NEURONAL DNA DOUBLE STRAND BREAK DAMAGE AND REPAIR FOLLOWING SUBLETHAL iGLuR
ACTIVATION, AND THE NEUROPROTECTIVE EFFECTS OF MELATONIN

A Dissertation
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in Neuroscience

By

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DNA double strand break (DSB) damage is among the most lethal forms of DNA damage, and its repair in mature neurons remains largely unexamined, particularly following neuronal excitation. A major cause of DSBs is mediated by endogenous reactive oxygen species (ROS) that lead to a state of oxidative stress. The brain’s large oxygen consumption, high metabolic rate and relative low levels of antioxidant defenses, make endogenous ROS the primary cause of neuronal DSBs. Since mature neurons do not divide, they cannot repair DSBs using accurate repair mechanism, and must rely on error-prone pathways to counter DSB damage. These factors suggest that post-mitotic neurons likely endure a slow accumulation of DNA errors over the years that can potentially lead to the onset various neuropathologies. Using a novel implementation of the neutral comet assay, a technique that examines DSBs, we show that neurons sustain DSB DNA damage following sub-lethal ionotropic glutamate receptor (iGluR) activity, leading to an increase in ROS and oxidative stress, and that they are repaired by a faulty repair mechanism. Due to the inherent error-prone quality of neuronal DSB repair, a viable approach in minimizing the deleterious effects associated with DSB damage is thus to minimize ROS generation in the first place. We show that neuronal pretreatment using the brain neurohormone melatonin completely prevents DSB generation, neuronal
ROS increase and mitochondria destabilization, caused by iGluR activation, making the use of this neurohormaone a potential strategy to prevent the onset of DNA damage in adult neurons.

Lastly, we begin to examine the role of two key proteins, ATM and DNA-PKcs, involved in DSB repair, and the effects that their inhibition has on neuronal survival.
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- Gil
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A-T</td>
<td>Ataxia telangiectasia disease</td>
</tr>
<tr>
<td>aa</td>
<td>Aminoacids</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>ATLD</td>
<td>Ataxia telangiectasia-like disorder</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related protein kinase</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair pathway</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs (DNA)</td>
</tr>
<tr>
<td>CS</td>
<td>Cockayne syndrome</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSR</td>
<td>Class switch recombination</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DIV</td>
<td>Day <em>in vitro</em></td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>Catalytic subunit of DNA-PK</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double-strand break</td>
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<tr>
<td>DSBR</td>
<td>Double-Strand Break Repair</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>H2AX</td>
<td>H2A histone family, variant X</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination repair pathway</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>iGluR</td>
<td>Ionotropic Glutamate receptor</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Ku</td>
<td>Ku70/80 heterodimer; Ku70: XRCC6; Ku80: XRCