brown *et al.*, 2003; Filippov *et al.*, 2003; Kronenberg *et al.*, 2003). Type-2B cells further differentiate into type-3 cells, which are DCX-positive and Nestin-negative and present a round-shaped nucleus (Brandt *et al.*, 2003). Type-3 cells also express the neuronal marker PSA-NCAM (Seki, 2002).

Within 3 days of cell division, this cell population can increase four- to five-fold, and newly generated cells enter a post-mitotic stage characterized by the expression of post-mitotic neuronal markers, like NeuN and calretinin (Brandt *et al.*, 2003; Kempermann *et al.*, 2003). The number of neuroblasts rapidly declines over the subsequent 4 days due to widespread apoptosis and then stabilizes at around 4 weeks, at which point approximately 20% of the newly generated

neurons have survived and are incorporated into the existing neuronal circuitry (Biebl *et al.*, 2000; Kempermann *et al.*, 2003; Kuhn *et al.*, 2005).

Indeed, within 2 weeks of their generation, new neurons begin to take on the morphological characteristics of the mature granule cells with a single dendritic tree extending towards the molecular layer and the formation of dendritic spines (Zhao *et al.*, 2006). Beginning less than 1 week after their generation, new granule cells extend axons, known as mossy fibers, through the hilus toward the CA3 and CA2 subregions of the hippocampal cornu Ammonis (Zhao *et al.*, 2006; Llorens-Martín *et al.*, 2015). Mossy fibers from new granule cells begin to form synapses with excitatory pyramidal cells in the CA3 and CA2 subregions (Toni *et al.*, 2008; Gu, Janoschka and Ge, 2013; Llorens-Martín *et al.*, 2015) as well as with hilar mossy cells (Toni *et al.*, 2008) by approximately 2-3 weeks post-mitosis.

The formation of synapses onto new neurons is known to develop within a week or two of their generation, with the first inputs coming from local GABAergic inhibitory interneurons (Espósito *et al.*, 2005; Ge *et al.*, 2006; Markwardt, Wadiche and Overstreet-Wadiche, 2009). Notably, at this stage, immature neurons transiently express a $Na^+/K^+/Cl^-$ co-transporter that actively transports Cl^- against its concentration gradient and into the cell. As a consequence, GABA signaling has an overall excitatory effect by allowing Cl^- to move according to its concentration gradient and depolarizing the intercellular space (Rivera *et al.*, 1999; Ganguly *et al.*, 2001; Ben-Ari, 2002; Ge *et al.*, 2006).

Following this stage, the final cell number is relatively established, and only a small number of cells are eliminated during the maturation stage (Kempermann *et al.*, 2003). Granule cells then begin their functional maturation, transitioning to normal membrane properties involving physical changes of size, length, thickness, and branching of dendrites and electrochemical changes including increased membrane capacitance and reduced membrane resistance (Van Praag *et al.*, 2002; Marín-Burgin *et al.*, 2012). Maturing granule cells eventually receive glutamatergic inputs from mossy cells in the hilus (Chancey *et al.*, 2014), and then from the entorhinal cortex via the perforant path a few weeks later (Espósito *et al.*, 2005; Ge *et al.*, 2006). Neuromodulatory input to adult-generated neurons occurs later (Vivar *et al.*, 2012; Deshpande *et al.*, 2013), as does the formation of mature inhibitory inputs from local inhibitory interneurons (Espósito *et al.*, 2005).

On the output side, adult-generated granule cells form synapses with several populations of neurons within the hippocampus, including the mossy cells in the hilus (Toni *et al.*, 2008), the pyramidal cells of the CA2 and CA3 regions (Toni *et al.*, 2008; Llorens-Martín *et al.*, 2015), as well as the inhibitory interneurons in the DG itself and the CA3 region (Restivo *et al.*, 2015; Drew *et al.*, 2016).

The maintenance of an endogenous pool of type-1 cells is key to the preservation of hippocampal neurogenesis throughout adulthood, and this process is therefore regulated by many factors. The transcription factor Sox2 appears to play a central role in regulating several intracellular signaling pathways (Steiner, Wolf and Kempermann, 2006). First, Sox2 controls the expression of Sonic hedgehog (Shh), which in turn promotes the proliferation of type-1 cells (Favaro *et al.*, 2009). In addition, Sox2 inhibits Wnt signaling, thereby maintaining the cells in a proliferative state (Kuwabara *et al.*, 2009).

Following cell proliferation, BDNF plays an important role in the early cell survival phase, activating proteins involved in cell survival and cell migration (Ortiz-López *et al.*, 2017).

Another study suggested that the protein Disrupted-in-schizophrenia (DISC1) may also be involved in newborn neuron migration in the SGZ (Duan *et al.*, 2007). The migration of newborn neurons in the DG may also be controlled by guidance cues, while signaling through the Reelin pathway may be involved in such regulation (Gong *et al.*, 2007).

Within the DG, the role of the NMDA receptor in adult neurogenesis has also been extensively studied. Although cell genesis in the DG does not require a functional NMDA receptor, global NMDA receptor-dependent activity is inversely correlated with the level of hippocampal proliferation (Jang, Song and Ming, 2008).

On the other hand, extrinsic factors that improve hippocampal neurogenesis include environmental enrichment (Kronenberg *et al.*, 2006; Leal-Galicia *et al.*, 2008; Zocher *et al.*, 2020), aerobic exercise (Kronenberg *et al.*, 2006; Nokia *et al.*, 2016; Leiter, Bernas, *et al.*, 2019; Leiter, Seidemann, *et al.*, 2019), diet (Poulose *et al.*, 2017; Ichwan *et al.*, 2021) and sexual behavior (Glasper and Gould, 2013). Although other extrinsic factors, such as stress and disease, exert a negative effect on hippocampal neurogenesis, aging is the key factor contributing to the decrease

in natural neurogenesis (Kuhn, Dickinson-Anson and Gage, 1996; Lazarov *et al.*, 2010).

Since new neurons are generated throughout the dorsoventral extent of the DG, the possibility that they might participate in more than one hippocampal function seems high. Indeed, adult-born neurons have been causally linked to many of the known functions and behaviors associated with the hippocampus.
Adult neurogenesis in the hippocampus seems to provide resilience to stress-induced anxiety (Snyder *et al.*, 2011; Hill, Sahay and Hen, 2015; Anacker *et al.*, 2018). Indeed, it is necessary for stress-induced social avoidance (Lagace *et al.*, 2010), as well as in social cognition (Garrett *et al.*, 2015).

1.6.3 NEUROGENIC NICHES

Although NSCs can be isolated from many areas of the adult nervous system, adult neurogenesis has only been consistently found in the SVZ and SGZ in vivo. It is hypothesized that the microenvironments of the SGZ and SVZ, known as the neurogenic niche, may have specific factors that are permissive to the differentiation and integration of new neurons (Morrison and Spradling, 2008).
During development and in regenerative tissues, stem cell maintenance and differentiation are often contingent upon their proximity to a basal lamina (BL). The SVZ BL is rich in laminin and collagen-1 and is associated with a perivascular "connective" tissue comprised of fibroblasts and macrophages (Mercier, Kitasako and Hatton, 2002). Mercier and colleagues proposed that the BL concentrates or modulates cytokines and growth factors derived from local cells. The BL is extensively interdigitated with all SVZ cell types, and type B cells have the most extensive contact. Perhaps this extensive attachment to the BL is important for type B cell maintenance of stem cell properties.

Proliferating cells and putative neural progenitors in both SGZ and SVZ are also closely associated with the vasculature, indicating that factors released from the blood vessels may have a direct impact on adult neural progenitors (Horner *et al.*, 2000; Alvarez-Buylla and Lim, 2004). Brain blood vessels in the SVZ are intimately

associated with the BL (Mercier, Kitasako and Hatton, 2002), while in the SGZ bursts of endothelial cell division are spatially and temporally related to clusters of neurogenesis (Palmer, Willhoite and Gage, 2000). Moreover, in the adult hippocampus, but not in nonneurogenic areas of the adult CNS, proliferating cell clusters are found near blood vessels, indicating the possibility that vasculature- or blood-derived factors are regulators of neurogenesis (Palmer, Willhoite and Gage, 2000). The finding that factors that promote endothelial cell proliferation also increase neurogenesis in the mammalian forebrain has suggested an important relationship between these two processes (Jin *et al.*, 2002; Zhu, Wang and Hansson, 2003).

In addition, regional differences in the astrocyte population have recently also been demonstrated to be important for the neurogenic microenvironment in the adult hippocampus. Adult hippocampal astrocytes actively regulate neurogenesis by promoting the proliferation of neural stem/progenitor cells and by instructing them to adopt a neuronal fate. In the SGZ, adult hippocampal progenitors are closely apposed to a dense layer of granule cells that includes both mature and newborn immature neurons. Within this microenvironment, there are also astrocytes, oligodendrocytes, and other types of neurons. Indeed hippocampal astrocytes may also play an important role in SGZ neurogenesis. They promote the neuronal differentiation of adult hippocampal progenitor cells and the integration of newborn neurons derived from adult hippocampal progenitors in vitro (Song, Stevens and Gage, 2002). Moreover, hippocampal astrocytes are thought to act through Wnt signaling (Lie *et al.*, 2005), instructing NSCs to adopt a neuronal fate. Concerning the neurogenic niche that generates the adult-born olfactory neurons, it has been found that astrocytes in the SVZ have similar effects on the proliferation and neuronal differentiation of neural stem/progenitor cells as their hippocampal counterparts (Lim and Alvarez-Buylla, 1999). Additionally, ependymal cells in the lateral ventricle regulate neurogenesis by secreting factors that bias/instruct the cell fate of the SVZ stem/progenitor cells (Lim *et al.*, 2000).

1.6.4 NEUROGENESIS IN OTHER AREAS OF THE CNS

Several additional areas of the CNS have emerged as containing newly generated neurons beyond early development. Animal studies have shown that these neurogenic areas include the hypothalamus (Evans *et al.*, 2002), striatum (Parent, Cicchetti and Beach, 1995; Suzuki and Goldman, 2003; Shapiro *et al.*, 2009), substantia nigra (Zhao *et al.*, 2003), cerebral cortex (Magavi, Leavitt and Macklis, 2000) and amygdala (Bernier *et al.*, 2002).

Some evidence showed that the new neurons in these novel neurogenic areas arise from migrating neural stem and progenitor cells, originating in the SVZ (Bernier *et al.*, 2002; Cao, Wenberg and Cheng, 2002; Dayer *et al.*, 2005; Inta *et al.*, 2008; Shapiro *et al.*, 2009; Huttner *et al.*, 2014). Other studies instead indicated that endogenous pools of neural stem and progenitor cells may exist within these regions (Parent, Cicchetti and Beach, 1995; Zecevic and Rakic, 2001; Evans *et al.*, 2002; Jhaveri *et al.*, 2018).

The generation of new neurons in these novel neurogenic areas can serve important functional roles. Neurogenesis in the hypothalamus has the potential to affect behavior and sexual function (Bernstein, Zuo and Cheng, 1993; Fowler *et al.*, 2002; Cheng *et al.*, 2004). It may also play a role in metabolism and fat storage (Kokoeva, Yin and Flier, 2005; Lee *et al.*, 2014).

In the amygdala, neurogenesis may play a role in fear conditioning and stress response (Shapiro *et al.*, 2009; Saul *et al.*, 2015).

However, the functional significance of new neurons is less well characterized in the striatum, substantia nigra, and cerebral cortex.

There are, however, studies that have failed to confirm these findings (Kay and Blum, 2000; Kornack and Rakic, 2001; Lie *et al.*, 2002), or have found such evidence only after damage (Collin *et al.*, 2005), growth factor infusion (Pencea *et al.*, 2001; Yoshikawa *et al.*, 2010; Zhu *et al.*, 2011), pharmacologic treatments (Rojczyk, Pałasz and Wiaderkiewicz, 2015), and environmental exposures (Kisliouk, Cramer and Meiri, 2014; Niwa *et al.*, 2016).

For example, a study reported the presence of migrating and maturing neurons in the cerebral cortex of mice after the induction of synchronous targeted apoptosis in layer 6 of the cortex and that these new cells persist for at least 28 weeks post-

injury (Magavi, Leavitt and Macklis, 2000). In another study, neuroblast migration and maturation were seen in damaged areas of the motor cortex of mice and some of these newly generated motor neurons showed long-term survival (more than 56 weeks) and developed projections into the spinal cord (Chen, Magavi and Macklis, 2004). The existence of a population of cortical progenitor cells was further confirmed after finding NG2-positive cells co-expressing DCX in the rat neocortex (Tamura *et al.*, 2007).

On the other hand, mixed results have been reported concerning the neurogenic potential of the primate cortex. In macaque monkeys, BrdU labeling showed a limited presence of new neurons in the neocortex up to 12 weeks after treatment (Gould *et al.*, 2001). However, a parallel study failed to replicate these findings and found no new neurons in the neocortex 10-23 days after BrdU injection (Kornack and Rakic, 2001).

With regards to humans, a study was able to isolate progenitor cells from the adult human frontal and temporal cortex, and in vitro treatment of these progenitor cells with FGF-2 and EGF led to the formation of multipotent neurospheres (Arsenijevic *et al.*, 2001). However, a study using accelerator mass spectrometry to measure C^{14} levels in post-mortem tissue from individuals exposed to radioactivity found that none of the NeuN-positive cells of the human cortex integrated the isotope (Bhardwaj *et al.*, 2006).

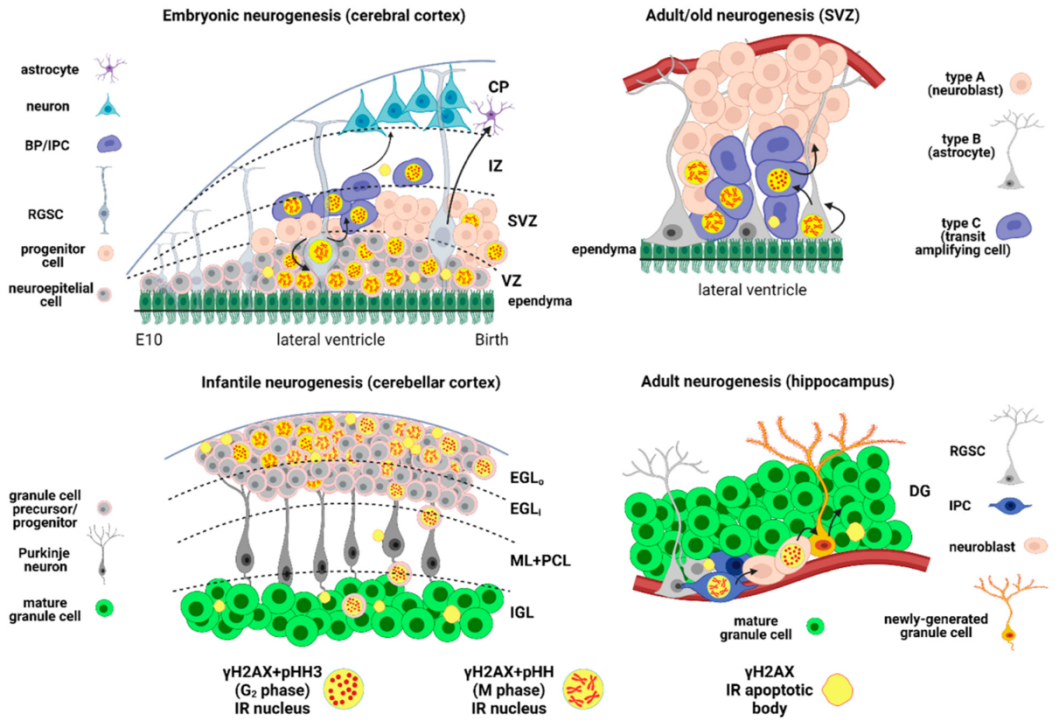


Figure 5. Simplified graphical summary of different phases of neurogenesis in the mouse and the immunocytochemical localization of γH2AX. Note that phosphorylation of H2AX mainly occurs in precursor and progenitor cells and radial glia stem cells (RGSCs) during the G2 and M phases. γH2AX is also detected in nuclei with apoptotic morphology and in apoptotic bodies (from Merighi *et al.*, 2021).

1.6.5 METHODOLOGIES TO INVESTIGATE ADULT NEUROGENESIS

For decades, exogenous DNA precursors, first ^{3}H-thymidine and, more recently, BrdU, which are incorporated into newly synthesized DNA by cells in the S-phase, have been used to study cell proliferation in the developing nervous system (Sauer and Walker, 1959; Sidman and Miale, 1959; Sidman, Miale and Feder, 1959) and have provided information about cell birth, migration, and various parameters of the cell cycle, including the size of the growth fraction and the lengths of the phases of the cell cycle. The general strategy employed assumes that when a 'pulse dose' of one of these exogenous tracers is given, only that proportion of the proliferative population that is in the S-phase at the time of the injection will incorporate the tracer and will thus be labeled.

The original techniques used for selectively labeling mitotic cells involved 'tagging' thymidine using a radioactive probe and a detection step with autoradiography or scintillation techniques (Taylor, Woods and Hughes, 1957; Messier, Leblond and Smart, 1958). ^{3}H-thymidine labeling and autoradiography were originally

developed by Woods, Taylor, and Hughes as DNA probes (Taylor, Woods and Hughes, 1957). In their work, they used autoradiographic analysis of chromosome preparations from the seedlings of *Vicia faba*, a plant of the pea and bean family, treated with ^{3}H-thymidine and revealed that only one of the two sister chromatids in each chromosome was radioactive in the cells of the roots collected after the second replication cycle. Thus, during replication, daughter chromosomes receive an original and a new strand. Later, the delivery of ^{3}H- thymidine and another radioactive nucleoside, ^{14}C-thymidine, with subsequent autoradiographic detection revealed features and mechanisms of DNA replication in pro- and eukaryotic cells, such as unwinding of the double helix, formation of the replication fork, spatial patterning of DNA replication, and creation of the lagging DNA strand through the intermittent synthesis of Okazaki fragments (Cavanagh *et al.*, 2011; Ligasová and Koberna, 2018). By tracing dividing cells and their progeny with autoradiographic detection, ^{3}H-thymidine was widely employed in developmental biology, regenerative biology, and stem cell research. For instance, this approach enabled birth dating of neurons within different cortical layers during corticogenesis in mammals (Angevine and Sidman, 1961), the identification of satellite cells as muscle stem cells and a cellular source for muscle regeneration (Reznik, 1969), and the discovery of the continuous production of new neurons in the walls of the lateral ventricles and the hippocampus in the adult mammalian brain (Altman, 1962, 1963). ^{3}H-thymidine is used for marking replicating DNA because, unlike the other nucleosides, ^{3}H-thymidine is a precursor of DNA but is not involved in RNA synthesis (Reichard and Estborn, 1951).

However, because of cost, logistic problems in handling radiolabeled substances, and the lengthy process of developing autoradiographs (3–12 weeks), nowadays studies are commonly performed with the thymidine analog BrdU, a pyrimidine 2'-deoxyribonucleoside compound having 5-bromouracil as the nucleobase. BrdU was originally used therapeutically as an antiviral and antineoplastic agent (Freese *et al.*, 1994; Begg *et al.*, 2000). Labeling was developed as an alternative approach for determining the proliferative index of tumors (Hoshino *et al.*, 1989; Struikmans *et al.*, 1997), and was introduced for studying cell proliferation in the developing nervous system by Nowakowski et al. (Nowakowski, Lewin and Miller, 1989). BrdU was synthesized already in 1955 by Beltz and Visser (Beltz and Visser, 1955),

however, more than 15 years were necessary to develop an efficient way to use it in non-isotopic detection in cellular DNA. The incorporation of BrdU into bacterial DNA was demonstrated for the first time in 1960 (Djordjevic and Szybalski, 1960), and into mammalian DNA in 1959 (Eidinoff, Gheong and Rich, 1959; Hakala, 1959). Until the discovery of specific antibodies, the detection systems of BrdU in DA were mainly based on autoradiography (Hakala, 1959). In 1971, specific antibodies raised against BrdU were produced, and the immunochemical detection of the incorporated BrdU was described in denatured DNA (Sawicki, Erlanger and Beiser, 1971). Four years later, the method of immunofluorescence for in situ detection of incorporated BrdU in nuclear DNA was developed (Gratzner *et al.*, 1975). BrdU immunohistochemistry offers several advantages over ^{3}H-thymidine autoradiography. First, BrdU can be revealed by immunohistochemistry using a monoclonal antibody directed against single-stranded DNA containing BrdU (Gratzner, 1982). Also, it allows for faster studies without handling radiolabeled material, and the detection of labeled cells throughout the relatively thick tissue sections required for stereological studies of the brain (West, Slomianka and Gundersen, 1991). This has led to the wide use of BrdU for studying adult neurogenesis. The ability to combine BrdU labeling with the detection of cell type-specific markers via specific antibody staining or reporter gene expression has become a gold standard for studying cell division and differentiation.

Other methods of detecting DNA synthesis involve using halogenated thymidine analogs IdU, CldU, and a relatively newer marker EdU. They mirror BrdU in targeting DNA and are also detected using specific antibodies (Yokochi and Gilbert, 2007). These halogenated derivatives may be multiplexed to probe proliferative cells (Tuttle *et al.*, 2010). The efficacy of DNA access and labeling is generally considered to be uniform for the halogenated thymidine analogs, however, detection efficiency may differ as determined by the efficacy of the antibodies targeting the unnatural bases. The use of these nucleoside analogs is usually restricted to some special cases. IdU and CldU were and still are used mainly for the double labeling of both DNA in cells (Aten *et al.*, 1992) and also on DNA fibers (Malínský *et al.*, 2001). The approaches based on the immuno-detection of halogen derivatives are very important procedures used for the detection of the replicational activity of cells, and serve as an important tool for the description of

cellular proliferation and cell cycle by microscopy or flow cytometry (Dolbeare *et al.*, 1983; Dolbeare and Selden, 1994; Lam *et al.*, 2014; Wu *et al.*, 2016). In this respect, it was, for example, proven that during the S phase five patterns can be observed in mammalian cells (O'Keefe, Henderson and Spector, 1992). Using the double labeling by IdU and CldU, it was, for example, demonstrated that the addition of exogenous deoxyribonucleotide triphosphates increases the speed of DNA replication only at the beginning of the S phase. Later in the S phase, this addition does not affect the speed of replication (Malínský *et al.*, 2001). The double labeling of cells with IdU and CldU was also applied to the analysis of cell proliferation, temporal analysis of DNA replication, or cell cycle kinetic (Aten *et al.*, 1992, 1994).

Because of the necessity to disrupt the structure of double-stranded DNA in the case of halogen derivatives of thymidine, other possibilities for the detection of replicating DNA were investigated. In 2008, Salic and Mitchinson published an alternative method for the labeling of DNA replication. They used EdU and click reaction with Alexa-azide stains for the detection of replicating DNA (Salic and Mitchison, 2008). The EdU was synthesized already at the end of the 1970s (Perman, Sharma and Bobek, 1976; Barr *et al.*, 1978). Not until 2008 did Salic and Mitchinson show that EdU is strongly incorporated into the DNA of mammalian cells. Thanks to the simplicity, quickness, and mainly because EdU detection does not require any DNA treatment, EdU quickly became a widely-used alternative for the detection of the replicational activity of cells. EdU is presently broadly used. Examples include studies focused on the analysis of DNA replication, S-phase progression, the monitoring of cells in subsequent cell cycles, cellular proliferation, and other processes connected with DNA synthesis (Poujol *et al.*, 2014; Endaya *et al.*, 2016). EdU is highly toxic in the commonly-used concentrations, and some studies even showed that EdU incorporation can lead to DNA breaks followed by cell death (Kohlmeier, Maya-Mendoza and Jackson, 2013; Zhao *et al.*, 2013), proving that EdU is extremely useful for short term experiments, but it is not suitable for long-term studies.

The toxicity of EdU led to the effort to find alternative marker nucleosides. Reported novel nucleotide analogs include (2'S)-2'-deoxy-2'-fluoro-5-ethynyluridine (F-ara-EdU), 5-(azidomethyl)-2'-deoxyuridine (AmdU), and 5-vinyl-

2’-deoxyuridine (VdU) (Solius *et al.*, 2021). Neef and Luedtke showed that F-ara-EdU exhibited only a minimal impact on genome function (Neef and Luedtke, 2011). The published data also indicated that F-ara-EdU is less toxic than EdU, and is therefore more suitable for long-term experiments. VdU is detected by a nonoverlapping bioorthogonal chemical reaction, therefore, it can be combined with other nucleotide analogs that are detected via the azide-alkyne click reaction and even to halogenated nucleotide analogs to produce multilabel marking of replicating DNA (Neef and Luedtke, 2011, 2014).

BrdU and tritiated thymidine can be delivered to most vertebrate species through intracerebroventricular (i.c.v), intravenous (i.v), intramuscular (i.m), or intraperitoneal (i.p) injection, or as an oral dose (Altman and Das, 1965; Taupin, 2007). The nucleoside analog molecule enters the bloodstream and permeates tissues. Once inside the tissue, the chemical is available to all cells. Dividing cells draw on the pool of nucleosides endemic to the extracellular environment (Taylor, Woods and Hughes, 1957; Altman, 1969). Nucleoside analogs compete with the cell's endogenous nucleosides for selection and incorporation into newly forming DNA.

BrdU is metabolized through dehalogenation when integrated into the DNA. Once dehalogenated, the uracil residue would be excised from the DNA by the uracil glycosylase repair system (Hume and Saffhill, 1986). BrdU is also metabolized rapidly through dehalogenation in the plasma (the half-life of BrdU in the plasma is reported to be around 8–11 min in humans) (Kriss *et al.*, 1963). The concentration of thymidine analog that reaches the brain is therefore only a fraction of the administered dose. Hence, i.c.v. injection leads to a higher concentration of BrdU in the brain than peripheral delivery and is employed when a higher and local concentration of BrdU in the brain is sought (Kaplan, 1983; Zhao *et al.*, 2003). However, i.p. injections are the most common mode of administration of BrdU in rodents and other small animals for studying adult neurogenesis, avoiding the need for surgery (Takahashi, Nowakowski and Caviness, 1994; Hayes and Nowakowski, 2000; Vega and Peterson, 2005; Zupanc, Hinsch and Gage, 2005; Luzzati *et al.*, 2006; Mandyam, Harburg and Eisch, 2007; D’Amico, Boujard and Coumailleau, 2011; Podgorny *et al.*, 2018). Larger animals,

such as monkeys, canines, or sheep, usually receive i.v. (Kornack and Rakic, 1999; Siwak-Tapp *et al.*, 2007; Lévy *et al.*, 2017) or i.m. injections (Cho *et al.*, 1997). Oral delivery may be preferred for studying adult neurogenesis in rodent studies, especially when long-term labeling of dividing cells is necessary, as the invasive procedure may induce stress, a condition reported to affect the rate of neurogenesis (Gould *et al.*, 1998). However, due to the circadian dependence of water intake, treatment with BrdU dissolved in drinking water marks different numbers of dividing cells during the light and dark phases of the day (Ševc *et al.*, 2015).

Nucleotide analogs resemble natural thymidine; therefore they readily absorb into the bloodstream after being injected, spread broadly in the body through the blood circulation system, and penetrate virtually all organs and tissues of an organism, including those separated by barriers (brain, testis, placenta). In particular, BrdU is transported to the brain, similarly to thymidine, via nucleoside transport systems present in the blood-brain barrier (Spector and Huntoon, 1984), which have low affinity and high capacity (Thomas and Segal, 1997), and might be eliminated through the cerebrospinal fluid (Spector and Berlinger, 1982; Thomas Née Williams and Segal, 1996; Thomas, Davson and Segal, 1997). It is estimated that BrdU is available for labeling in the adult brain for approximately a 2h period, after systemic injection. After 2 h, the level of BrdU available for labeling in the brain drops sharply (Hayes and Nowakowski, 2000).

Independently from the route of delivery, the thymidine analog dosage is of great importance when labeled cells are evaluated quantitatively and a comparison between the experimental and control groups is necessary. A saturating dose, which is defined as the dose of a nucleotide analog necessary to label most cells in the S-phase, satisfies this condition. The saturating dose of a nucleotide analog depends on the species used, the organ studied, and the life stage analyzed. Current estimations of the saturating dose of BrdU determined by quantification of labeled cells in the hippocampal DG after a single i.p. delivery are 150 mg/kg body weight in mice (Mandyam, Harburg and Eisch, 2007) and 200 mg/kg (Eadie, Redila and Christie, 2005) or 300 mg/kg (Cameron and Mckay, 2001) body weight in rats.

2 AIMS

Earlier observations in neuroscience research suggested that no new neurons are formed in the mature CNS. However, evidence now indicates that, at least in some species, newly formed cells can be observed in specific areas of the adult mammalian brain.

Previous observations carried out in this laboratory demonstrated that in the old mouse brain the histone H2AX was γ-phosphorylated in the absence of known DNA damage (Barral *et al.*, 2014). The areas in which γH2AX was detected comprised, among others, the hippocampal DG, the SVZ, and the OB. Remarkably, these are the areas of "classic" neurogenesis in rodents and other species. Yet γH2AX was also widely detected in neurons (and glia) of the cerebral cortex, which, apart from some possible exceptions yet to be fully accepted by the scientific community, does not contain proliferating neural cells and is thus populated by post-mitotic neurons. This observation was puzzling, so it was the detection of colocalization of γH2AX with BrdU, indicating that the epigenetic modification was somewhat associated with DNA synthesis.

Because neurogenesis in old mammals is still a cause of discussion in the scientific community, I wanted to shed some light on a few different aspects of cell life in the aging mouse brain.

To do so, the study has been divided into three parts:

- Phosphorylation of the histone H2AX is an early response to DNA damage and a marker of aging and disease in several cells and tissues. However, little is known about in vivo phosphorylation of H2AX in neurons of adult and old animals.

  Specifically, I wanted to better understand the functional significance of H2AX phosphorylation in aging and its correlation with the entrance of the cell into the S phase of its cycle, as demonstrated by the incorporation of exogenously administered BrdU. More precisely, I wanted first to clarify whether or not H2AX phosphorylation was indeed related to a DDR in the

different populations of positive neurons described by Barral and colleagues (Barral *et al.*, 2014). To do so, I experimentally induced a DDR by exposing mice to X-rays and studied the response of H2AX and other components of the DDR to ionizing radiations. Second, I wanted to assess if the phosphorylation of H2AX could be related to apoptosis and if the latter event was associated with DNA synthesis. To do so, I studied the activation of CASP3, the main effector caspase, using specific antibodies against the cleaved form of the enzyme and performed double/multiple IMF experiments to clarify the functional relationship between γH2AX, activation of CASP3, and DNA synthesis, detected with the use of BrdU incorporation.

- BrdU integrates into the DNA of dividing cells during the S phase of the cell cycle. Some investigators have pointed out that, in the brain of old animals, BrdU may be incorporated into cells that are synthesizing DNA for reasons other than mitosis. Indeed, studies of damaged tissues have reported BrdU labeling in the absence of mitosis, particularly in cells that are dying or undergoing DNA repair (Kuan *et al.*, 2004; Menu Dit Huart *et al.*, 2004). However, to my knowledge, no examples of this have been reported in the intact old brain. Thus, I wanted to investigate the connection between BrdU incorporation, mitosis, and DNA repair in the brain of the adult mouse.

- Accumulating evidence reveals that cell cycle kinetics plays a very important role in the regulation of neurogenesis. In the neurogenic niches of the adult brain, it has been proposed that the duration of the cell cycle regulates differentiation, suggesting also that there are differences in the replicative kinetics of the proliferating cell types among different niches (Farioli-Vecchioli and Tirone, 2015). Moreover, it has been proposed that senescence causes a lengthening of the cell cycle in some cells of the mouse brain (Tropepe *et al.*, 1997). However, the length of the cell cycle has not been calculated. More recently, it has also been shown that cell cycle re-initiation is involved in the brain aging process and that the brain

appears to lose telomere length in both replicative and non-replicative cells though at different rates (Ain *et al.*, 2018).

Here, I wanted to study the length of the cell cycle and the duration of its phases in cells of the cerebral cortex, hippocampus, and SVZ of 24-month-old mice.

3 MATERIALS AND METHODS

3.1 ANIMALS AND THYMIDINE ANALOGS ADMINISTRATION

All animal experiments were authorized by the Italian Ministry of Health (n. 65/2016 PR and n. 130/2012-B) and by the Bioethics Committee of the University of Turin. Animal procedures were carried out according to the guidelines and recommendations of the European Union (Directive 2010/63/UE) as implemented by current Italian regulations on animal welfare (DL n. 26-04/03/2014).

The number of animals was kept to the minimum necessary for statistical significance, and all efforts were made to minimize their suffering.

Ten 24-month-old CD1 mice were used for the analysis of the cell cycle length, while irradiation experiments were performed on six 24-month-old B6/129 mice.

X-ray irradiation was carried out at Enea (Rome) in the general framework of another project aiming to characterize the response of a tumor cell line to ionizing radiations. As mouse brains and intestines (used as positive controls) were not necessary for the project, they were kindly donated for our study.

This explains why a different strain of mice was used in these studies, also considering that the laboratory did not hold the prescribed authorizations to carry on irradiation experiments. Although there is extensive literature on different radiation susceptibility among mouse strains in tissues and organs other than the brain (see e.g. (Jackson, Vujaskovic and Down, 2010; Gridley *et al.*, 2011)), I found little information regarding putative differences in the brain if not related to mortality rates in mice (Yang *et al.*, 2016) or strain in rats (Cacao, Kapukotuwa and Cucinotta, 2018). In addition, we have not used mice of different strains for either irradiation studies or cell cycle studies.

We divided these mice into three groups of two animals each: control (not irradiated), short survival, and long survival (irradiated and left to survive for different periods thereafter) (Fig. 6). In the beginning, all animals were injected with BrdU intraperitoneally at a dose of 100 mg per kg/BW. Only irradiated animals were then exposed to a 10 Gray dose 15 (short survival) or 30 (long survival) min before suppression. X-ray irradiation was performed with a Gilardoni CHF 320G X-ray generator (Gilardoni S.p.A., Mandello del Lario, Italia). Animals

were put in a small plastic box and left free-moving, while placed under the radiation beam. All animals belonging to the same experimental group were irradiated together. According to the specifics of the X-ray machine, 10 min long sessions were necessary to reach a 10 Gray dose of irradiation.

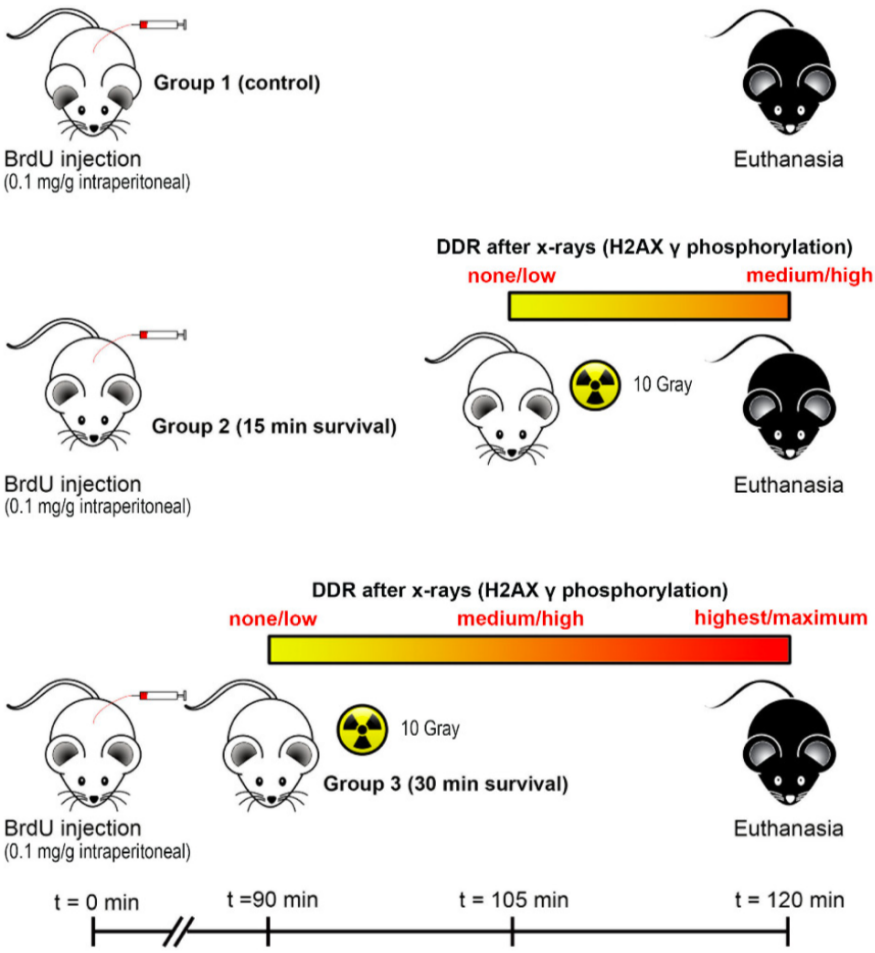


Figure 6. Schematic layout of the X-ray irradiation and control experiments. Experiments are designed so that all animals are sacrificed 2 hours after BrdU injection. The colored bars represent, pictorially, H2AX γ phosphorylation.

To study the length of the cell cycle, we used a double labeling procedure whereby animals were injected with two sequential intraperitoneal doses of equimolar (65 mM) IdU and BrdU (Lossi, Mioletti and Merighi, 2002; Burns and Kuan, 2005; Martynoga *et al.*, 2005). The technique is a modification of the original protocol of Hayes and Novakowski (Hayes and Nowakowski, 2002) who used ^{3}H thymidine and BrdU as markers. BrdU was given at a dosage of 50 mg per Kg/body weight (BW) after having been dissolved in sterile 0.9% NaCl and administered at 20 mg/mL (65 mM). Hence, to achieve 50 mg per Kg/BW, the solution is injected at 2.5 µL/g BW.

To accelerate and assure that all the BrdU is dissolved, sonication with gentle heating (e.g. 37 °C) can be used. To achieve 65 mM of IdU, one needs 23 mg/mL (i.e. 57.5 mg/kg BW). This way, injections can also be delivered at 2.5 mL/Kg BW. IdU was prepared as indicated above for BrdU, but it is difficult to dissolve in water, therefore it is necessary to add 0.007 M NaOH to the solution. In addition, IdU, it may take longer to dissolve than BrdU. To accelerate the process, stronger agitation and/or sonication may be needed (see Duque and Rakic, 2015). Mice were divided into three groups, based on the length of the interval between IdU and BrdU injections: 24h (1 mouse), 72h (5 mice), and 120h (4 mice).

In general terms, the numbers of animals used in this study were remarkably low. Yet, I would like to stress that obtaining 24-month-old mice is quite difficult as many subjects initially included in the experimental groups died before reaching the required age to be included in this work.

Aside from this, statistical analysis was fully supportive of my findings as also discussed in one of the publications stemmed from this work in which, among others, the results of X-ray irradiations were reported (Gionchiglia et al. 2021).

Regarding data on the cell cycle kinetics, it will be necessary to increase the number of animals in the 2-hour survival group and to add a group of animals with longer survival (e.g. 240 h) to publish the observations stemming from the thesis.

3.2 SAMPLING AND TISSUE PROCESSING

Under deep anesthesia with sodium pentobarbital (30 mg/Kg; Thiopental), animals were perfused through the left ventricle with cold Ringer solution [phosphate buffer (PB) 0.01M pH 7.4-7.6, 0.8% NaCl, 0.025% KCl, 0.05% $NaHCO_3$] followed by fixative (4% paraformaldehyde in PB 0.2M pH 7.4). Brains and intestines (as positive controls for successful incorporation of BrdU and IdU) were then removed and post-fixed for two additional hours in the same fixative. Tissues were washed, dehydrated through a graded ethanol series, and embedded in paraffin wax. Randomly chosen transversal sections of the jejunum (7-10 µm) were collected on slices to be further processed for immunocytochemistry. Brains were serially cut in parasagittal sections (10µm) that were collected and mounted in triplets on poly-L-lysine (PLL; Sigma, Cat# P8920) pre-coated slides. We assigned

equally spaced sections to different series to be further processed for immunolabeling procedures (Fig. 7).

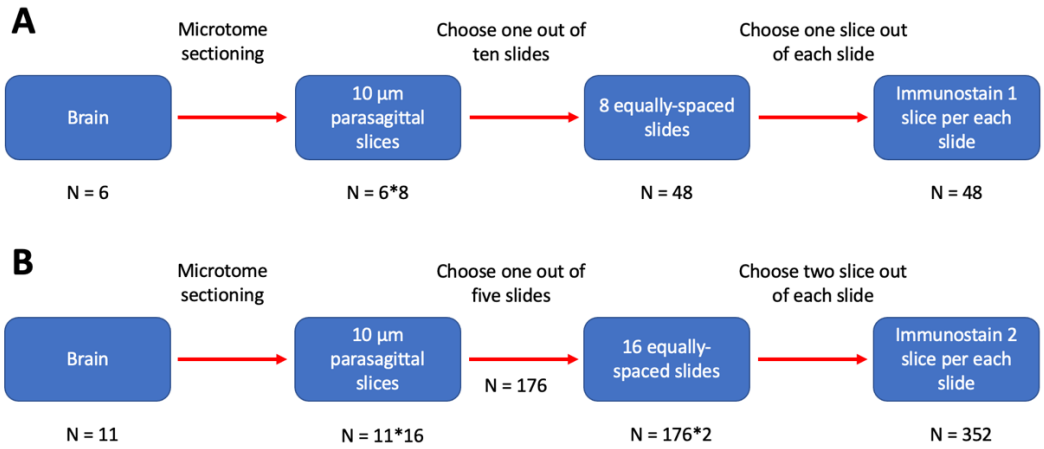


Figure 7. Method for quantitative analysis of nucleotide analog immunolabeling procedures. Single staining with BrdU (**A**), and double stainings with IdU and BrdU (**B**).

For each series, one out of ten sections were used for analysis, for a total of eight equally-spaced sections for each procedure, except for double stainings with BrdU and IdU where 32 sections were used for each animal. All selected sections contain all neocortical layers, the hippocampal formation, and the SVZ surrounding the lateral ventricles (Fig. 7).

3.3 IMMUNOFLUORESCENCE

To perform single and double immunostaining, paraffin was removed in xylene, and sections were rehydrated with serial passages in decreasing concentrations of ethanol. Sections were then subjected to microwave antigen retrieval (3 min at 95-99°C in sodium citrate buffer, 10mM pH 6), washed 5 min in phosphate-buffered saline (PBS) 0.01 M pH 7.4, and blocked for 1 hour in PBS containing 1% ovalbumin (Sigma, Cat# A5503) and 0.3% Triton X-100 (Sigma, Cat# X100). Sections were then incubated overnight at room temperature in primary antibodies at optimal titer. After being rinsed 3 x 5 min in PBS, sections were incubated for 1 hour with appropriate secondary antibodies (anti-rabbit or anti-mouse Alexa Fluor® 488 (1:200) or Alexa Fluor® 594 (1:500); Thermo Fisher, Cat# A11034, #

A11029, # A11037, # 11032) diluted in the same diluent used for primary antibodies. After several washes in PBS, sections were counterstained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, Cat# D9564) and mounted in a fluorescence-free medium (Fluoroshield; Sigma, Cat# F6182).

Only for stainings with BrdU and IdU, a different protocol was used: after paraffin removal, sections were incubated for 2 min with 1% $NaBH_4$ (Carlo Erba, Cat# 478953) dissolved in ddH_2O, rinsed with ddH_2O and processed for microwave antigen retrieval. After microwave treatment, sections were washed for 5 min with PBS and blocked for 1 hour in PBS containing 5% bovine serum albumin (BSA; Sigma, Cat# A9647), 1% Agar (Oxoid, Cat# L11), 4% normal goat serum (NGS; Sigma, Cat# G9023), and 0,5% Triton-X. Sections were then incubated for 48 hours at 4°C with a mix of primary antibodies, diluted in the blocking solution. After being rinsed 3 x 10 min in PBS, sections were incubated for 30 min with the blocking solution, and then for 1 hour with appropriate secondary antibodies. After 3 x 5 min washes, sections were counterstained with DAPI and mounted with Fluoroshield.

Sections were then photographed using either a Leica DM6000 wide-field fluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a 20X, 40X or 63X lens or a confocal microscope (Leica SP8, Leica Microsystems, Wetzlar, Germany) with a 63X oil lens.

3.4 PRIMARY ANTIBODIES AND CONTROLS

Primary antibodies used in this study were:

1:10 monoclonal mouse anti-BrdU (Bio-Rad Laboratories, Cat# MCA2483); 1:10 monoclonal rabbit anti-BrdU (Antibodies Online, Cat# 6941451); 1:40 monoclonal mouse anti-IdU (Origene, Cat# TA190129); 1:200 monoclonal mouse anti-γH2AX (Abcam, Cat# ab18311); 1:1500 polyclonal rabbit anti-γH2AX (Calbiochem, Cat# DR1017); 1:10 polyclonal rabbit anti-pHH3 (Abcam, Cat# ab26127); 1:1000 polyclonal rabbit anti- 53BP1 (Abcam, Cat# ab172580); 1:10 polyclonal rabbit anti-cCASP3 (Abcam, Cat# 2302); 1:20 monoclonal mouse anti-NeuN (Millipore, Cat# MAB377).

Primary antibodies were diluted in PBS containing 2% BSA and 2% PLL (Sigma Cat# P0879).

Positive controls for BrdU and IdU stainings were performed by using the corresponding mouse's intestine.

Negative controls were performed by omitting the primary antibody.

Cross-reactivity analyses were performed to test the selectivity of BrdU and IdU antibodies. To do so, the brains and intestines of P8 animals injected with BrdU were stained with anti-IdU antibodies, and vice versa (Appendix 1).

3.5 QUANTITATIVE STUDIES

To study single or double immunostaining with BrdU and/or IdU, sections were observed with a 40X lens. For each forebrain section, three areas were selected: the cerebral cortex, the hippocampus, and the SVZ. All immunoreactive cells for BrdU and/or IdU were counted in these areas, and colocalization of the two markers was recorded directly by the observer.

To count γH2AX-immunoreactive nuclear foci, forebrains were single labeled and five random photographs of the cerebral cortex, hippocampus, and SVZ were taken using a 63X or 100X lens for each section. The entire hippocampal formation was analyzed, as wall as all neocortical layers. Foci of all cells within each photograph were automatically counted using the Foci Counter program (Version 1, University of Konstanz, GE - http://focicounter.sourceforge.net/) according to the software manual. The image threshold was adjusted to achieve a clear identification of the nuclei in the "select" window and was maintained unaltered among different images.

To study all other staining reactions, five random microscopic fields of each section of the cerebral cortex, hippocampus, and SVZ were photographed with a 40X lens. All immunoreactive cells, as well as all DAPI fluorescent nuclei, were manually counted in each photograph.

3.6 STATISTICS

All statistical analyses were carried out with the use of GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Normality was ascertained with the D'Agostino and Pearson normality test. Animals treated with the same irradiation protocol were compared using either the unpaired t-test with Welch's correction or the unpaired two-tailed Mann-Whitney test.

With normally distributed data, the three experimental groups were compared using either a one-way ANOVA followed by Tukey's multiple comparison test, when standard deviations (SDs) were equal, or the Brown-Forsythe and Welch ANOVA tests followed by Dunnett's T3 multiple comparison tests, when SDs were unequal. When data did not pass the D'Agostino and Pearson normality test, the three experimental groups were compared using the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons tests.

All data were reported as mean ± 95% confidence interval (CI) and values of $p < 0.05$ were considered statistically significant.

4 RESULTS

4.1 γ PHOSPHORYLATION OF H2AX

4.1.1 X-RAY IRRADIATION INDUCES A STRONG γ PHOSPHORYLATION OF H2AX

Although H2AX γ phosphorylation was originally discovered to be associated with DDR (Rogakou *et al.*, 1998), the molecule has multiple functions (Turinetto and Giachino, 2015; Merighi *et al.*, 2021). Therefore I first aimed to establish if γH2AX was a reliable marker of DNA damage in the old mouse brain. To do so I exposed the brain to X-ray irradiation and left mice to survive for 15 or 30 min thereafter. The specificity of immunostaining was confirmed with current IF controls. Then, I analyzed the expression of γH2AX and the presence of foci of immunoreactivity in the nuclei of irradiated cells. In control and irradiated mice, I observed γH2AX labeled cells in the cerebral cortex, the hippocampus, and the SVZ. Some scattered cells were also rarely detected in other areas of the brain, but this was an occasional observation. Control mice compared to irradiated ones showed a lower number of γH2AX immunoreactive cells (Fig. 8A, D, G). In the SVZ positive nuclei were quite diffusely distributed, whereas in the cerebral cortex (Fig. 6B) and hippocampus they were mainly scattered throughout the gray matter.

I then counted the volumetric density of γH2AX immunoreactive nuclei and calculated the percentage of γH2AX immunoreactive cells (about 4-8%) versus the total number of cells marked with DAPI (Fig. 9, black bar). Remarkably, in both irradiated groups, γH2AX expression was considerably higher compared to the control group and most nuclei showed a foci distribution (Fig. 8 B, C, E, F, H, I).

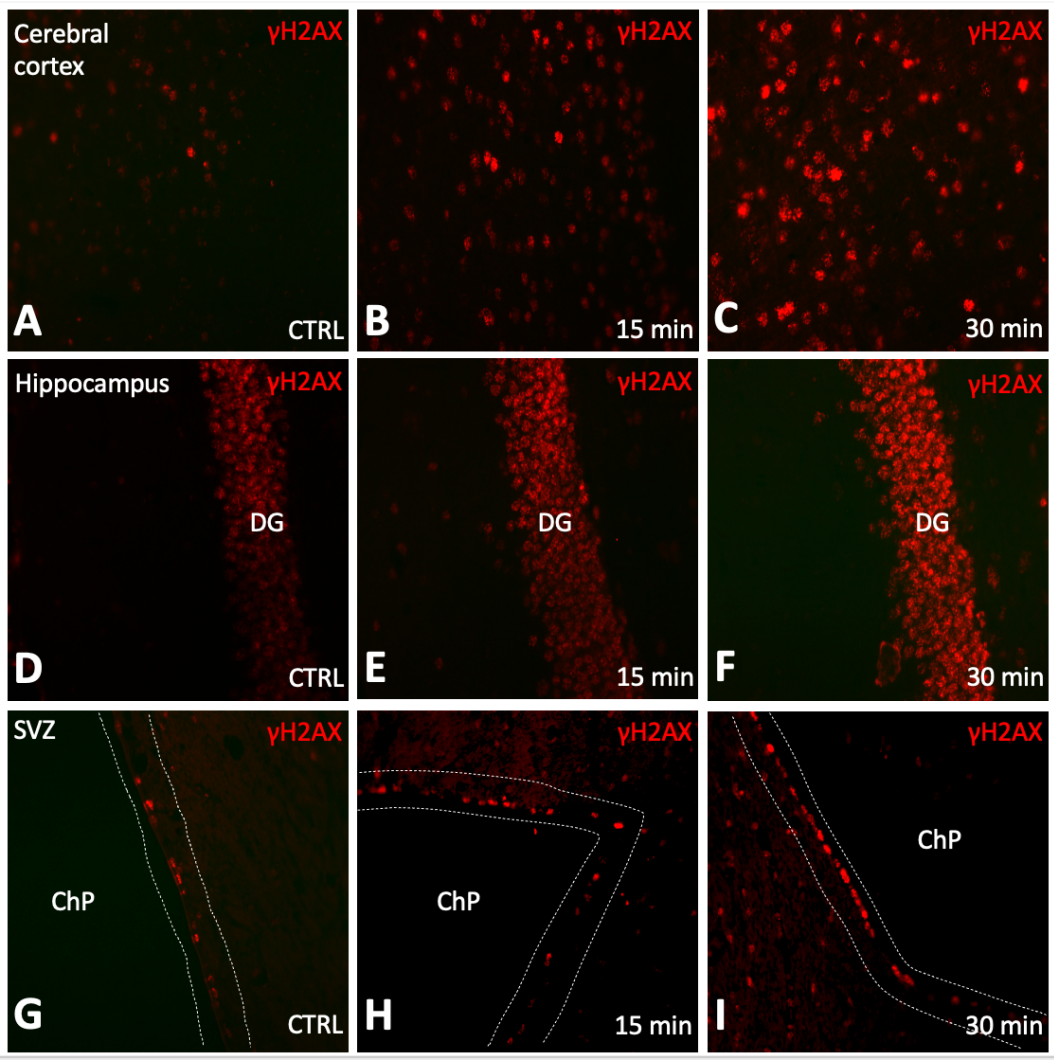


Figure 8. Exemplification images of γH2AX immunoreactive cells in control and irradiated animals. (**A-C**) γH2AX immunoreactive cells in the cerebral cortex of control mice, short- and long-survival animals. (**D-F**) γH2AX immunoreactive cells in the hippocampus of control mice, 15 min and 30 min groups. (**G-I**) γH2AX immunoreactive cells in the SVZ of control mice, short- and long-survival animals. Abbreviations: ChP = Choroid plexus of lateral ventricle; CTRL = control; DG = dentate gyrus of the hippocampus. SVZ: subventricular zone of the lateral ventricle. Original magnification 40X.

Quantitative analysis showed that, following X-ray irradiation, there was a significant increase in the volumetric density and the percentage of γH2AX positive cells, compared to controls, in the three areas of the forebrain under investigation (Fig. 9). However, this expression somewhat varied between areas. The increase of γH2AX immunoreactive cells was statistically different between the two groups of irradiated mice only in the SVZ (about 31-38%), whereas there was no statistical difference in the cortex and hippocampus.

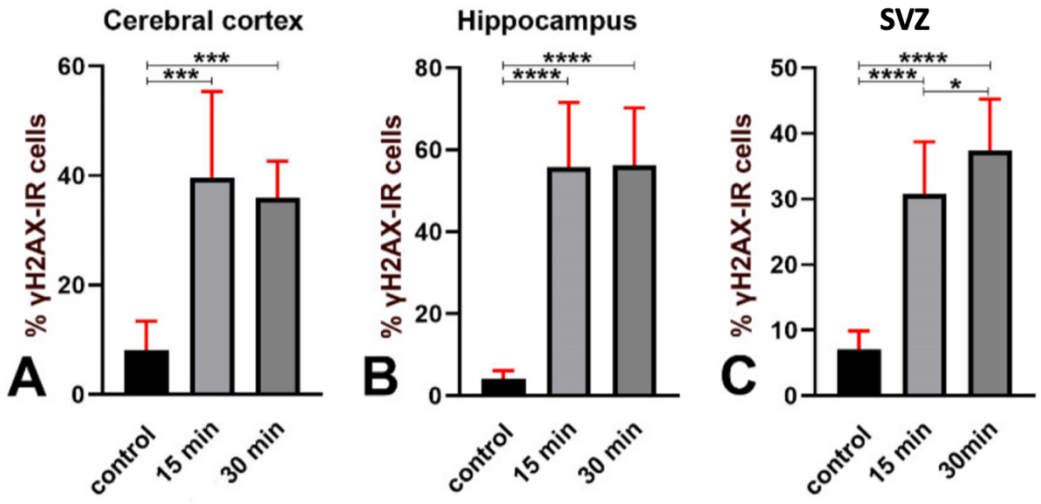


Figure 9. Quantitative analysis of γH2AX immunoreactive nuclei. Percentages of γH2AX immunoreactive nuclei versus the total number of DAPI-labeled nuclei in (**A**) the cerebral cortex (Kruskal-Wallis and Dunn's multiple comparisons test), (**B**) hippocampus (Ordinary one-way ANOVA followed by Tukey's multiple comparisons test), and (**C**) SVZ (Ordinary one-way ANOVA followed by Tukey's multiple comparisons test) in control and irradiated animals. * $0.05 > p \geq 0.01$, *** $0.001 > p \geq 0.0001$, **** $p < 0.0001$. T-Bars are 95% CI.

These data demonstrate that γH2AX immunoreactive cells make up a small fraction of the total cell population throughout the forebrain of old untreated animals. Moreover, they show that H2AX is massively phosphorylated after X-ray irradiation and that the percentage of γH2AX positive cells varies among the forebrain areas under study, with a maximum of 55% in the hippocampus. Furthermore, γ phosphorylation of H2AX was already visible 15 min after irradiation and the percentage of γH2AX positive nuclei remained statistically unchanged 15 min later in the cortex and hippocampus. On the other hand, in the SVZ there was a statistically significant increase in both the percentage and the volumetric density of γH2AX positive cells between 15 min and 30 min survivors.

I then calculated the number of foci per nucleus with the Foci Counter software. This analysis was performed only on the two groups of irradiated mice. γH2AX immunoreactive cells of control animals were not taken under consideration because, even though there were nuclear foci, their number and brightness were below the threshold to be counted by the software.

The number of foci/nucleus was statistically different between the short and long survival groups not only when all forebrain was examined (Fig. 10A), but also among the three areas taken under consideration (Fig. 10D-F). Moreover, a statistical difference was also observed among forebrain areas in the two groups of irradiated mice (Fig. 10B, C).

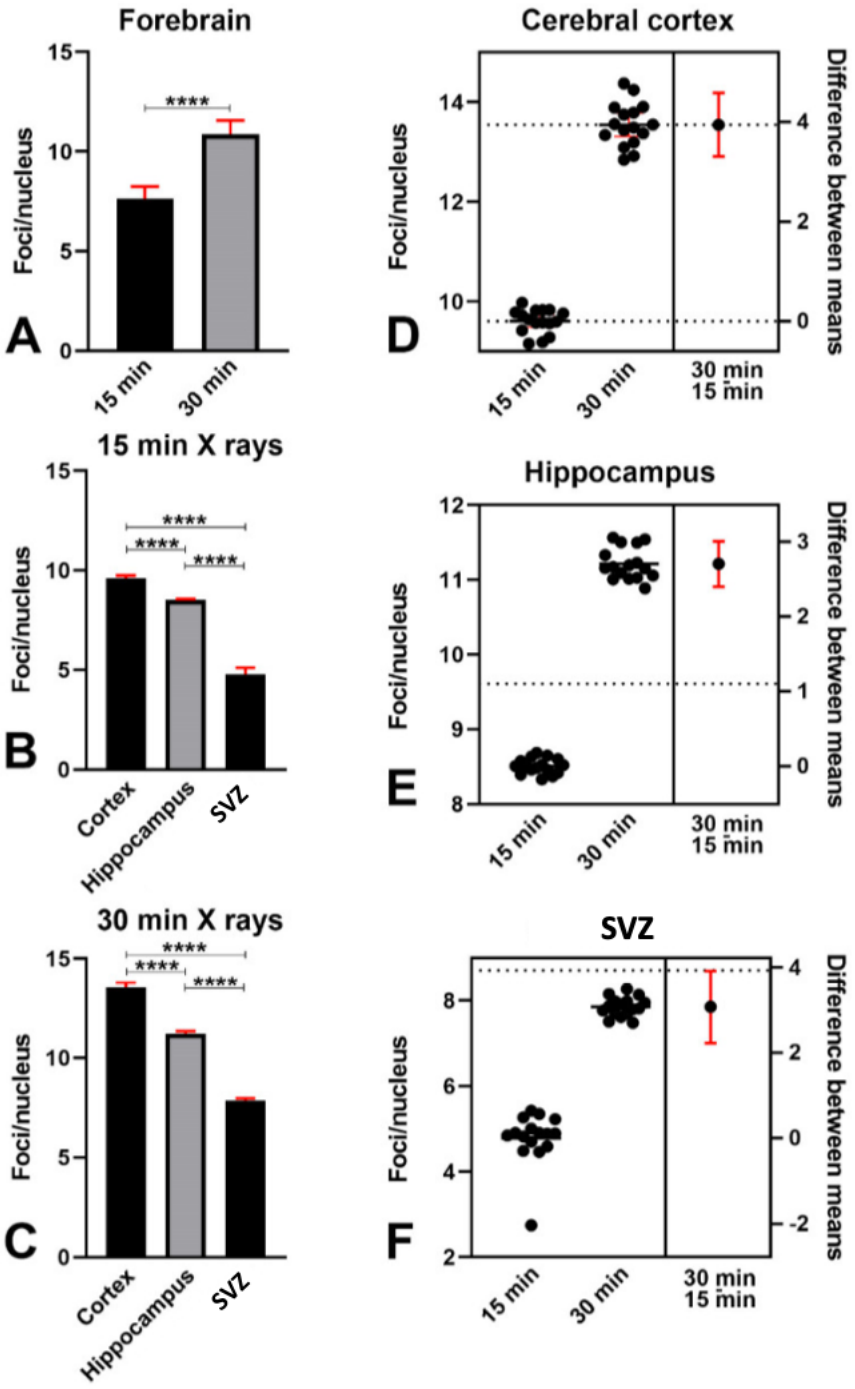


Figure 10. Analysis of γH2AX foci. Number of γH2AX immunoreactive foci per nucleus in short- and long-term survival groups. (**A**) Difference in the mean number of immunoreactive foci/nucleus in the forebrain at 15 min and 30 min survival. (15 min: 7.633 ± 0.616; 30 min: 10.87 ± 0.69; Mann–Whitney test, two-tailed exact p-value < 0.0001). (**B**) Difference in the

mean number of foci/nucleus among forebrain areas in the 15 min survival group. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test (cerebral cortex: 9.609 ± 0.129; hippocampus: 8.510 ± 0.054; OB/SVZ/RMS 4.779 ± 0.3275 – adjusted p values for all comparisons < 0.0001). (**C**) Difference in the mean number of foci/nucleus among forebrain areas in the 30 min survival group. Ordinary one-way ANOVA followed by Tukey's multiple comparisons test (cerebral cortex: 13.55 ± 0.23; hippocampus 11.21 ± 0.12; OB/SVZ/RMS 7.852 ± 0.12; adjusted p values for all comparisons < 0.0001). (**D**) Differences in the mean number of foci/nucleus in the cerebral cortex after short- and long-term survival. Unpaired t-test with Welch's correlation; two-tailed p-value < 0.0001. (**E**) Differences in the mean number of foci/nucleus in the hippocampus after short- and long-term survival. Unpaired t-test with Welch's correlation; two-tailed p-value < 0.0001. (**F**) Differences in the mean number of foci/nucleus in the SVZ after short- and long-term survival. Unpaired t-test with Welch's correlation; two-tailed p-value < 0.0001. **** p < 0.0001. T-Bars are 95% CI.

These data confirm that γ phosphorylation of H2AX is the early response to the DNA damage induced by X-rays and that there is a relationship between the time of survival and the appearance of γH2AX immunoreactive nuclear foci. Foci are quantitatively related to the degree of DNA damage (Rogakou *et al.*, 1999; Fernandez-Capetillo *et al.*, 2004) and to the time required for the cell to activate the DDR mechanisms (Adams *et al.*, 2010). From the results of these experiments, I could conclude that γ phosphorylation of H2AX in response to irradiation increases over time and that the three brain areas under study have different levels of vulnerability to irradiation. As these results were somewhat predictable based on the existing literature, the present observations confirm the usefulness of γH2AX as a marker of DNA damage in the intact brain of control mice.

4.1.2 EXPRESSION OF 53BP1 PARALLELS THAT OF γH2AX REINFORCING THE NOTION THAT A TRUE DDR OCCURS IN γH2AX IMMUNOREACTIVE CELLS

To further confirm that the γH2AX response to X-rays was indeed due to a DDR, I have performed tumor suppressor P53-binding protein 1 (53BP1) double-immunostaining with γH2AX. 53BPI is one of the main mediators of DDR that plays a pivotal role in orchestrating the choice of the DSB repair pathway (Mirza-Aghazadeh-Attari *et al.*, 2019). Therefore, the occurrence of double-labeled cells would be confirmative of the activation of DDR.

I observed that irradiation not only induced an increase in γ phosphorylation of H2AX (as demonstrated in the previous section) but also a strong rise in the number of 53BP1 positive cells (Fig. 11). I also noticed that most 53BP1 immunoreactive cells were also positive for γH2AX and that both labels mainly occurred in foci (Fig. 11B, C). Remarkably, γH2AX+53BPI colocalization was also well detectable in the forebrain of control mice, as well as in its three areas under investigation here (Fig. 11A and black bars in Fig. 11D-G).

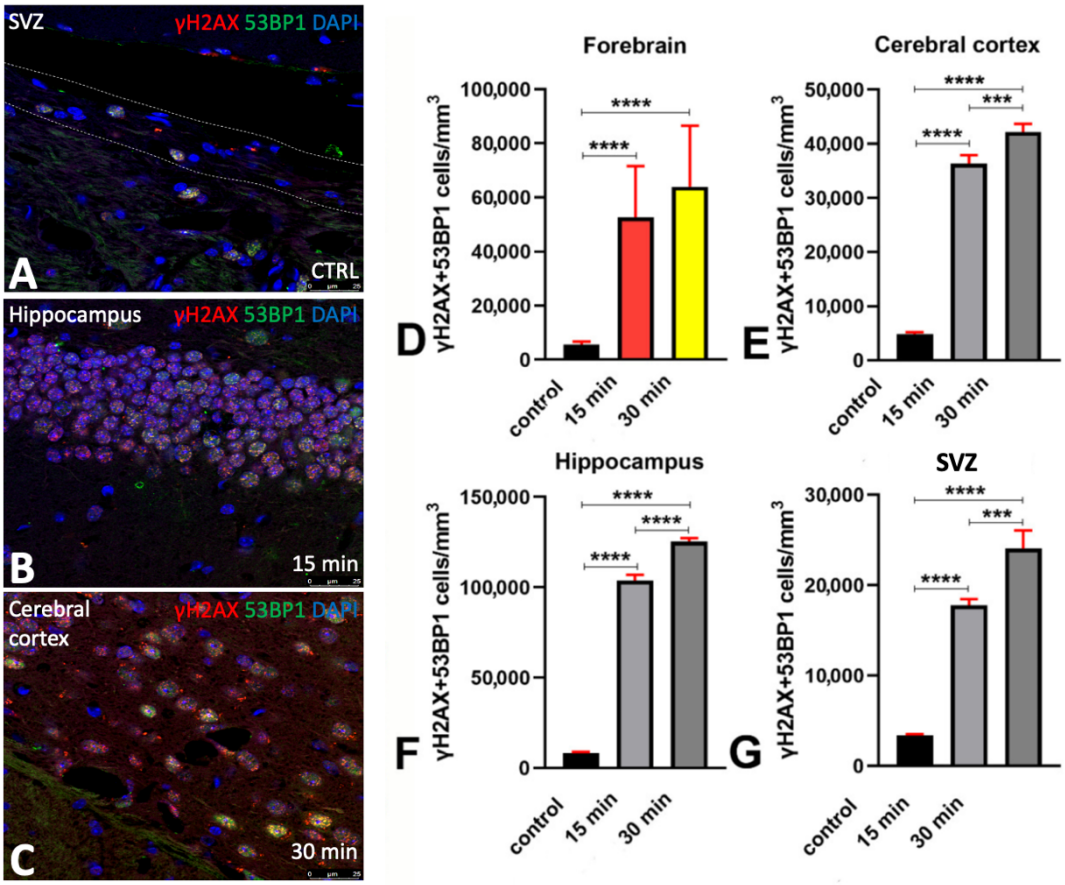


Figure 11. γH2AX + 53BP1 double-labeling cells in the forebrain of control and irradiated animals. (**A**) Exemplification image of the pattern of immunostaining in the SVZ of a control mouse. Note that in double-labeled cells the immunoreactive foci are either singularly labeled for γH2AX (red) or 53BP1 (green), or double-labeled (yellow). (**B**) Exemplification image of the pattern of immunostaining in the hippocampus of a short-term survival mouse. Note that some of the immunoreactive cells, which have increased compared to control animals, show distinct foci of γH2AX (red) or 53BP1 (green). (**C**) Exemplification image of the pattern of immunostaining in the cerebral cortex of a long-term survival mouse. Note that most cells are double-labeled and display numerous foci of colocalization (yellow). (**D-G**): quantitative analysis of the volumetric density of double-labeled cells, in the whole forebrain (Ordinary one-way ANOVA followed by Tukey's multiple comparisons test), cerebral cortex (Welch's ANOVA and Dunnett's T3 multiple comparison tests), hippocampus (Welch's ANOVA and Dunnett's T3 multiple comparison tests), and SVZ (Welch's ANOVA and Dunnett's T3 multiple comparison tests). Data are expressed as mean ± 95% CI. *** 0.001 > p ≥ 0.0001, **** p < 0.0001. T-Bars are 95% CI.

Therefore, these data demonstrated that γ phosphorylation of H2AX occurs together with a strong expression of 53BP1, therefore proving definitive evidence that expression of γH2AX is a reliable indication of the activation of the cell DDR in both control and X-ray-irradiated mice.

4.1.3 OCCURRENCE OF cCASP3 LABELED CELLS IN THE FOREBRAIN OF THE AGING MICE AND ITS INCREASE AFTER X-RAY IRRADIATION

Apoptotic cell death is an important homeostatic phenomenon during development but it occurs throughout life and it is still unclear whether it may be important in the reduction of the number of cells in the aging brain (Ryu *et al.*, 2016). In addition, irradiation may lead to cell death and DNA fragmentation, i.e. to an apoptotic form of cell demise. Before analyzing the occurrence and distribution of cleaved (active) caspase 3 (cCASP3), I wanted to establish if irradiation induced a measurable cellular death under the experimental conditions tested here. To do so, after staining with DAPI, I measured the volumetric density of the cells in the three experimental groups of mice under investigation. Remarkably, the Kruskal–Wallis test, followed by Dunn's multiple comparison test, demonstrated that differences between groups were not statistically significant (Fig. 12).

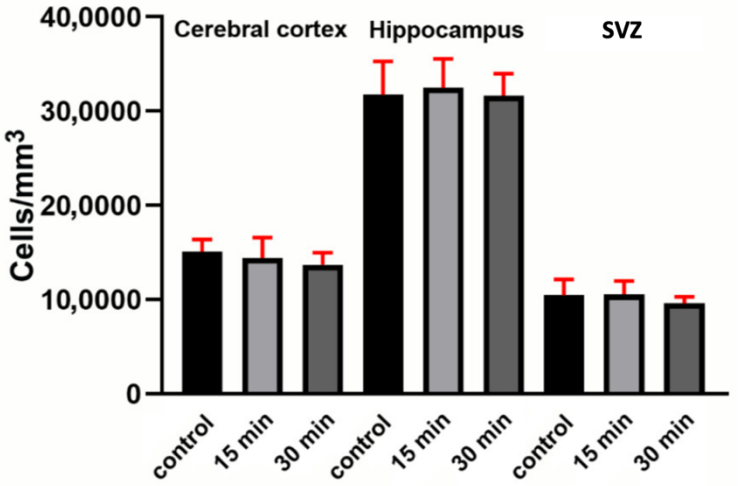


Figure 12. Determination of cell density in forebrain areas. Volumetric cellular density in the cerebral cortex, hippocampus, and SVZ after nuclear labeling with DAPI. Data are expressed as mean ± 95% CI. Bars are 95% CI.

These results show that, at the dosage employed and in the temporal window of this study, irradiation did not provoke a measurable reduction in the number of cells/volume in the three forebrain areas investigated, even though they indicate a slight tendency toward reduction in the cerebral cortex.

I then analyzed the distribution of cCASP3 immunoreactive cells. Microscopic observation showed that cCASP3-positive cells displayed either the classic form of apoptotic bodies or that of intact nuclei with a foci distribution of the fluorescent signal (Fig 12 A-C). Remarkably, immunopositive cells were detected not only after X-ray irradiation but also in control mice. Statistical analysis revealed an increase in the number of cCASP3 immunoreactive cells starting from 15 min after irradiation in the cerebral cortex and the SVZ, which further increased after 30 min (Fig. 13). On the other hand, this increment was only visible after 30 min in the hippocampus.

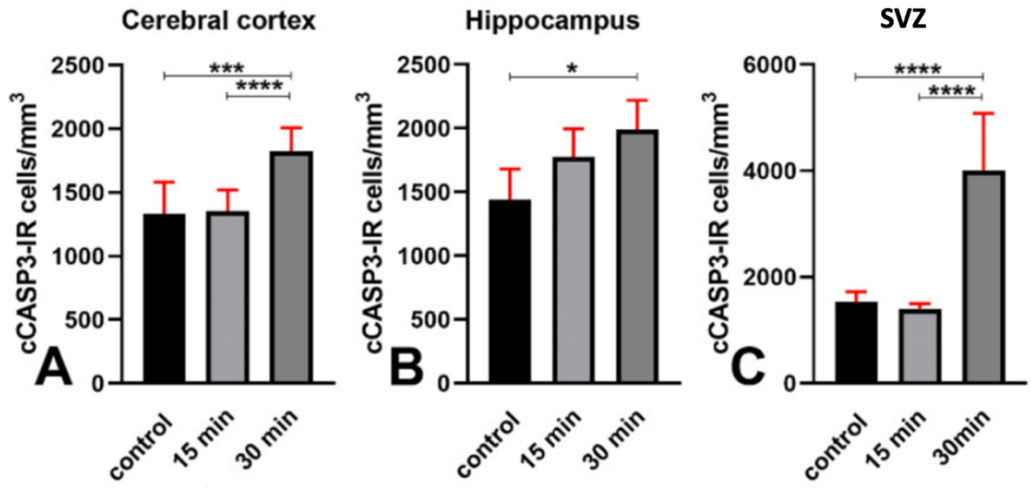


Figure 13. cCASP3 labeled cells in the forebrain of control and irradiated animals. Volumetric density of cCASP3 immunoreactive cells in (**A**) the cerebral cortex, (**B**) the hippocampus, and (**C**) SVZ of control and irradiated mice. Kruskal-Wallis and Dunn's multiple comparisons test. Data are expressed as mean ± 95% CI. * $0.05 > p \geq 0.01$, *** $0.001 > p \geq 0.0001$, **** $p < 0.0001$. Bars are 95% CI.

Therefore, I concluded that the number of cCASP3 immunoreactive cells/volume is not changed after 15 min irradiation in all the three areas of the forebrain under study, but rises over time in the following 15 minutes and that the hippocampus

exhibits different behavior in the apoptotic response to irradiation compared to the cerebral cortex and SVZ.

4.1.4 CO-EXPRESSION OF γH2AX AND cCASP3 IN CONTROL MICE AND AFTER X-RAY IRRADIATION

The microscopic observation of double staining reactions for γH2AX and cCASP3 suggested that the number of double-labeled cells, which were already clearly detectable in control mice, was augmented after X-rays (Fig. 14 A-C).

To confirm this observation, I calculated the volumetric density (Fig. 14 D-F) and the percentage of double-positive cells (cCASP3+ γH2AX double-labeled cells / cCASP3 single-labeled cells) in the three brain areas under study.

In control mice, these percentages ranged from about 43% in the SVZ to about 69% in the cortex and hippocampus and were not statistically different between the three areas.

From this data, it emerges that, under physiological conditions, there is a small number of cCASP3+γH2AX double-positive cells in the forebrain, representing only a fraction of the total number of cCASP3 labeled cells.

I have also observed that in the short survival group only the SVZ showed an increase in the volumetric density of double-labeled cells of 1.9 folds, while there was no statistical difference in the cerebral cortex and the hippocampus (Fig. 14 D-F).

Conversely, in the long survival group, all areas of the forebrain displayed an increment in the volumetric density of double-labeled cells (cortex: 2.2 times; hippocampus: 2.1 times; SVZ: 3.6 times) (Fig. 14 D-F).

We concluded that the volumetric density of cCASP3+γH2AX double-positive cells increases after irradiation, demonstrating that X-rays not only induce a strong rise in the number of γH2AX immunoreactive cells but also an apoptotic response, that is also visible in the SVZ compared to the other areas of the forebrain.

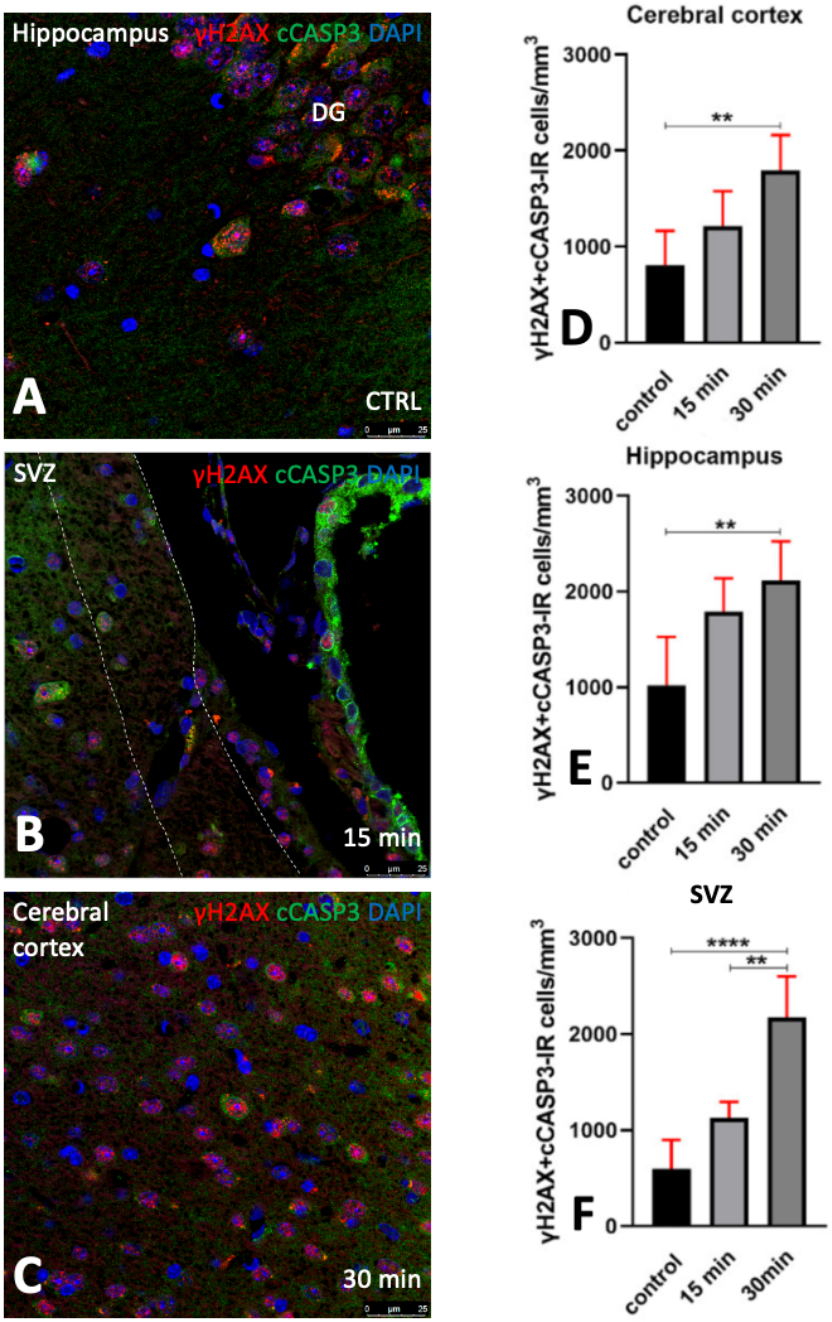


Figure 14. γH2AX + cCASP3 labeled cells in the forebrain of control and irradiated animals. (**A**) Exemplification image of the pattern of immunostaining in the hippocampus of a control mouse, (**B**) in the SVZ of a short-term survival mouse, and (**C**) in the cerebral cortex of a long-term survival mouse. Note the pattern of immunostaining in foci for γH2AX (red) and with a condensed apoptotic morphology for cCASP3 (green). (**D-F**): volumetric density of γH2AX+cCASP3 immunoreactive cells in the cerebral cortex, hippocampus, and SVZ. Kruskal-Wallis and Dunn's multiple comparisons test. Data are expressed as mean ± 95% CI. ** 0.01 > p > 0.001, **** p < 0.0001. T-Bars are 95% CI.

I then tested whether double-labeled cells could be caused by chance, in the case of independent expression of γH2AX and cCASP3. To do so, I calculated the predicted percentage of co-labeling and compared it with our observed

percentages (Table 1). I discovered that the observed percentages were much higher than the predicted random percentages of co-labeling in any of the experimental conditions tested, meaning that the colocalization of the two markers did not happen by chance.

Forebrain Areas—Animal Groups	% γH2AX	‰ cCASP3	PCP (%)	OCP (%)
Cerebral cortex-Control	8.055	6.296	5.0714	68.75
Cerebral cortex-15 min survival	39.65	10.66	42.267	85.9
Cerebral cortex-30 min survival	35.97	14.55	52.336	89.17
Hippocampus-Control	4.106	4.394	1.804	68.75
Hippocampus-15 min survival	55.78	6.517	36.352	89.06
Hippocampus-30 min survival	56.22	7.079	39.798	95.31
SVZ/RMS/OB—Control	7.036	10.48	7.374	43.21
SVZ/RMS/OB-15 min survival	30.72	10.29	31.611	87.81
SVZ/RMS/OB-30 min survival	37.39	23,6	88.240	93.76

Table 1. Predicted co-labeling percentage (PCP) and observed co-labeling percentage (OCP) of γH2AX+cCASP3 immunoreactive nuclei in the forebrain areas of control and irradiated mice.

Altogether, it seems possible to attest that CASP3 activation and γ phosphorylation of H2AX influence each other or that they are both influenced by a common upstream process.

Histone H2B phosphorylated on Ser14 is another biochemical hallmark of apoptotic cells (Ajiro, 2000). In fact, during apoptosis, histone H2B undergoes phosphorylation at Ser14 following CASP3 activation. Immunofluorescent staining did not show any evidence of the onset of phosphorylated H2B in any of our experimental conditions (data not shown).

4.2 CALCULATION OF CELL CYCLE PARAMETERS IN UNTREATED MICE AND EFFECTS OF IRRADIATION ON BrdU INCORPORATION

4.2.1 CALCULATION OF CELL CYCLE PARAMETERS

For a correct analysis of the cell cycle parameters using one of the available double labeling methods, it is necessary to use different intervals of administration of the two tracers to (i) estimate the S-phase length, (ii) estimate the G2-phase length, and (iii) estimate the G1-phase length. It would then be finally possible to calculate the duration of the whole cycle. Alternatively, it is possible to combine the labeling with the S-phase markers with the immunocytochemical detection of specific markers of the different phases of the cell cycle.

The double labeling methods that are commonly utilized to reveal the progression of cells through the nuclear cycle are based on the use of two DNA labels delivered at different time intervals (Hayes and Nowakowski, 2000; Lossi, Mioletti and Merighi, 2002; Vega and Peterson, 2005; Encinas *et al.*, 2011) (Table 2) – for a recent review of the existing double labeling methods see also (Solius *et al.*, 2021). These methods rely on the notion that because the cells tagged with the first label exit the S-phase and unlabeled cells enter the S-phase, the proportion of cells that have incorporated both labels gradually decreases as the time between the administration of the two labels increases.

The time point at which all cells labeled with the first label leave the S-phase is shown by the declination line's intersection with the time axis – see Fig. 13 (Solius *et al.*, 2021), providing an estimate of the length of the S-phase ($\mathbf{t_S}$). If the interval between the administration of the two tracers is made longer, the proportion of the double-labeled cells starts rising as the cells that have incorporated the first label enter a subsequent S-phase of their cycle. If the interval between the administration of the tracers is further protracted, the proportion then decreases again (see Fig. 15). The duration of the cell cycle ($\mathbf{t_C}$), or the period between two successive S-phases, is the period during which the peak is attained.

S-phase markers	Calculation of t_S	Calculation of t_C	Abs	Ref
IdU CldU	$t_S = t \times \frac{IdU^+ + DLC}{IdU^+}$	$* t_C = t + (t_S \times \frac{CldU^+}{CldU^+ + DLC})$	a	(Brandt, Hübner and Storch, 2012)
IdU BrdU	$t_S = 0.5 \times \Delta t \times \frac{DLC}{IdU^+}$	See paper	b	(Burns and Kuan, 2005)
CldU IdU	$t_S = t \times \frac{IdU^+ + DLC}{IdU^+}$	See paper	a	(Encinas *et al.*, 2011)
H^3thymidine and BrdU	$t_S = t \times \frac{BrdU^+ + DLC}{Thy^+}$	See paper	N/A	(Hayes and Nowakowski, 2002)
IdU and BrdU	$t_S = t \times \frac{BrdU^+ + DLC}{IdU^+}$	$t_C = \frac{t_S}{LI} \times GF$	c	(Lossi, Mioletti and Merighi, 2002)
EdU and BrdU	$t_S = t \times \frac{DLC}{EdU^+}$	See paper	d	(Harris, Zalucki and Piper, 2018)
IdU and BrdU	$t_S = t \times \frac{DLC}{IdU^+}$	See paper	a	(Martynoga *et al.*, 2005)
IdU and BrdU	$t_S = t \times \frac{DLC}{IdU^+}$	$t_C = \frac{t_S}{LI} \times GF$	a	(Shibui *et al.*, 1989)

Table 2. Common double labeling protocols to study neuron proliferation. *Abbreviations*: BrdU, CldU, EdU, IdU (5-bromo-2'-deoxyuridine, 5-chloro-2'-deoxyuridine, 5-ethynyl-2'-deoxyuridine, 5-iodo-2'-deoxyuridine); DLC = double-labeled cells; GF = growth fraction; LI = labeling index (% of S phase cells). * to calculate t_C, the interjection interval should be longer than t_S and t_C-t_S, but shorter than the expected duration of $2t_C$-t_S. a = marker antibodies specifically recognize their respective antigen; b = antibody against IdU cross-reacts with BrdU while antibody against BrdU is specific; c = antibody against IdU cross-reacts with BrdU but under the conditions use it yields IdU specific labeling - antibody against BrdU is specific; d = antibody against EdU cross-reacts with BrdU while antibody against BrdU is specific.

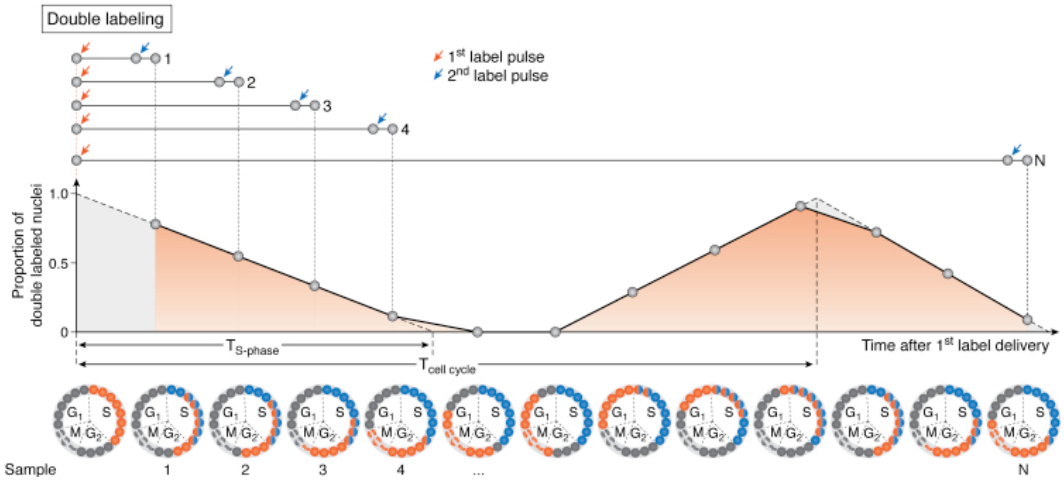


Figure 15. Schematic representation of the variation in the markers' ratio against the length of the interval between the administration of the two DNA markers (Solius *et al.*, 2021). The model conforms to that represented in Appendix 2 Fig. 25.

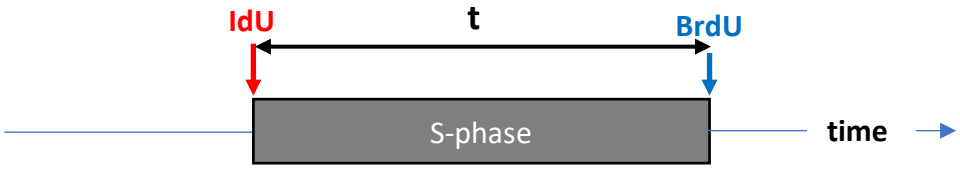


Figure 16. Schematic diagram of the relationship between the length of the S-phase of the cell cycle and the interjection interval (t) between IdU and BrdU. As one of the conditions for a correct application of double labeling formulas is that the cell population is in an asynchronous state, that is, cells in the population have equal chances of being at any point in their cycle, in the case shown the number of IdU^+ cells will be equal to half of the sum of BrdU+ cells and double-labeled cells.

Therefore it immediately appears that the choice of the time interval between the administration of the two DNA labels is crucial for the reliability of the experiments. Previous unpublished experiments carried out in this laboratory with a repeated pulse labeling procedure (Contestabile *et al.*, 2009) aimed to calculate the cell cycle parameters of the SVZ cells in the brains of mice of comparable ages to those of this study and led to the observation that these cells had an unusually long cell cycle. For this reason, I here analyzed three groups of mice with intervals between the two label pulses of 24, 72, and 120 hours. Table 3 below reports the absolute numbers of IdU^+ (1^{st} marker), $BrdU^+$ (2^{nd} marker), and $IdU^+/BrdU^+$ double-labeled cells in the hippocampal DG, SVZ, and cerebral cortex.

Tracer administration interval	Labeled cells	Hippocampus	SVZ	Cerebral Cortex
24 hours	IdU^+	4.00	11.00	13.00
	$BrdU^+$	6.00	13.00	15.00
	$IdU^+/BrdU^+$	3.00	9.00	10.00
72 hours	IdU^+	6.33	14.33	15.67
	$BrdU^+$	7.00	15.33	16.00
	$IdU^+/BrdU^+$	2.00	4.33	7.33
120 hours	IdU^+	7.25	16.00	17.25
	$BrdU^+$	7.75	15.50	17.00
	$IdU^+/BrdU^+$	1.50	3.00	4.75

Table 3. Mean absolute numbers of IdU^+, $BrdU^+$, and $IdU^+/BrdU^+$ cells in the three forebrain areas of 2-year-old mice.

As the formulas to calculate the cell cycle parameters are valid only under certain specific conditions (Wimber and Quastler, 1963) – see also Appendix 2 and Discussion – and the S-phase and cell cycle durations were not approximately known in advance, the method developed by Burns and Kuan (Burns and Kuan, 2005) was used to calculate t_S. This method is based on counting the number of IdU^+, $BrdU^+$, and double-labeled cells and plotting the ratio against the injection interval time. It is then reasoned that when the interval between the two injections equals the length of the S-phase, cells labeled by the first pulse of IdU and cells labeled by the second pulse of BrdU will be completely segregated, thereby giving a ratio of 0.5. This is because all cells labeled with IdU at beginning of the S-phase will be also labeled by BrdU given at the end of the S-phase as well as cells that were in the S-phase when BrdU is administered.

Therefore, from Table 3 data, I calculated the ratios of $\frac{IdU^+}{BrdU^+ + IdU^+/BrdU^+}$ and reported them in Table 4 below.

From these data, $\mathbf{t_S}$ was calculated directly using the following equation, modified from(Burns and Kuan, 2005), as, in these experiments, the anti-IdU and anti-BrdU antibodies specifically recognized their respective antigen, whereas in (Burns and

Kuan, 2005) the first antibody used recognized both IdU and BrdU, and the second was specific for BrdU (Table 6).

$$Ts = 0.5 \times \Delta t \times \frac{BrdU^{+} + IdU^{+}/BrdU^{+}}{IdU^{+}}$$

Or, for better precision, I calculated t_S by computing the equation of the linear regression analysis (Fig. 15 and Table 5)

Time (hours)	$\frac{IdU^{+}}{BrdU^{+} + IdU^{+}/BrdU^{+}}$		
	Hippocampus	**SVZ**	**Cerebral Cortex**
24	0.44	0.50	0.52
72	0.70	0.73	0.67
120	0.78	0.86	0.77

Table 4. Markers' ratio at different time intervals of injections in the brain areas under study.

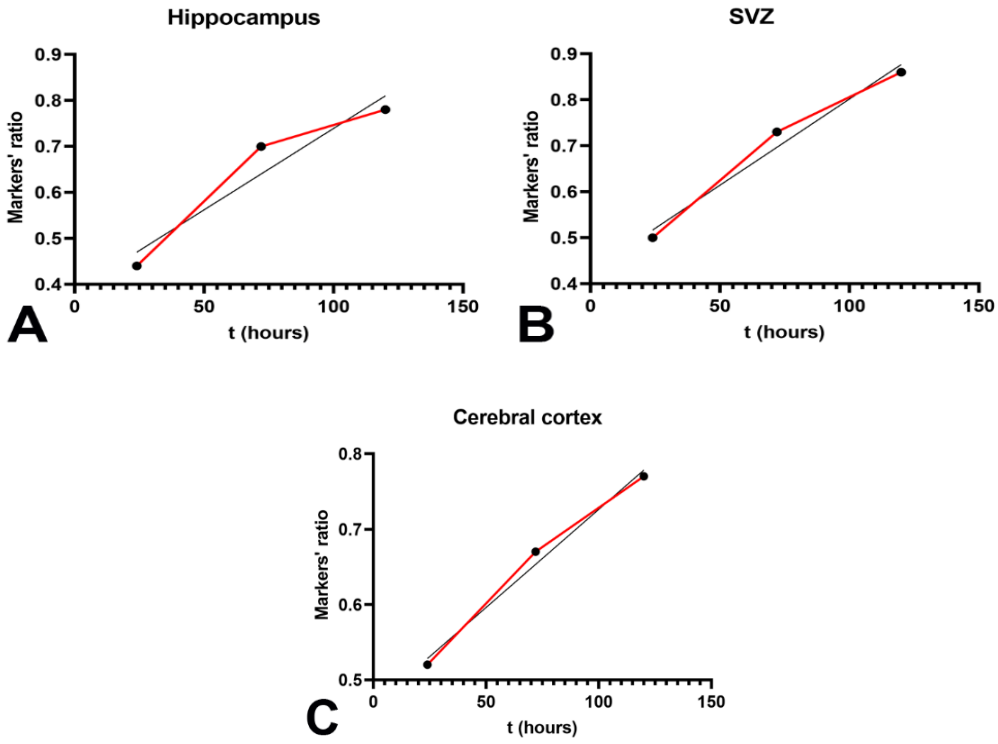


Figure 17. Linear regression analysis. Markers' ratio and linear regression in the hippocampal DG (**A**), SVZ (**B**), and cerebral cortex (**C**).

Hippocampus	SVZ	Cerebral cortex
$y = 0.003542x + 0.3850$	$y = 0.003750x + 0.4267$	$y = 0.002604 + 0.4658$

Table 5. Linear regression equations of the ratio between the IdU-only labeled cells and the total number of BrdU labeled cells (BrdU-only + double-labeled cells) in the hippocampus, SVZ, and cerebral cortex.

The results of the simple linear regression curves show that the slopes in all three areas under study are not significantly non-zero (*hippocampus*: F 10.70; DFn, DFd 1,1; P value 0.1888. *SVZ*: F 38.88; DFn, DFd 1,1; P value 0.1012. *cerebral cortex*: F 75.00; DFn, DFd 1,1; P value 0.0732). Therefore, one can assume that the three cell populations studied here do not substantially differ from each other and that the injection intervals used drop within a plateau of the curve, or, at least, the difference of the slopes in the 24-72 hour and 72-120 hour intervals is minimal.

S-phase length (t_S) (hours)	Burns and Kuan	Linear regression
Hippocampus	27	31.57
SVZ	24	19.95
Cerebral cortex	23.08	13.38

Table 6. Duration of the S-phase of the cell cycle in cells of the hippocampal DG, SVZ, and cerebral cortex was calculated according to the method of Burns and Kuan (2005) or using the linear regression equations of Table 4 above.

Considering the very small slope of the regression lines of the three cell populations, it was reasonable that **t_C was remarkably long**, with values of 174.2 (hippocampus), 252 (SVZ), and 205.69 (cerebral cortex).

Therefore, based on the theory reported in Appendix 2, I used **Eq. 2** (Wimber and Quastler, 1963) to calculate **t_{G2}** at the interjection interval **t** of 72 hours (Table 7). To do so, for reliability, the value of t_S obtained after linear regression analysis was used and it was assumed that λ equals 1 or 0.75, 0.5, and 0.25 (25%, 50%, or 75% of newly generated cells undergoing death – see Discussion).

G2-phase length (t_{G2}) - hours	**Hippocampus**	**SVZ**	**Cerebral cortex**
λ = 1	114.43	126.05	132.62
λ = 0.75	127.91	139.32	141.74
λ = 0.5	174.86	198.10	211.24
λ = 0.25	235.72	269.97	277.21

Table 7. Duration of the G2-phase of the cell cycle in cells of the hippocampal DG, SVZ, and cerebral cortex.

In the three populations $t_S<t_C/2$ and $t_{G2}>t_S$. Therefore, they fit several cases represented in Appendix 2 Fig. 26. The figure below (Fig. 18) reports the indication of the intervals between the tracer injections used in the present experiments and shows the curves that are compatible with the results of the experiments of this thesis.

However, if one inspects Fig. 18 carefully, it immediately appears that the graph represented in B cannot be taken into consideration, as the corresponding circular diagram shows that there are no double-labeled cells in this type of population for the interinjection intervals used.
Also, one has to consider that in graph C there is a slope that can be calculated according to the following equation (Wimber and Quastler, 1963):

$$Markers'ratio = 1 \textbf{ (Eq. 6)}$$

Eq. 6 reaches a minimum when $\mathbf{t_C} = \mathbf{T_S}$ and a maximum when $\mathbf{t} = \mathbf{t_{G2}} - \mathbf{t_X}$ (Wimber and Quastler, 1963).

I have then calculated the theoretical values of **t** from the linear regression equations for a markers' ratio 1 when $t = t_{G2} - t_X$ (the survival time after the injection of the 2nd marker, i.e. 2 hours) and reported them in Table 8 considering different yield of cells (λ) after mitosis.

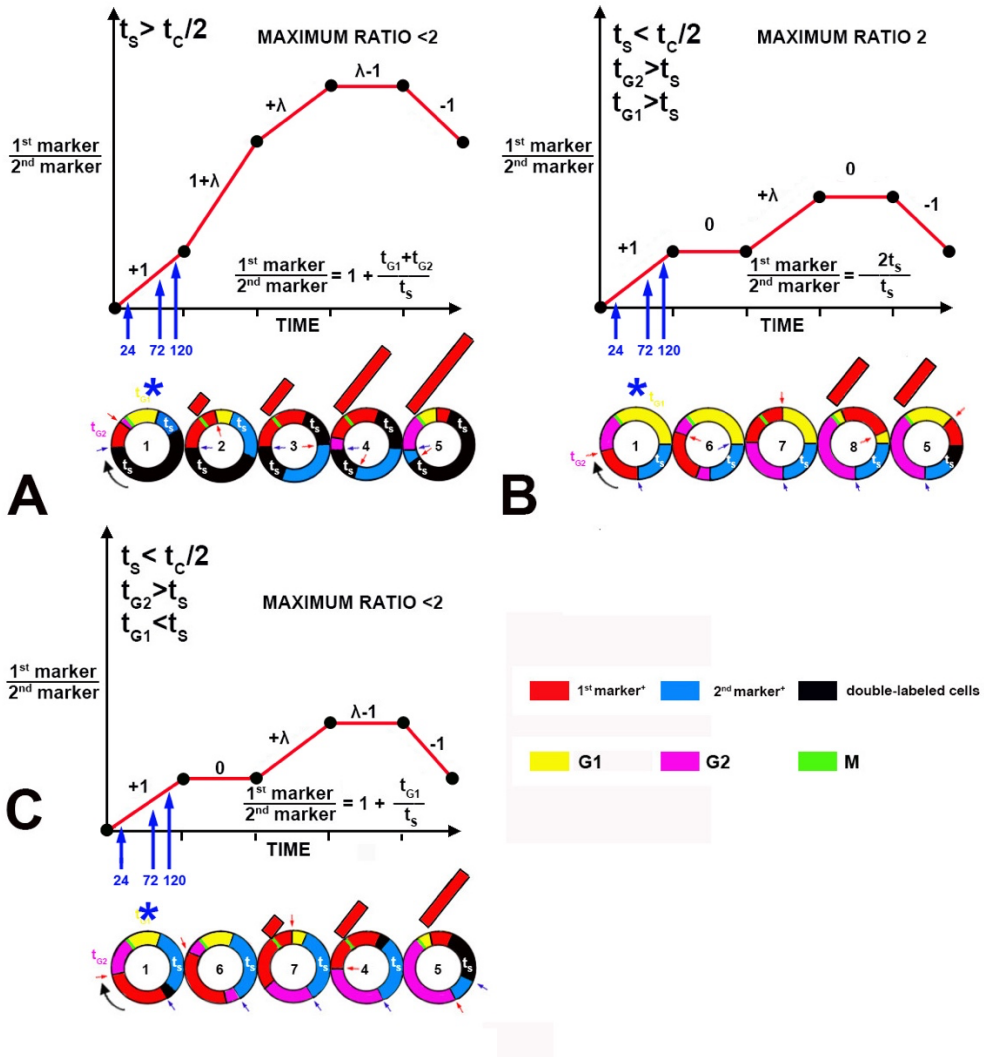


Figure 18. Quantitative analysis of cell cycle parameters in double labeling experiments. Dispersion graphs showing the slopes of the curves representing the variations of the markers' ratio with time and circular diagrams illustrating the double labeling sequences of the three different steady-state populations that better represent the experimental data. The asterisks indicate the circular diagrams corresponding to the interjection intervals (blu arrows).

$\frac{1^{st}marker}{2^{nd}\ marker} = 1$	t (hours)		
	Hippocampus	**SVZ**	**Cerebral cortex**
regression line	205.70	174.43	151.52
Eq. 6 (λ = 1)	114.43	126.05	132.62
Eq. 6 (λ = 0.75)	127.91	139.32	141.74
Eq. 6 (λ = 0.5)	174.86	198.10	211.24
Eq. 6 (λ = 0.25)	235.72	269.97	277.21

Table 8. Theoretical interinjection intervals (t) that are necessary to obtain a markers' ratio = 1 (curve plateau) when mitosis gives rise to one additional cell (λ = 1 absence of cell death) or when cell death reduces the yield of new cells.

Finally, given the very slow slope of the curves for all the three cell populations examined, it seems that at the different values of **t** in the experiments carried out in this thesis, curves are still rising and thus it is not possible to calculate $\mathbf{t_{G1}}$ using Eq. 3 (graph in A) or 5 (graph in C).

4.2.2 INCREASE IN CELLULAR BRDU INCORPORATION AFTER X-RAY IRRADIATION

I have investigated the effects of X-ray irradiation on BrdU incorporation into the forebrain, as a previous study from this laboratory indicated that in the brain of 24-month-old mice there were cells that could be labeled with anti-BrdU antibodies after administration of the thymidine analog and different patterns of survival (Barral *et al.*, 2014). In all mouse groups studied here, I observed that BrdU-positive nuclei were present in all the forebrain areas under investigation (Fig. 19).

Moreover, at microscopic observation, I noticed that BrdU-immunoreactive cells showed a punctate reaction pattern, with clear foci of positivity. At the light microscope, control animals showed quite a few BrdU immunoreactive cells, even though their number was lower than in the irradiated mice.

I then calculated the volumetric density of BrdU-positive cells and observed that the highest number was in the SVZ, followed by the hippocampus and, lastly, the cerebral cortex (Fig. 20). In addition, both the short and long-survival groups showed an increase in the number of BrdU immunoreactive cells compared to control animals.

Quantitative analysis (Fig. 20) showed that, following X-ray irradiation, there was a statistically significant increase in the volumetric density of positive cells.

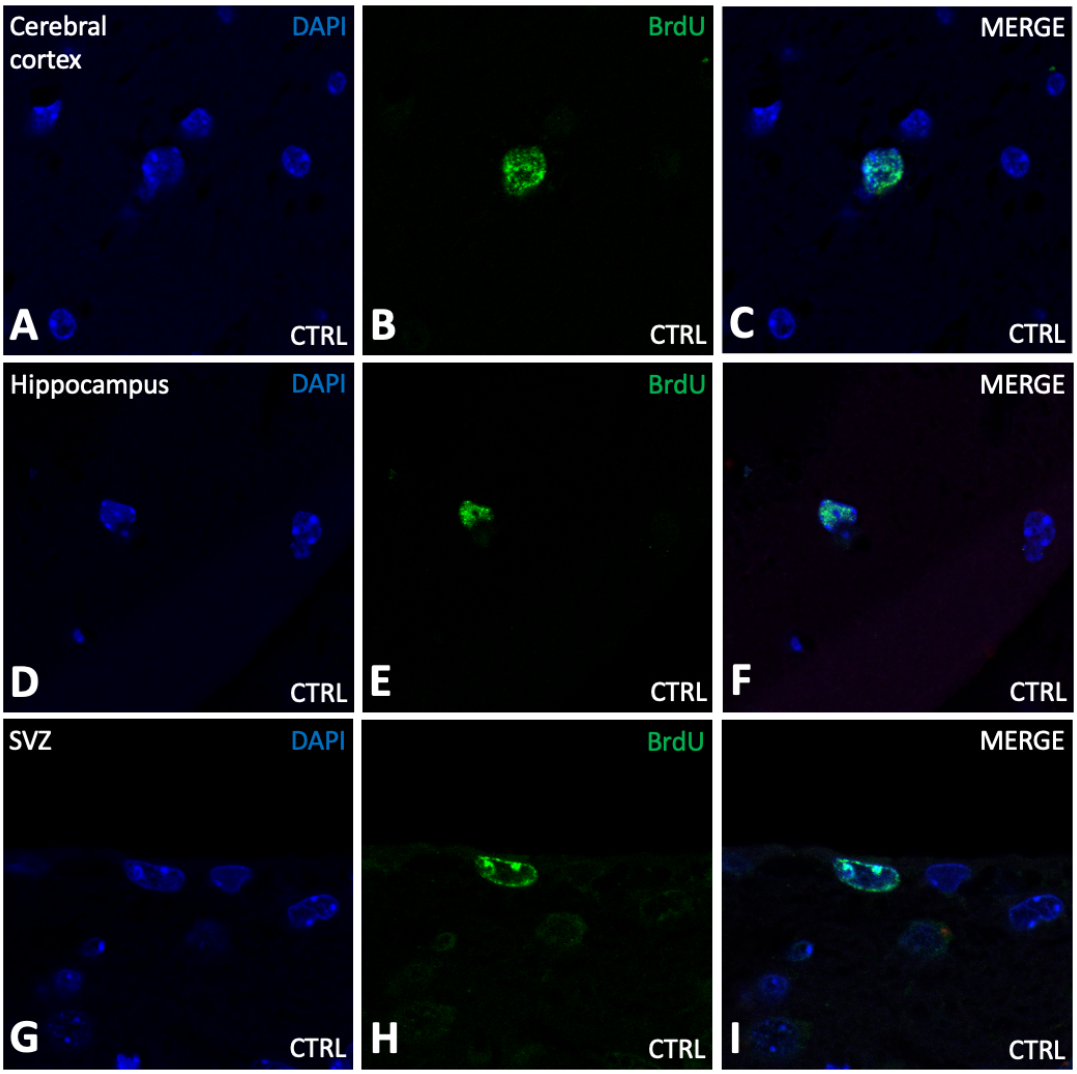


Figure 19. Exemplification images of BrdU labeling cells. BrdU immunoreactive cells in the cerebral cortex (**A**), hippocampus (**B**), and SVZ (**C**) of control animals.

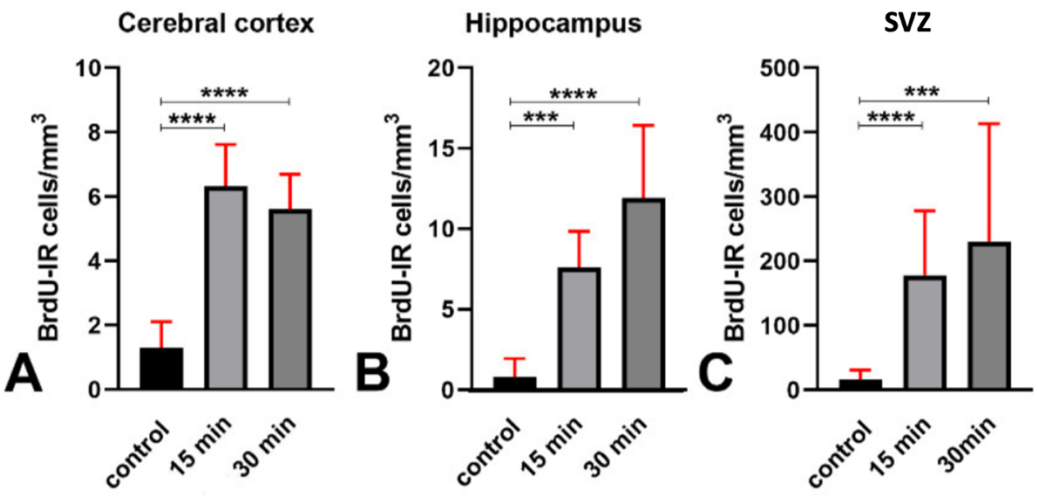


Figure 20. BrdU labeling cells in the forebrain of control and irradiated animals. Quantitative analysis of the volumetric density of BrdU immunoreactive cells in the cerebral cortex (**A-C**),

hippocampus (**D-F**), and SVZ (**G-I**). Kruskal-Wallis and Dunn's multiple comparisons test. Data are expressed as mean ± 95% CI. *** 0.001 > p ≥ 0.0001, **** p < 0.0001. T-Bars are 95% CI.

Nonetheless, the increment differs between the three areas of the brain under study (Fig. 20). In fact, irradiation led to a 5-fold rise in the cerebral cortex, while the increase was 14-fold in the SVZ and 15-fold in the hippocampus.

Altogether, these observations converged to demonstrate that X-ray irradiation leads to a statistically significant increase in the volumetric density of BrdU immunoreactive cells, which was already evident after 15 min survival.

4.2.3 BrdU INCORPORATION AND PHOSPHORYLATION OF HISTONE H3 OCCUR IN INDIVIDUAL CELLS AND INCREASE CONCURRENTLY AFTER X-RAY IRRADIATION

To obtain more information on the relationship between BrdU incorporation and mitosis, I then analyzed the colocalization of BrdU and pHH3 at the single-cell level.

From microscopic observation, I noticed that pHH3 immunoreactivity displayed a pattern in foci, which is typical of cells in the G2 or G2/M phases of their cycle (Fig. 21A-C) (Hendzel *et al.*, 1997). It is known from the literature that in proliferating cells pHH3-immunoreactivity also occurs in the M phase, intensely staining mitotic chromosomes and such a pattern of staining has also been previously described in the mouse embryonic and postnatal brain when the occurrence of phosphorylated H2AX was investigated (Barral *et al.*, 2014). However, I never observed pHH3-immunoreactivity suggestive of staining of mitotic chromosomes.

Statistical analysis of the volumetric density of BrdU+pHH3 immunoreactive cells showed an increase in the number of double-positive cells in the cerebral cortex and the SVZ, after X-ray exposure (Fig. 21E, G). On the other hand, the hippocampus did not exhibit a statistical increment in the number of BrdU+pHH3 double-labeled cells/mm^3 at the two time points under study (Fig. 21F).

Taking the whole forebrain under consideration, it is noteworthy that, in control mice, BrdU+pHH3 immunoreactive cells represented 5.55% of the total number of

single-labeled BrdU positive cells, while this percentage increased drastically in the irradiated mice (specifically, 24.41% in the short survival group, and 25.64% in the long survival group). Therefore, one can substantiate that, at least a subpopulation of the cells synthesizing new DNA (i.e. BrdU+) is likely to progress to mitosis (i.e. they display the pHH3 focal pattern of G2 or G2/M cells).

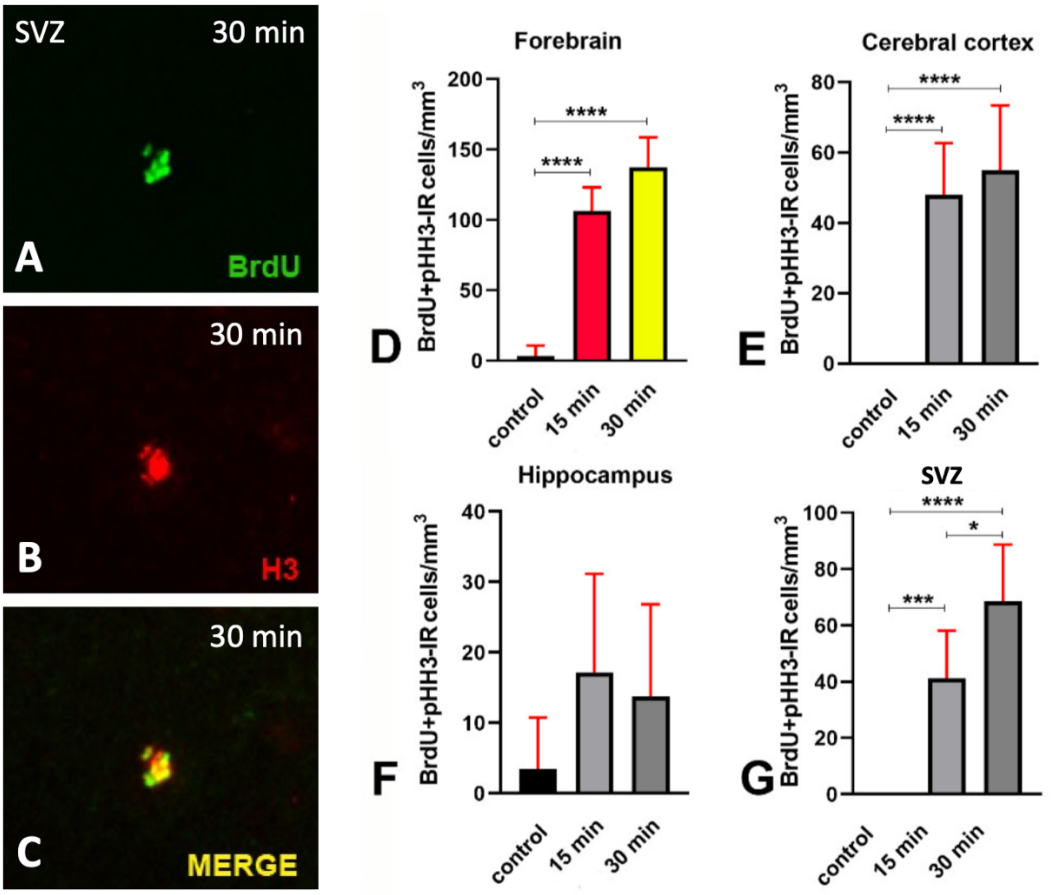


Figure 21. BrdU + pHH3 double-labeling cells in the forebrain of control and irradiated animals. (**A-C**) Exemplification images of the pattern of immunostaining in the SVZ of a 30 min survival animal. (**D-G**) Quantitative analysis of the volumetric density of double-labeled cells in the whole forebrain (Welch's ANOVA and Dunnett's T3 multiple comparison tests), cerebral cortex (Ordinary one-way ANOVA followed by Tukey's multiple comparisons test), hippocampus (Welch's ANOVA and Dunnett's T3 multiple comparison tests), and SVZ (Ordinary one-way ANOVA followed by Tukey's multiple comparisons test). Data are expressed as mean ± 95% CI. * 0.05 > p ≥ 0.01, *** 0.001 > p ≥ 0.0001, **** p < 0.0001. T-Bars are 95% CI.

4.2.4 COLOCALIZATION OF BrdU AND γH2AX IN BASAL CONDITIONS AND IN RESPONSE TO X-RAY IRRADIATION

It is widely accepted that cells with damaged DNA may attempt to de novo synthesize their genetic material during the DDR (Tripathi *et al.*, 2015). Hence they

may incorporate BrdU in their nuclei if the thymidine analog is available during their response to DNA damage.

To prove or disprove this hypothesis, I analyzed the colocalization of BrdU and γH2AX in control and X-ray irradiated mice (Fig. 22A-C).

The microscopic observations of double-labeled preparations showed the occurrence of low numbers of BrdU/γH2AX immunoreactive cells in control mice. They were scattered in the gray matter of the cerebral cortex, the DG, and the SVZ and dispersed among the much higher number of γH2AX single-labeled cells. Remarkably, double-labeled cells displayed a typical pattern of immunoreactivity in foci (Fig. 22A-C) Quantitatively they were very few in control mice, and statistical analysis of their volumetric density demonstrated that, in parallel with the increment of BrdU immunoreactive cells shown in section 5.1, irradiation with X-rays induced a significant rise in the number of these cells (Fig. 22D-G).

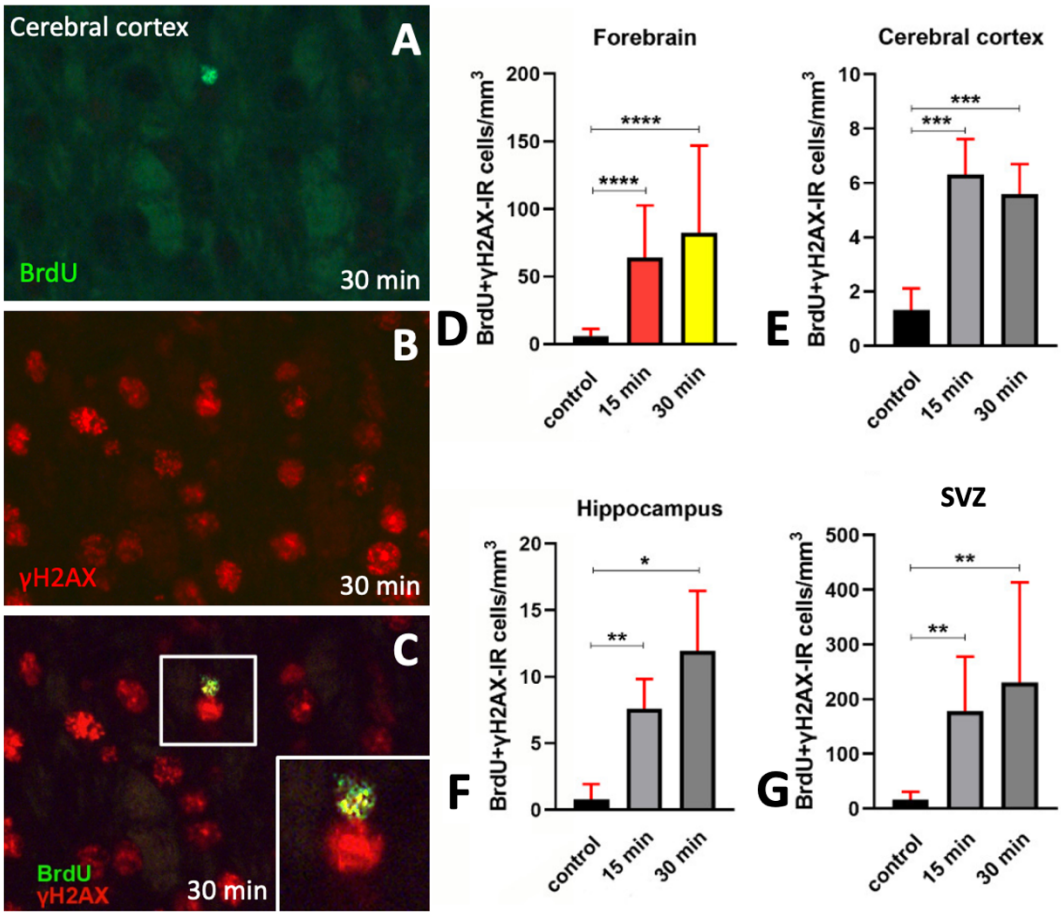


Figure 22. BrdU + γH2AX double-labeling cells in the forebrain of control and irradiated animals. (**A-C**) Exemplification images of the pattern of immunostaining in the cerebral cortex of a long-term survival animal. (**D-G**) Quantitative analysis of the volumetric density of double-labeled cells in the whole forebrain, cerebral cortex, hippocampus, and SVZ. Kruskal-Wallis

and Dunn's multiple comparisons test. Data are expressed as mean ± 95% CI. * 0.05 > p ≥ 0.01, ** 0.01 > p ≥ 0.001, *** 0.001 > p ≥ 0.0001, **** p < 0.0001. T-Bars are 95% CI.

In control animals, I calculated the volumetric density of BrdU+γH2AX positive cells singularly in each of the three forebrain areas and observed that the highest number was in the SVZ, followed by the cerebral cortex and the hippocampus. On the other hand, in both the short and long-survival groups, irradiation led to a 5-fold rise in the cerebral cortex, 8-fold in the SVZ, and 12-fold in the hippocampus. Altogether, the data obtained from this set of experiments showed that the increase of BrdU incorporation was paralleled by an increment in the γ phosphorylation of H2AX in response to X-ray irradiation. At the same time, however, double-labeled cells were spotted also in control animals, demonstrating that expression of γH2AX occurred in at least a subpopulation of the cells that incorporated the BrdU administered two hours before sacrifice.

4.2.5 AN INCREASE OF 53BP1 LABELED CELLS ACCOMPANIES BrdU INCORPORATION AFTER X-RAY IRRADIATION

To confirm that, during the DDR, cells with damaged DNA were incorporating BrdU in an attempt to synthesize new genetic material and repair the damage, I investigated the colocalization between BrdU and 53BP1, which, as mentioned previously, is involved in the early DNA damage-signaling pathway (Mirza-Aghazadeh-Attari *et al.*, 2019).

By direct microscopic observation of BrdU+53BP1 double-labeled preparations (Fig. 23A-C), I noticed that irradiation, in parallel with the above-described increment in BrdU uptake, also induced a very strong rise in the number of 53BP1 immunoreactive cells, with the appearance of double-labeled cells as early as 15 min after X-rays.

I then analyzed these preparations quantitatively and confirmed the results of the microscopic data.

In particular, statistical analysis of the volumetric density of BrdU+53BP1 immunoreactive cells showed an increase in the number of double-positive cells

in the cerebral cortex and the SVZ 15 min after X-ray exposure (Fig. 23E, G). On the other hand, the hippocampus did not exhibit a statistical increment in the number of BrdU+53BP1 double-labeled cells/mm3 at the two time points under study (Fig. 23F).

These observations, as well as the increase in the colocalization between BrdU and γH2AX, confirm that the DDR triggered by irradiation is accompanied by an attempt to synthesize new DNA.

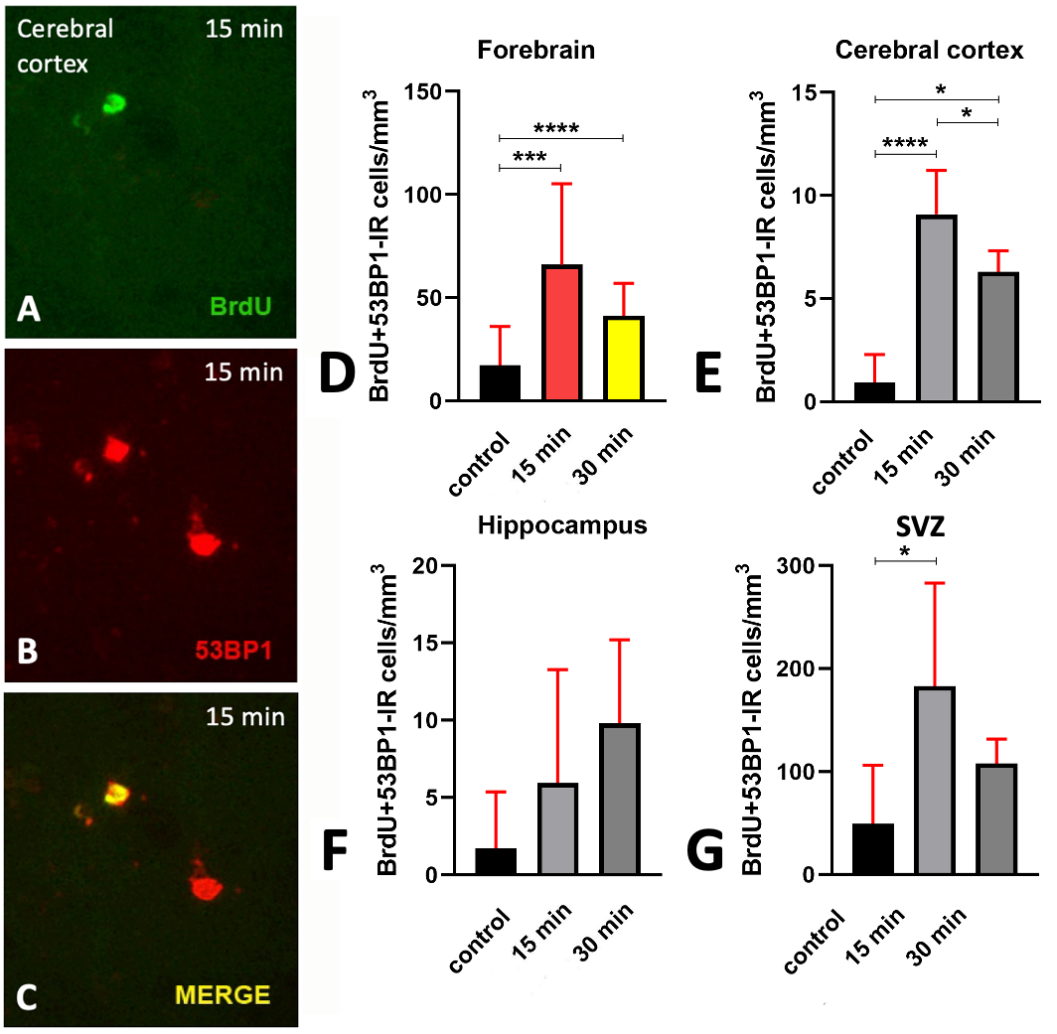


Figure 23. BrdU + 53BP1 double-labeling cells in the forebrain of control and irradiated animals. (**A-C**) Exemplification images of the pattern of immunostaining in the cerebral cortex of a short-term survival animal. (**D-G**) Quantitative analysis of the volumetric density of double-labeled cells in the whole forebrain, cerebral cortex, hippocampus, and SVZ. Abbreviations: Data are expressed as mean ± 95% CI. * 0.05 > p ≥ 0.01, *** 0.001 > p ≥ 0.0001, **** p < 0.0001. T-Bars are 95% CI.

4.3. APPENDIX 1: ASSESSMENT OF THE SUCCESS OF DNA MARKERS INJECTIONS AND ANTI-DNA MARKERS ANTIBODIES SPECIFICITY

An antibody has a specific amino acid sequence (the Fab region) that dictates its affinity for a specific antigen. Cross-reactivity between antigens occurs when an antibody raised against one specific antigen has a competing high affinity toward a different antigen, therefore the antibody can recognize a protein that is different from the one it was raised against. This is often the case when two antigens have similar structural regions that the antibody recognizes (Chadwick, 2008).

Although the chemical structures of thymidine and its analog BrdU are nearly identical, anti-BrdU antibodies have very little cross-reactivity with thymidine itself (Magaud *et al.*, 1989; Aten *et al.*, 1992). However, many anti-BrdU antibodies recognize the other halogenated thymidine analogs CldU and IdU, due to their structural similarities (Kimoto *et al.*, 2008; Liboska *et al.*, 2012).

To test whether anti-BrdU may be reacting to an antigen other than incorporated BrdU, namely IdU, I injected P8 animals with only IdU and labeled the cerebellum and the small intestine with either BrdU or IdU, as a positive control. By direct microscopic observation, I noticed that positive cells were only visible when slices were labeled with IdU (Fig. 24B, D), but not when labeled with BrdU (Fig. 24A, C).

On the other hand, to test whether the anti-IdU antibody used in these experiments does not cross-react with BrdU, P8 animals were injected with BrdU, and cerebellum and small intestine slices were labeled with IdU and BrdU as positive control. The microscopic observations only showed BrdU-positive cells (Fig. 24E, G), while IdU-positive cells were not present in these preparations (Fig. 24F, H).

Altogether these observations demonstrate that two primary antibodies are specific for their targets, but also that all animals used in our experiments were injected successfully with the two markers.

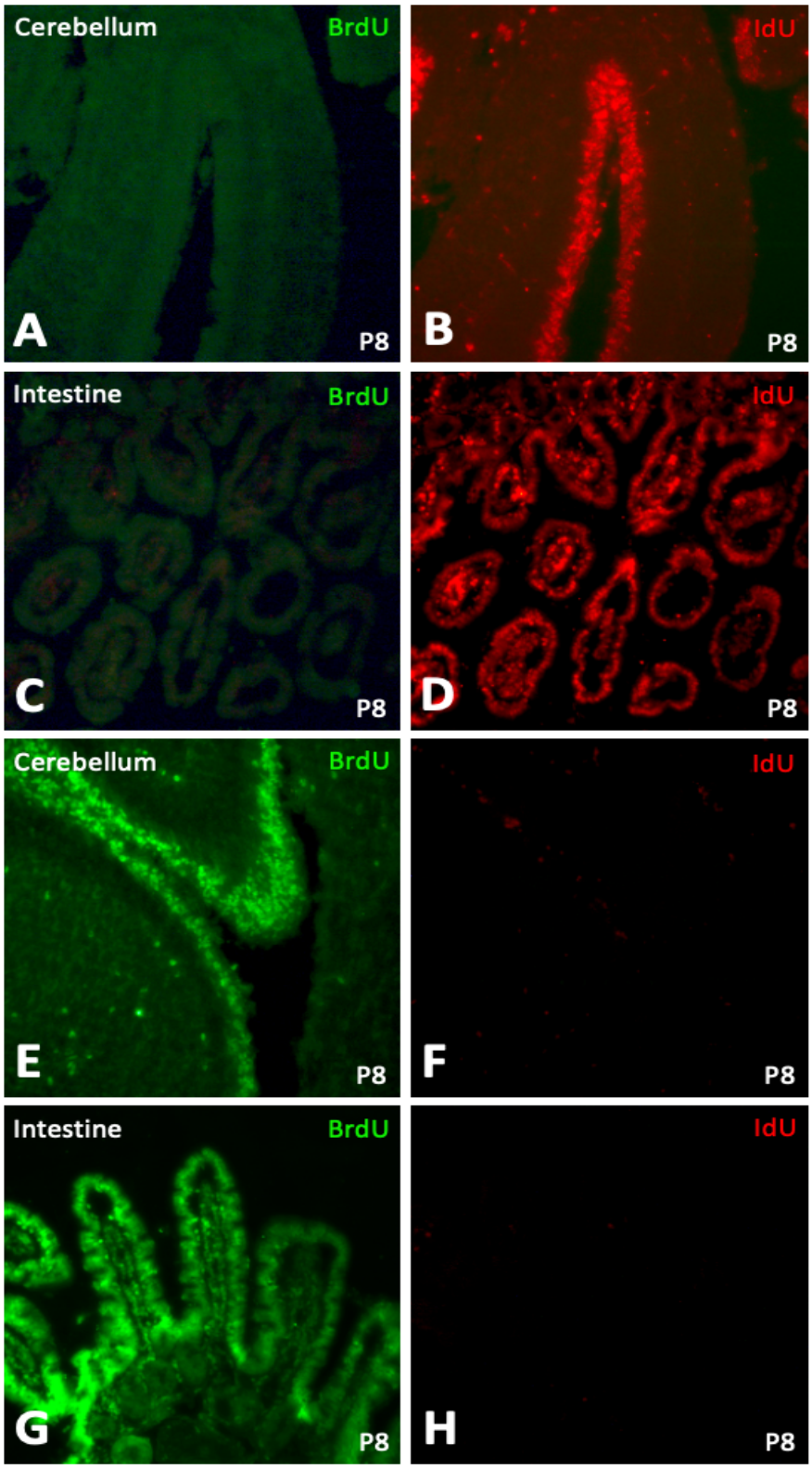


Figure 24. BrdU and IdU cross-reactivity reactions. (**A-D**): BrdU cross-reaction test in the cerebellum (**A**) and small intestine (**C**) of P8 mouse injected with IdU, and its positive controls (**B, D**). (**E-H**): IdU cross-reaction test in the cerebellum (**F**) and small intestine (**H**) of P8 mouse Injected with BrdU; **E** and **G** are the BrdU positive controls.

4.4 APPENDIX 2: THEORETICAL BACKGROUND OF CELL CYCLE ANALYSIS

The analysis of the cell cycle performed in this thesis is based on the theory beyond the use of the double labeling method to calculate the cell cycle parameters as described by Wimber and Quastler in 1963 (Wimber and Quastler, 1963). These two authors have used a ^{3}H- and ^{14}C-thymidine double-labeling technique to study cell proliferation in the root tips of a clone of *Tradescantia paludosa* (inch plant). For simplicity, and for a better comprehension of the Discussion, their original formulae have been modified by using the terms 1st and 2nd S-phase markers to indicate, respectively, ^{3}H- thymidine and ^{14}C-thymidine. The analysis is conditional on several characteristics of the cell population under study. (i) The population is supposed to be in a **steady state**, i.e., the size of the population is constant and equal numbers of cells are lost and born. (ii) The population must be in an **asynchronous state**, that is, cells have equal chances of being at any point in the nuclear cycle. (iii) **Maturation, death, or loss of cells from the population must occur after division and before DNA synthesis**. (iv) Finally, **the population is assumed to have only minor variations in generation time**.

If a cell population with the characteristics listed above is pulse-labeled with two different S-phase markers at a given interval of time (**t**) it will be possible to detect three groups of labeled cells. At the first labeling pulse, a fraction of cells proportional to the duration of the S-phase ($\mathbf{t_s}$), will be labeled. During the interval between treatments "worth of cells" will have passed out of DNA synthesis, and hence, will only be labeled with the 1st marker and not with the 2nd maker subsequently applied. There will also be a doubly-labeled group of cells in proportion to the difference $\mathbf{t_s - t}$ if $\mathbf{t_s > t}$.

If no labeled cells have divided, then the ratio between the cells singly labeled with the 1st marker and ***all*** the cells labeled with the 2nd marker yields $\mathbf{t_s}$ from the following equation (1):

$$Marker's\ ratio = \frac{t}{t_s} \quad \textbf{(Eq.1)}$$

where the marker ratio is the number of cells singly labeled with the first S-phase marker divided by the number of the remainder of the labeled cells. i.e., all the cells single- or double-labeled with or the 2nd marker[1].

In practice, the S-phase markers must be made available to the cell population for a certain amount of time for them to be incorporated into the newly synthesized DNA and this may cause a slight overestimate of t_s.

When the duration of $\mathbf{t_S}$ is longer than half of the total nuclear cycle length there are five different possibilities according to the type of cell population and the interval **t** between the administration of the two DNA tracers. Fig. 25 modified from Wimber and Quastler (1963) represents pictorially these populations and the three additional populations that may be encountered when $\mathbf{t_S}$ is shorter than half of the total nuclear cycle length.

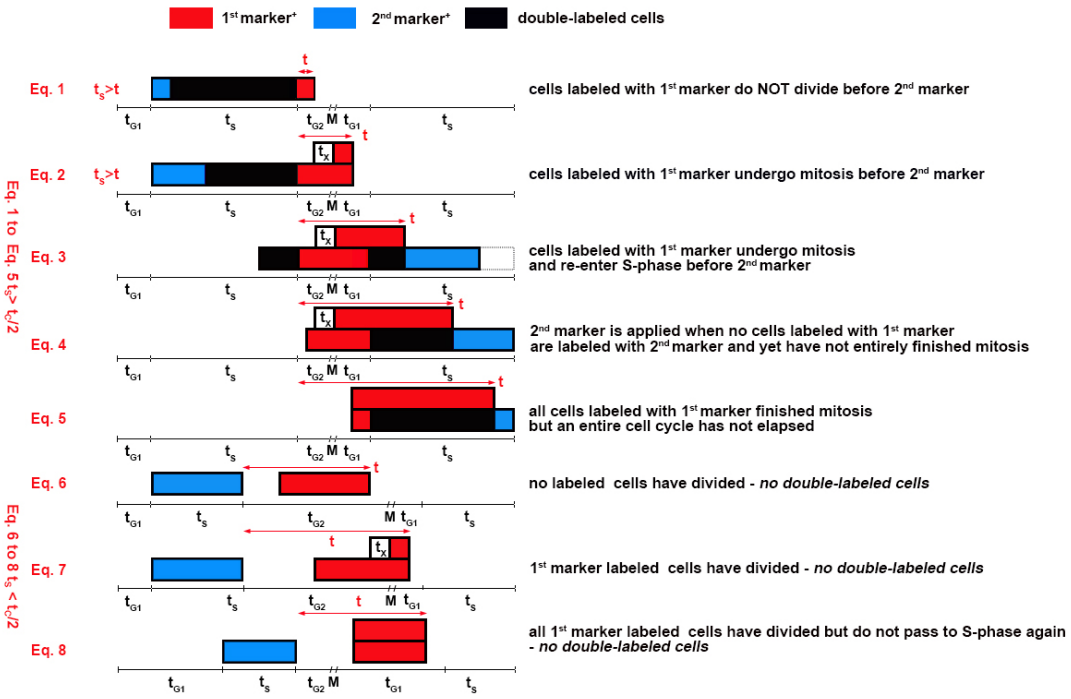


Figure 25. Graphic representation of the scenarios encountered when one applies different double-labeling sequences to the study of cell proliferation. Diagrams illustrate the marker ratio under various double-labeling protocols with a progressive increase of the interval between tracer administration (**t**) in five cell populations where $\mathbf{t_S}$ is longer than half of the total nuclear cycle length (diagrams 1-5) or in the three cell populations where $\mathbf{t_S}$ is shorter

[1] In the cases discussed here the two radiolabeled markers can be easily recognized and do not obviously display the problems related to possible cross-reactivity of the antibodies that are now used for cell cycle studies. These issues are argumented in the Discussion of the thesis.

than half of the total nuclear cycle length (diagrams 6-8). Fig. 26A-E (again modified from (Wimber and Quastler, 1963) graphically shows the relative rates of changes in the markers' ratio with time corresponding to the equations discussed below and represented pictorially in Fig. 25.

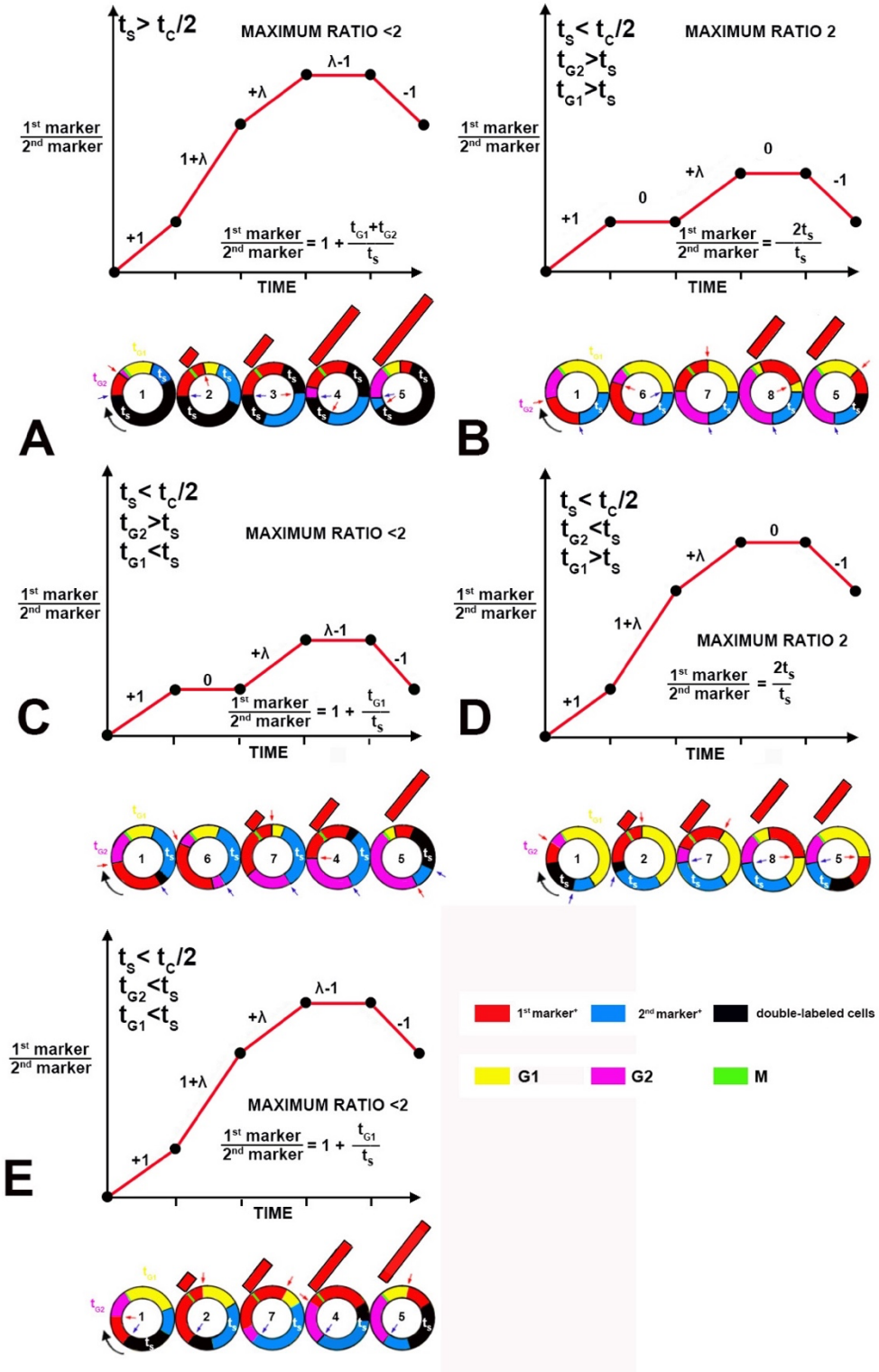


Figure 26. Dispersion graphs showing the slopes of the curves representing the variations of the markers' ratio with time and circular diagrams illustrating the double labeling sequences in different steady-state populations. In each graph, there is an indication of the main features of the population (top left) and the formula to calculate the value of the maximum ratio of the two markers. λ is the yield of cells at mitosis (see text).

In circular diagrams, cells pass through the nuclear cycle in a clockwise direction (black curved arrows). The small red arrows indicate the injection point of the 1st marker, and the small blu arrows that of the 2nd marker. The numbers inside the cell cycle diagrams indicate the corresponding equations in Fig. 22. The red rectangles outside the circles indicate the cells that have left the cycle after mitosis.

As shown in Fig. 25, there are five possible populations in which **t_S is greater than ½ t_C**. For a proper analysis, it is of paramount importance to appropriately set the interval between the injections of the two markers (**t**) according to the nuclear cycle features.

The first population (see Eq. 1 above) is represented graphically in Fig. 26A. The graphic shows the values of the markers' ratio with time with the corresponding slopes indicated by the red line. The dot on the origin of the X-Y axes marks the time point of injection of the 1st tracer and each dot on the curve marks the different time points of the injection of the 2nd tracer.

If the period between tracers injection is extended so that some labeled cells from the first labeling have passed through mitosis at the time of the second labeling as illustrated (Fig. 25 – Eq. 2) the duration of G2+prophase-metaphase (**t_{G2}** for simplicity) may be calculated according to the following equation:

$$Markers'ratio = \frac{t+\lambda(t-t_{G2}+t_X)}{t_S} \quad \textbf{(Eq.2)}$$

where **t** is the interval between tracer treatments, λ is the yield of new cells per mitosis and **t_X** is the time between the 2nd tracer injection and fixation of tissue. If λ is assumed to be anything other than 1, two sets of data must be applied to this equation to find λ and t_{G2}. In some cases it is possible to roughly estimate t_{G2} by counting labeled mitoses; if none are seen then $t_{G2} > t\text{-}t_X$ and if half of the mitoses are labeled then $t_{G2} \cong t\text{-}t_X$.

G1 + ana-telophase (**t_{G1}** for simplicity) may be calculated if the interval between the injections of the two tracers is extended so that some cells that were labeled during the first treatment have passed through mitosis and enter into S-phase again. In a steady state system half or more of the cells labeled with the first tracer only will be committed to enter the S-phase again (Fig. 25 – Eq 3). In this case, t_{G1} may be estimated from the formula:

$$Markers'ratio = \frac{t_{G1}+t_{G2}+\lambda(t-t_{G2}+t_X)}{t_S} \quad \textbf{(Eq.3)}$$

If the 2nd marker is applied when none of the 1st marker labeled cells are labeled with the 2nd marker and yet the 1st marker labeled cells have not finished passing through division (Fig. 25 - Eq 4), then the following equation is applicable:

$$Markers'ratio = 1 + \frac{t_{G1}+t_{G2}-t+\lambda(t-t_{G2}+t_X)}{t_S} \quad \textbf{(Eq.4)}$$

Lastly, if all 1st marker-labeled cells have passed through mitosis and yet an entire nuclear cycle has not elapsed (Fig. 25 – Eq. 5), then the following equation can be used to calculate t_{G1}:

$$Markers'ratio = (1 + \lambda) + \frac{t_{G1}+t_{G2}-t}{t_S} \quad \textbf{(Eq.5)}$$

There are other four populations to be discussed in which t is shorter than half of the total nuclear cycle (Fig. 25 – Eq. 6-8). A characteristic of these populations is that after double labeling they do not yield double-labeled cells for the reasons indicated in the figure. Since – as reported in the following paragraph – double-labeled cells were detected in all experiments of this thesis, they will not be discussed further.

5 DISCUSSION

Since 1962 when Altman suggested the presence of newly generated neurons in the adult brain, huge progress has been made in understanding the cellular and molecular processes that govern the proliferation, differentiation, and integration of new neurons in the preexisting circuits.

Adult neurogenesis has been identified and confirmed in two brain regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus of the hippocampal formation (Capilla-Gonzalez, Herranz-Pérez and García-Verdugo, 2015). Newborn neurons in the SVZ migrate through the RMS where they become granule and periglomerular neurons in the OB. Cells born in the adult SGZ migrate into the deep layer of the dentate gyrus and fully differentiate into excitatory dentate granule cells (Cameron *et al.*, 1993). More recently, other areas of the brain have been shown to contain newly generated neurons beyond early development, such as the cerebral cortex (Magavi, Leavitt and Macklis, 2000). The finding that the production of new neurons continues throughout life in the adult mammalian brain has generated enormous interest and substantial advances in the field of neuroscience. Moreover, cell proliferation and apoptosis have been found to occur in spatial and temporal overlapping windows during prenatal and postnatal neurogenesis, however, the relationship between the two events remains an issue of debate regarding neurogenesis in adult and old mammals, including humans (Ryu *et al.*, 2016).

Previous work carried out in this laboratory provided a demonstration of the occurrence in vivo of DNA repair activity not only during development but also in specific areas of the old mouse brain (Barral *et al.*, 2014). However, it did not provide an in-depth analysis of γH2AX in the aging normal and pathological brain, which would allow us to better understand DNA repair activity in postmitotic neurons.

Since there are still many issues of debate in the scientific community regarding the topic, I here wanted to elucidate a few different aspects of the cell life in the aging mouse brain.

5.1 CELL CYCLE ANALYSIS

Analysis of the cell cycle is a complex procedure, particularly in the absence of data giving preliminary information on the duration of the S phase (Wimber and Quastler, 1963). Most studies on cell cycle parameters in neurons focused on the neural stem and progenitor cells (NPCs) during the development of the cerebral cortex. Using a cumulative labeling procedure with EdU, it was e.g. demonstrated that the basal progenitors (BPs) have a substantially longer G1 phase than the apical progenitors (APs) and that the previously observed G1 lengthening of neurogenic NPCs in the ventricular zone at E14 reflected an increasing contribution of BPs. These cells exhibited a substantially longer S phase than APs, suggesting a greater time investment into quality control of replicated DNA in the population of expanding NPCs (Arai *et al.*, 2011). Remarkably, NPCs are highly proliferative cells (the growth faction is 98-99%) and their t_S and t_C in Arai et al. study ranged from 1.8-8.0 and 15.8-29.4 hours, respectively.

In this laboratory, to study cell proliferation in the old mouse brain SVZ a method was previously employed that combined a cumulative labeling procedure with repeated pulse delivery of BrdU at 2, 4, and 6 hours to calculate t_C and t_S (Solius *et al.*, 2021) (Fig. 27) in combination with pHH3 immunostaining to calculate the duration of the G2 and M phases (Contestabile *et al.*, 2009).

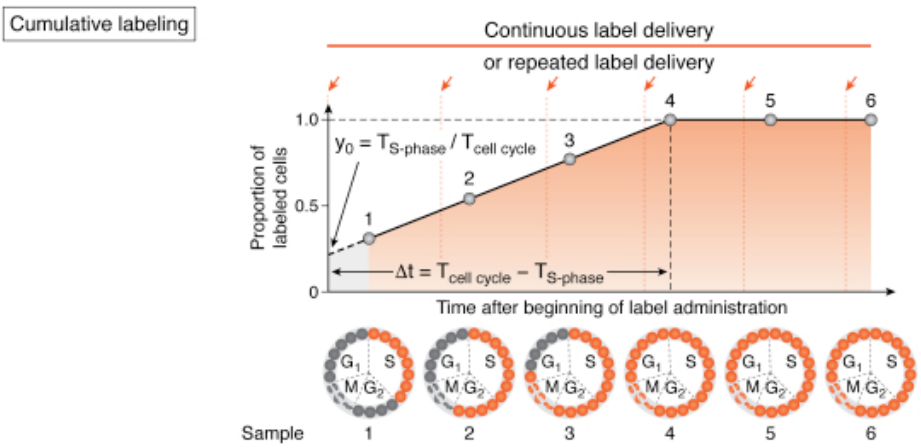


Figure 27. Explanation of the principle of the cumulative labeling method. Using this method, an intercept between the linear regression line of the slope and the plateau enables the determination of the time interval (Δt) equal to $t_C - t_S$. If one normalizes cell counts to the maximal value, an intercept between the y-axis and the continuation of the slope (y_0) enables

the estimation of the ratio t_S/t_C with the creation of the equation system below where Δt is the value of **t** when 100% of the cells are labeled:

$$\begin{cases} \Delta t = t_C - t_S \\ y_0 = \dfrac{t_S}{t_C} \end{cases}$$

(from Solius et al., 2021).

These unpublished experiments yielded - as expected - a very low value of the growth fraction (1.4-3.8%) with a surprisingly high t_C (about 220 hours), a quite short t_S (1.8 hours – corresponding to the value for neuronally committed NPCs in (Arai *et al.*, 2011), a surprisingly long t_M of 4.2 hours, t_{G2} of 11.8 hours (about 7.5 times that calculated in (Arai *et al.*, 2011), and t_{G1} of 195 hours (more than 10 times the average of the values calculated in (Arai *et al.*, 2011).

The equation of the regression line was y = 0.004740x + 0.008 and had a slope that was not significantly different from 0 (F 120.3; DFn, DfD 1, 1; P value 0.0579).

These observations were puzzling and suggested that SVZ cells in 24-month-old mice were proliferating very slowly and/or were undergoing an aberrant cell cycle re-entry (Omais, Jaafar and Ghanem, 2018).

One major drawback of the cumulative labeling method is that it requires the estimation of the population growth fraction and this is subjected to a certain degree of error, particularly in the presence of very low values as above. It also requires the use of pHH3 immunocytochemistry (Contestabile *et al.*, 2009) or other nuclear phase markers to calculate t_{G2} and t_M adding further elements of disadvantage.

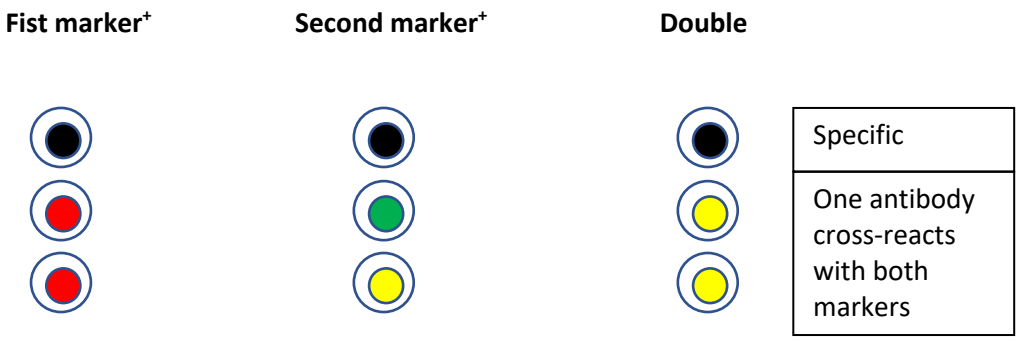
In this thesis, I used a double-labeling method to analyze the cell cycle duration of three different populations of cells in the old mouse forebrain as it does not require calculating the population growth fraction. Also, the intervals between the tracer administration were considerably elongated to a maximum of 120 hours, taking into account the t_C calculated in cumulative labeling experiments. I examined the hippocampal DG, the SVZ of the lateral ventricles, and the cerebral cortex as in previous studies on the old mouse brain cells in these areas were demonstrated to incorporate BrdU in single injection experiments (Barral *et al.*, 2014; Gionchiglia *et al.*, 2021).

The first point of discussion regards the choice of the labeling method and the formulas to calculate the cell cycle parameters.

Table 1 of section 5.1 reports some of the protocols commonly used in the studies on neuron proliferation.

There are some issues to be considered when comparing these methods and the relative formulas to calculate t_S and t_C. First, one has to consider that they can all be used when the condition of **t_s> t is fulfilled.** If the interinjection interval (t) is shorter than t_S a significant error is introduced as discussed in Appendix 2.

Second, **formulae differ if the antibodies are specific or not** because the formulas in which the antibody against the 1st marker cross-reacts with the 2nd marker do not consider the 2nd marker-only positive cells as they also appear to be double-labeled. The two conditions are exemplified graphically below:



For this reason, it is mandatory to assess the antibody specificity in double-labeling experiments (see Appendix 1).

To do so and to evaluate the effective absorption of the DNA labels into the blood flow, I double stained the small intestine and the cerebellum of each IdU/BrdU injected mouse for proliferating cells concluding that the two primary antibodies were specific for their targets and that all animals used in experiments were injected successfully with the two markers (Appendix 1).

After double labeling experiments, I lead to the conclusion that there are no major differences in the cell cycle dynamics in the three populations of forebrain cells, although the DG and SVZ are today considered classic neurogenetic niches in the adult brain, whereas virtually all neurons of the adult cerebral cortex are postmitotic (Jurkowski *et al.*, 2020).

I was also able to calculate t_C from the linear regression curves of Fig. 16 with values of 174.2 (hippocampus), 252 (SVZ), and 205.69 (cerebral cortex). Regarding SVZ cells, the difference in t_C calculated here (252 hours) and after cumulative labeling (220 hours) can be likely explained by the dissimilar degree of errors between the two procedures and/or considering that the interjection intervals used in both types of experiments were still too short to provide more accurate data.

Yet both procedures converge to demonstrate an aberrant long duration of the cell cycle in the SVZ cells and the current study extends this observation to those in the hippocampal DG and cerebral cortex.

Previous and current experiments on the cells that are capable to incorporate the S-phase markers are prone to two different (and not necessarily mutually exclusive) interpretations: from one side the incorporation of the DNA tracer(s) may be related to a very long-lasting cell cycle, and from the other that it may be linked to an attempt to repair the damaged DNA in cells which, at least in part, express some molecules related to the DNA damage response (DDR) – see section 4.2. The observation of $IdU^+/BrdU^+$ cells in the experiments of this thesis demonstrates that some cells labeled with the first marker indeed entered again a phase of DNA synthesis at the time of the administration of the second tracer and lead to hypothesizing that the three populations under study behave as represented in Fig. 18 of section 5.1. If this is the case, the interpretation of data is made even more complex by the existence of a plateau in which the ratio of the two markers remains unchanged. It would be also possible to hypothesize that, at the interinjection intervals examined, the very low slope of the linear regression curves (which are not statistically different from 0) indeed indicates that cells are attempting to repair their DNA albeit very slowly rather than being truly proliferating.

Another issue of complexity is related to the concurrent presence of caspase-dependent apoptosis in these populations (Gionchiglia *et al.*, 2021), as will be discussed in section 5.2.

5.2 γH2AX IN THE NORMAL AGING BRAIN AND RESPONSE TO X-RAY IRRADIATION

H2AX is a histone H2A variant that is structurally similar to other H2A species except for the presence of a unique COOH terminal tail, containing a serine four residues from the C terminus. H2AX, as with all other histones, serves to structure and stabilize the DNA. It also has a very specific function in the complex DNA damage detection and repair machinery of higher eukaryotes. The phosphorylation of H2AX at position Ser139 (γH2AX) is one of the first signals for the detection of DNA double-strand breaks and an essential step for the initialization of DNA repair. This histone modification spreads beyond the DSB into neighboring chromatin, generating a DDR platform that protects against end disassociation and degradation, minimizing chromosomal rearrangements. Many DNA damage response and DNA repair proteins also accumulate around the sites of DSBs, where they can be visualized as DNA damage-induced foci. The focus formation of many of these DNA damage repair proteins requires H2AX, thus implying that H2AX plays an important role at the early stage of DNA damage response.

Although phosphorylation of H2AX is an initial step in DDR, the histone has also been implicated in cell proliferation and apoptosis, however, there are no data in the literature correlating the expression of γH2AX and cCASP3, the main effector protease in apoptosis, in the aging mouse brain. To better understand this correlation I studied the two markers in the normal aging brain of 24-month-old mice and after X-ray irradiation, which causes DNA damage and strongly induces H2AX phosphorylation.

First, I demonstrated that X-ray irradiation induces strong phosphorylation of H2AX and the appearance of nuclear foci, which are a sign of DDR in irradiated cells (Rogakou *et al.*, 1999). The same study has also shown that H2AX is rapidly γ phosphorylated after exposure of cells to ionizing radiation, with half-maximal amounts reaching 1–3 min and the maximum 10–30 min after irradiation. Based on these findings, I left irradiated mice to survive for 15 or 30 min, to allow H2AX phosphorylation to reach a peak. This was confirmed after calculating the volumetric density of γH2AX immunoreactive cells and the percentage of γH2AX-

positive nuclei versus the total number of nuclei after labeling with DAPI (Fig. 9). Both parameters showed that there was no statistically significant increase between short- and long-term survivors, except for the SVZ, where the numbers of immunoreactive cells increased further at 30 min survival. After calculating the number of γH2AX immunoreactive foci per nucleus in short- and long-term survivors, I remarkably saw a statistical difference between 15 and 30 min survivors, with a higher number of γH2AX foci/nucleus in long-term survivors, proving that DNA damage was still in progress between the two time points (Fig. 8). γH2AX+53BP1 double labeling experiments, which showed a significant increase in the volumetric density of double-labeled cells already after 15 min after irradiation, further confirmed that X-ray irradiation causes DDR in cell nuclei (Fig. 11).

With regards to the untreated mice, I detected cells immunoreactive for γH2AX, demonstrating that the early components of the DDR are also present in untreated mice, but in smaller numbers compared to irradiated animals. These findings confirmed what was already discovered in a previous work of our group, showing that γH2AX was detectable in the brains of adult mice that were not subjected to any known DNA injury (Barral *et al.*, 2014). Herein, I calculated the percentage of γH2AX immunoreactive cells versus the total number of nuclei of the forebrain areas under study and saw that about 4-8% of the total cell population was labeled by the marker (Fig. 9). Double labeling experiments for γH2AX and 53BP1 showed that, as for irradiated animals, a large number of nuclei expressed both markers, proving that cells in the forebrain of untreated mice were also undergoing a DDR (Fig. 11).

Then, after demonstrating that irradiation induced a robust DDR with intense phosphorylation of H2AX and upregulation of 53BP1, I wanted to investigate the fate of the cells with damaged DNA, studying the correlation between γH2AX and cCASP3 in irradiated mice. γH2AX+cCASP3 double-labeling experiments showed that irradiation increased the colocalization of the two markers.

These results, together with the observation that the percentage of colocalization between the two labels after X-rays were higher than those predicted by chance (Table 1), I hypothesized that three different scenarios might be possible: γ phosphorylation of H2AX may cause CASP3 activation; CASP3 activation may lead to H2AX phosphorylation, or an upstream process may be responsible for the activation of both molecules. However, since cCASP3 immunoreactive cells are only a fraction of the γH2AX-positive nuclei, it seems odd that activation of CASP3 might precede H2AX phosphorylation. Thus, I theorize that X-ray irradiation is the upstream process responsible for the increase in the volumetric density of γH2AX+cCASP3 immunoreactive cells and the percentage of colocalization of the two markers.

Therefore, X-ray irradiation leads to a DDR with rapid phosphorylation of H2AX, expression of 53BP1, and the subsequent activation of CASP3. The latter may be probably due to the mid-level X-ray dosage (10 Gray) used in my study (apoptosis increases up to 16 Gray in previous studies – see section 1.3.1 RADIATION-INDUCED DNA DAMAGE). For this reason, activation of CASP3 did not result in a measurable reduction of the volumetric density in any of the forebrain areas under study (Fig. 12), and cCASP3 was detected only in a small percentage of γH2AX immunoreactive cells.

In untreated mice, expression of cCASP3 was analogous to what was observed for the irradiated animals, showing a similar pattern of immunostaining and quantitative estimations, but a lower number of cells (Fig. 13 and 14).

Previous studies have shown that IR induces DNA damage (Azzam, Jay-Gerin and Pain, 2012; Lomax, Folkes and O'Neill, 2013; Vignard, Mirey and Salles, 2013) and may initiate neuronal apoptotic pathways leading to neuronal cell death (Gobbel *et al.*, 1998) via various mechanisms, among which activation of CASP3 (Ivanov and Hei, 2014). However, there have been no direct observations on the relationship between phosphorylation of H2AX and CASP3 activation in the intact adult brain.

Prior experiments have shown that γH2AX not only formed at the onset of the DDR but was also involved as the earliest epigenetic modification during apoptosis (Rogakou *et al.*, 2000). Careful examination of single cells by confocal immunofluorescence microscopy revealed a previously unnoticed staining pattern

for γH2AX during apoptosis induced by TRAIL that differs from the well-established DDR focal appearance. This specific pattern, called the apoptotic ring, looks like a nuclear annular staining early in apoptosis. This ring is, in three-dimensional space, a thick intranuclear shell consisting of epigenetic modifications including histone H2AX and DDR proteins, but it excludes the DNA repair factors usually associated with γH2AX in the DDR nuclear foci (Solier and Pommier, 2009). Moreover, the γH2AX ring coincides with the cellular site of localization of another phosphorylated histone, the H2B (Solier and Pommier, 2009). Notably, I have been unable to observe the presence of the apoptotic γH2AX ring, nor the phosphorylation of H2B in any of my experimental conditions, thus confirming that activation of CASP3 is a secondary event to DDR and the onset of γH2AX is not a primary consequence of apoptosis.

5.3 INCORPORATION OF BRDU IN THE NORMAL AGING BRAIN AND RESPONSE TO X-RAY IRRADIATION

^{3}H-thymidine and the later developed halogenated thymidine analogs, BrdU, IdU, CldU, as well as EdU, are permanently integrated into the DNA of dividing cells during the S phase of the cell cycle. Because of this, these markers of DNA synthesis have revolutionized the ability to study the dynamics of the cell cycle as well as to identify dividing cells and follow their fate, processes that are particularly complex in the nervous system. However, even though BrdU is truly an S phase marker, it has been often misused to identify proliferating cells, especially in the study of mammalian adult neurogenesis (Taupin, 2007; Duque and Rakic, 2015). Therefore, it is imperative while studying neurogenesis with BrdU to distinguish cell proliferation and neurogenesis from other events involving DNA synthesis, like DNA repair, abortive cell cycle reentry, and gene duplication. Indeed, previous studies have reported the detection of DNA repair by BrdU immunohistochemistry in vitro on irradiated fibroblasts (Beisker and Hittelman, 1988; Selden *et al.*, 1993, 1994). Moreover, damaged or degenerating terminally differentiated postmitotic neurons in the adult brain can reenter the cell cycle, activate cell cycle-associated proteins, cyclins, and ubiquitins, and initiate abortive DNA synthesis without cell division, before undergoing cell death, as shown in the

study of Kuan et al. (Kuan *et al.*, 2004), where they reported that the combination of hypoxia and ischemia in mice induced terminally differentiated neurons to reenter the cell cycle and incorporate BrdU.

However, to my knowledge, no examples of BrdU incorporation during DNA repair and abortive cell cycle reentry have been reported in the intact adult brain.

To understand the connection between BrdU incorporation, mitosis, and DNA repair, I studied the expression of BrdU and different markers of DDR and proliferation in the brain of 24-month-old mice under normal conditions and after X-ray irradiation, as a positive control of DNA damage.

First, I wanted to investigate the connection between BrdU incorporation and mitosis. To do so, after experimentally injuring the DNA with X-rays, I examined the colocalization of BrdU and pHH3, a marker of dividing cells displaying a nuclear distribution in foci or a marker of mitotic chromosomes in the G2/M phases of the cell cycle (Hendzel *et al.*, 1997), in both untreated animals and irradiated ones.

Then, I studied whether the incorporation of BrdU was associated with DDR, observing the colocalization between BrdU, γH2AX, and 53BP1, which are both involved in the early DNA damage-signaling pathway.

I demonstrated that irradiation induced not only an increase in the volumetric density of BrdU immunoreactive cells (Fig. 20) but also a rise of BrdU+pHH3 double-labeled cells (Fig. 21) and in the fraction of BrdU immunoreactive cells that also stained for pHH3. On the other hand, the results of BrdU+ γH2AX double-labeling experiments show a statistically significant rise of both BrdU+ γH2AX (Fig. 22), which is also confirmed by the increase of BrdU+53BP1 immunoreactive cells (Fig. 23) after X-rays. Therefore, I hypothesize that the incorporation of BrdU in the forebrain cells of irradiated mice might be caused by two different mechanisms, either happening concurrently or individually: the attempt to respond to the DNA damage caused by X-rays with a de novo synthesis of DNA, and/or the start of an aberrant mitotic response. As IR causes a raise of H2AX phosphorylation and colocalization of γH2AX foci with BrdU foci indicates stalled or collapsed replication forks (Tripathi *et al.*, 2015), I believe that expression of pHH3 and its colocalization with BrdU derives from a mitotic aberrant response or a mitotic catastrophe (Fig. 27).

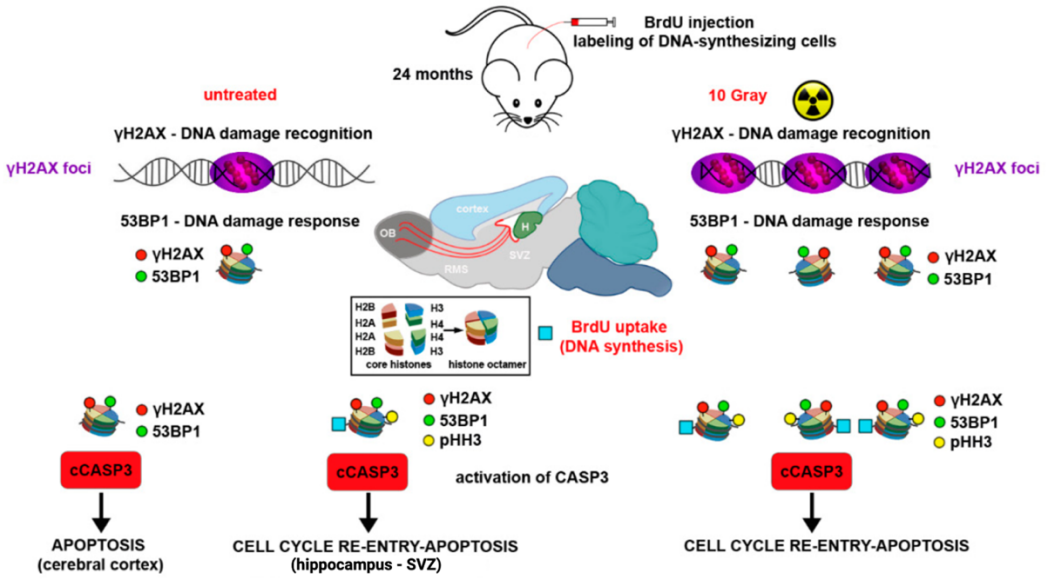


Figure 27. Schematic representation of the occurrence of γH2AX in the forebrain of untreated and X-ray irradiated mice. (from (Merighi *et al.*, 2021)).

In untreated animals, the pattern of immunostaining and the quantitative trends for BrdU, pHH3, γH2AX, and 53BP1 were analogous to those observed after irradiation, however, the number of immunoreactive cells was consistently smaller (Fig. 20, 21, 22 and 23). In addition, in these animals, there were clear differences among forebrain areas, most likely caused by the nonsynchronous occurrences of the DNA damaging events, contrarily to the irradiated mice where the damage was induced at the same time all over the forebrain.

In particular, the cerebral cortex is one of the main areas of expression of γH2AX in not-irradiated mice and about 50% of γH2AX-positive cells are also labeled by 53BP1 (Fig. 11E), confirming previous observations carried out in this laboratory (Barral *et al.*, 2014). Moreover, the pattern in foci of γH2AX and 53BP1 (Fig. 11A-C), and the high percentage of γH2AX+BrdU immunoreactive cells (Fig. 22E) indicate that a DDR is occurring. In the previous study, the percentage of cells expressing γH2AX and 53BP1 was constant throughout the telencephalon, suggesting that the whole cerebral cortex of aging animals is prone to DNA damage (Barral *et al.*, 2014). Additionally, I observed BrdU+γH2AX (Fig. 22E), BrdU+53BP1 (Fig. 23E), and γH2AX+cCASP3 immunoreactive cells (Fig. 14D), while there was no colocalization between BrdU and pHH3 (Fig. 21E). Therefore, I can

confirm that BrdU incorporation in the cerebral cortex of adult animals is caused by the presence of naturally occurring DDR with phosphorylation of γH2AX, recruitment of 53BP1, and activation of CASP3, leading to apoptosis (Fig. 27). The volumetric density of cCASP3-positive cells is higher than that of BrdU immunoreactive cells, suggesting that at least a fraction of cells labeled with cCASP3 may quickly undergo apoptosis, having accumulated too many unrepaired SSBs. Other cells of the aging cerebral cortex, in which DSBs are formed, may instead incorporate BrdU in an attempt to repair their DNA, and, if failing, undergo CASP3-dependent apoptotic death (Fig. 27). Given the important role of γH2AX in the cell cycle checkpoint response (Fernandez-Capetillo *et al.*, 2002), I also hypothesize that BrdU immunoreactive cells that are negative to γH2AX are already committed to death.

In the hippocampus, phosphorylation of γH2AX is accompanied by the expression of 53BP1 (Fig. 11F), proving that it is linked to DDR, and a fraction of them co-expressed cCASP3 (Fig. 14E), demonstrating that these cells are committed to apoptosis. Another fraction of γH2AX positive cells incorporate BrdU (Fig. 22F), of which, a small part also expresses pHH3 (Fig. 21F). Thus, it is possible to suggest that cells in the aging hippocampus expressing the DDR markers γH2AX and 53BP1, as well as activated CASP3, have accumulated an amount of DSBs which is incompatible with survival. Previous works have shown evidence of re-entry into the cell cycle by neurons in aging and diseases such as Alzheimer's disease (Jurk *et al.*, 2012; Simpson *et al.*, 2015, 2016). This aberrant cell cycle re-entry is lethal and can lead to the apoptotic death of the neuron. A recent study has shown that amyloid-β42 oligomers, one of the core pathological players of Alzheimer's disease, promoted an increase in senescence-associated DNA damage, γ phosphorylation of H2AX and genomic DNA lesions (Li *et al.*, 2022). Moreover, increased levels of γH2AX have been reported in the neuronal nuclei of Alzheimer's disease patients, but also in their buccal cell nuclei, which have been found to reflect systemic changes in pathology in other tissues of ectodermal origin, such as the nervous system (Siddiqui *et al.*, 2020). So, I hypothesize that the incorporation of BrdU in aged hippocampal cells may be linked to an aberrant re-entry into the cell cycle before undergoing apoptotic death (Fig. 27).

In the old mouse SVZ, similarly to the hippocampus, the expression of γH2AX is accompanied by 53BP1 (Fig. 11G), cCASP3 (Fig. 14F), and BrdU (Fig. 22G). However, in this area, BrdU immunoreactive cells did not express pHH3 (Fig. 21G). Thus, the occurrence of BrdU+γH2AX double-labeled cells and the absence of BrdU+pHH3 immunoreactive cells in the SVZ suggest that H2AX in the SVZ is phosphorylated in cells that are in the S phase of their cycle. Therefore, in these cells, BrdU incorporation may be linked to a DDR during abortive mitosis (Giunta, Belotserkovskaya and Jackson, 2010), leading to cCASP3 activation and apoptosis (Fig. 27). However, after different types of injury (olfactory bulbectomy, brain irradiation, kainic acid-induced seizure) in young adult mice, it was concluded that BrdU is not significantly incorporated during DNA repair and that labeling is not detected in vulnerable or dying postmitotic neurons, even when a high dose of BrdU is directly infused into the brain (Bauer and Patterson, 2005).

The link between cell cycle reactivation and apoptosis of terminally differentiated neurons has recently been reviewed (Xia *et al.*, 2019), highlighting the role of cyclins and CDKs. Previous studies from this laboratory have demonstrated that the death of premigratory immature granule cell neurons was linked to activation of DNA checkpoint and alteration of the normal cell cycle, whereas in postmigratory mature neurons, apoptosis was dependent upon CASP3 activation (Lossi *et al.*, 2004).

Therefore, additional studies will be necessary to prove or disprove my hypothesis, as literature data are scanty and discordant.

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7 APPENDIX 3: PUBBLICATIONS DERIVED FROM THIS THESIS

1. Biomedicines. **2021** Sep 6;9(9):1166. doi: 10.3390/biomedicines9091166

Association of Caspase 3 Activation and H2AX γ Phosphorylation in the Aging Brain: Studies on Untreated and Irradiated Mice.

***Gionchiglia N**, Granato A, Merighi A, Lossi L.*

Phosphorylation of H2AX is a response to DNA damage, but γH2AX also associates with mitosis and/or apoptosis. We examined the effects of X-rays on DNA integrity to shed more light on the significance of H2AX phosphorylation and its relationship with activation of caspase 3 (CASP3), the main apoptotic effector. After administration of the S phase marker BrdU, brains were collected from untreated and irradiated (10 Gray) 24-month-old mice surviving 15 or 30 min after irradiation. After paraffin embedding, brain sections were single- or double-stained with antibodies against γH2AX, p53-binding protein 1 (53BP1) (which is recruited during the DNA damage response (DDR)), active CASP3 (cCASP3), 5-Bromo-2-deoxyuridine (BrdU), and phosphorylated histone H3 (pHH3) (which labels proliferating cells). After statistical analysis, we demonstrated that irradiation not only induced a robust DDR with the appearance of γH2AX and upregulation of 53BP1 but also that cells with damaged DNA attempted to synthesize new genetic material from the rise in BrdU immunostaining, with increased expression of cCASP3. Association of γH2AX, 53BP1, and cCASP3 was also evident in normal nonirradiated mice, where DNA synthesis appeared to be linked to disturbances in DNA repair mechanisms rather than true mitotic activity.

Keywords: DNA; DNA damage; H2AX; aging; apoptosis; caspase 3; cell proliferation; forebrain; ionizing radiations; neurons.

2. Molecules. **2021** Nov 27;26(23):7198. doi: 10.3390/molecules26237198

The Phosphorylated Form of the Histone H2AX (γH2AX) in the Brain from Embryonic Life to Old Age.

Merighi A, **Gionchiglia N**, Granato A, Lossi L.

The γ phosphorylated form of the histone H2AX (γH2AX) was described more than 40 years ago and it was demonstrated that phosphorylation of H2AX was one of the first cellular responses to DNA damage. Since then, γH2AX has been implicated in diverse cellular functions in normal and pathological cells. In the first part of this review, we will briefly describe the intervention of H2AX in the DNA damage response (DDR) and its role in some pivotal cellular events, such as regulation of cell cycle checkpoints, genomic instability, cell growth, mitosis, embryogenesis, and apoptosis. Then, in the main part of this contribution, we will discuss the involvement of γH2AX in the normal

and pathological central nervous system, with particular attention to the differences in the DDR between immature and mature neurons, and to the significance of H2AX phosphorylation in neurogenesis and neuronal cell death. The emerging picture is that H2AX is a pleiotropic molecule with an array of yet not fully understood functions in the brain, from embryonic life to old age.

Keywords: DNA damage; H2AX; aging; apoptosis; cerebral cortex; mitosis; neurogenesis; neurons; subventricular zone.

8 APPENDIX 4: RESPONSES TO REVIEWERS' COMMENTS

Reviewer #1 Dr. François BOUSSIN CEA, France

In an interesting study, Nadia Gionchiglia has investigated the DNA damage response in the neurogenic niches of the aged mouse brain.

I have only minor suggestions:

- This is a well-written manuscript, beginning with a well-documented introduction, however, I would have appreciated a description of previously published data on the effects of radiation on the embryonic and adult mouse brain (cell cycle checkpoints, DNA damage foci, apoptosis, etc...). This would have been useful for the interpretation of the data.

In response to this comment, I have added sections 1.3.2. CELL CYCLE AND RADIATION and 1.3.3 RADIATION-INDUCED CELL DEATH to the Introduction.

- The manuscript reports a high amount of work using robust— but time-consuming— techniques. However, the number of animals/groups may appear surprisingly quite low. A little discussion of this choice is somehow lacking.

In response to the comment, I would like to stress that obtaining 24-month-old mice is quite difficult as many subjects initially included in the experimental groups died before reaching the required age to be included in this work. Aside from this, although the number of animals is undoubtedly low, statistical analysis was supportive of my findings. Additional animals are still being collected to further substantiate the finding of this thesis. I have briefly discussed my choice in the Material and methods sections of the thesis (3.1 ANIMALS AND THYMIDINE ANALOGS ADMINISTRATION) as follows:

"In general terms, the numbers of animals used in this study were remarkably low. Yet, I would like to stress that obtaining 24-month-old mice is quite difficult as many subjects initially included in the experimental groups died before reaching the required age to be included in this work.

Aside from this, statistical analysis was fully supportive of my findings as also discussed in one of the publications stemmed from this work in which, among others, the results of X-ray irradiations were reported (Gionchiglia et al. 2021).

Regarding data on the cell cycle kinetics, it will be necessary to increase the number of animals in the 2-hour survival group and to add a group of animals with longer survival (e.g. 240 h) to publish the observations stemming from the thesis."

- Along the same lines, the study is limited to aged mice.

In response to the comment, I have briefly discussed the reasons for my choice on page 73 of the revised thesis as follows: X-ray irradiation was carried out at Enea (Rome) in the general framework of another project aiming to characterize the response of a tumor cell line to ionizing radiations. As mouse brains and

intestines (used as positive controls) were not necessary for the project, they were kindly donated for our study.
This explains why a different strain of mice was used in these studies, also considering that the laboratory did not hold the prescribed authorizations to carry on irradiation experiments. Although there is extensive literature on different radiation susceptibility among mouse strains in tissues and organs other than the brain (see e.g. Jackson et al. 2010; Griedley et al., 2011), I found little information regarding putative differences in the brain if not related to mortality rates in mice (Yang et al., 2016) or strain in rats (Cacao et al., 2018). In addition, we have not used mice of different strains for either irradiation studies or cell cycle studies.

I would have been interested in a comparison with young mice using the same technical approaches in order to appreciate the real significance of the findings.

I do agree that such a comparison would have been very interesting, yet my main goal was the study of the aging brain and there is extensive literature on the duration of the cell cycle in embryonic, postnatal, and young adult mice, as well as on the effects of X-ray irradiation on the neurons from these mice.

- It would have been also highly informative to extend the study to longer times after irradiation.

Again, I do agree with this comment, but also in this case the existing literature is quite wide and, on top of this, it was not my primary goal to study the neuronal response to irradiation but, specifically, the H2AX response of aged neurons to X-rays.

- P115: contrary to what is written, 10 Gy is a very high dose leading to a high level of apoptosis in neurogenic niches.

I do agree that in neurogenic niches a 10 Gray administration is a very high dose, but regarding adult neurons, I found no clear data in the literature and I based my assumption on some data in vitro (reported in section 1.3.1). In any case, I have changed the text as follows:
"Therefore, X-ray irradiation leads to a DDR with rapid phosphorylation of H2AX, expression of 53BP1, and the subsequent activation of CASP3. The latter may be probably due to the mid-level X-ray dosage (10 Gray) used in my study (apoptosis increases up to 16 Gray in previous studies – see section 1.3.1 RADIATION-INDUCED DNA DAMAGE)."

- There is no characterization of PHH3- and BrdU-positive cells. Immunophenotyping would have helped for the interpretation of the data.

This is correct. Unfortunately, positive cells were very few, and although I did some double/triple labeling experiments with neuronal and or glial markers, the results were not satisfactory.

- Finally, it would have been interesting to discuss more the implications of one of the main results of the study that in the old mouse brain, BrdU incorporation after irradiation is linked to a DDR during abortive. There again a comparison

with young animals would have been interesting to discuss the importance of these findings.

In response to this observation, I have added the following to the end of discussion in the revised thesis:

"However, after different types of injury (olfactory bulbectomy, brain irradiation, kainic acid-induced seizure) in young adult mice, it was concluded that BrdU is not significantly incorporated during DNA repair and that labeling is not detected in vulnerable or dying postmitotic neurons, even when a high dose of BrdU is directly infused into the brain (Bauer and Patterson, 2005).

The link between cell cycle reactivation and apoptosis of terminally differentiated neurons has recently been reviewed (Xia et al 2019), highlighting the role of cyclins and CDKs. Previous studies from this laboratory have demonstrated that the death of premigratory immature granule cell neurons was linked to activation of DNA checkpoint and alteration of the normal cell cycle, whereas in postmigratory mature neurons, apoptosis was dependent upon CASP3 activation.

Therefore, additional studies will be necessary to prove or disprove my hypothesis, as literature data are scanty and discordant."

<u>Reviewer # 2 Dr. Feng Ru TANG, National University of Singapore</u>

In this Ph.D. Thesis, the candidate aimed to 1) clarify whether or not H2AX phosphorylation was indeed

related to a DDR in the different populations of neurons by experimentally inducing a DDR with X-rays and investigating the response of H2AX and other components of the DDR to ionizing radiations; 2) assess if the phosphorylation of H2AX could be related to apoptosis and if the latter event was associated with DNA synthesis by investigating the activation of CASP3, the main effector caspase, with specific antibodies against the cleaved form of the enzyme, performing double/multiple IMF experiments to clarify the functional relationship between γH2AX, activation of CASP3, and DNA synthesis, detected with the use of BrdU incorporation; 3) investigate the connection between BrdU incorporation, mitosis, and DNA repair in the brain of the adult mouse; 4) study the length of the cell cycle and the duration of its phases in cells of the cerebral cortex, hippocampus, and SVZ of 24-month old mice.

In general, this is a well-written Ph.D. thesis and addresses important issues γH2AX may be involved in brain DDR, apoptosis, neurogenesis, and aging I have the following comments and suggestions for the candidate to consider clarifying and doing in the future:

Major:

1) To make scientific experimental conclusions, one or two animals in each group are certainly not enough for statistical analysis.

I have responded above to a similar observation made by Reviewer #1.

2) To find a peak γH2AX expression after irradiation in this specific mouse strain, 2-time points of 15 and 30 mins are not enough, 1, 2, or 4 and 24h after irradiation may be needed to confirm your argument as the peak of in vivo formation of gamma-H2AX and 53BP1 DNA repair foci may be very different in

different strains and species of animals, different tissues and cell types, different sources of radiation, and different doses/ dose rates of radiation, etc. For instance, the peak of in vivo formation of gamma-H2AX and 53BP1 DNA repair foci in blood cells after radioiodine therapy of differentiated thyroid cancer was at 2h (Lassmann et al., 2010, J Nucl Med 2010;51:1318–25. doi:10.2967/jnumed.109.071357).

I do agree with this observation. However, I was primarily interested in showing that there was an H2AX response in the old mouse brain detectable with IMF procedures, rather than in finding the peak of γH2AX expression.

3) How did the candidate define γH2AX immunopositive cells in Fig. 8 in the control and experimental mice, i.e., the definition was based on γH2AX foci number, size, or others? What are the average foci number and size in the so-called γH2AX immunopositive cells? Any difference in the average foci number and size between the control and experimental mice? The candidate should add pictures for the control, 15 and 30min after irradiation for all three areas selected so that readers can compare Fig 8 and Fig 9 to see the difference in the expression of γH2AX foci.

In response to these observations, Fig. 8 was modified as required by the reviewer.

5) In the methods, “five random photographs of the cerebral cortex, hippocampus, and SVZ were taken using a 63X or 100X lens for each section”. The candidate may need to clarify which layer(s) and cerebral cortex was chosen and was the dorsal or ventral hippocampus analyzed.

The required information was added in the method section (3.5 QUANTITATIVE STUDIES).

Minor:

1. What is the rationale to perform irradiation experiments in six 24-month-old B6/129 mice and theanalysis of the cell cycle length in ten 24-month-old CD1 mice? Any strain difference in doing thetwo studies in the same strain?

I have already addressed this issue in response to the comments made by reviewer # 1.

2. In Fig 22, could the candidate add pictures for control and 15min after irradiation so that readers could see the difference in the expression of BrdU and γH2AX among the three groups?

Unfortunately, I cannot provide single pictures that can qualitatively show, i.e. by simple by-eye inspection, the quantitative differences calculated after direct cell counting and statistical analysis, due to the fact that positive cells are very much scattered throughout the brain tissue.

3. In Fig 23B, how did the candidate define 53BP1 foci since different sizes of 53BP1 products appeared in Fig 23B? The pictures for the control and 30min after irradiation should be added.

Foci were defined based on their morphology, rather than size, in contrast with diffuse nucleoplasm staining. The heavy stain of the two cells in Fig.23B is indeed the result of the clumping of several foci of 53BP1 immunoreactivity. That 53BP1

immunoreactivity occurs in foci is clearly demonstrated in e.g. Fig 11 and Fig. 6 in Gionchiglia et al., 2021. Again, double-positive cells are very scattered so it is difficult to provide single images exemplificative of the quantitative data.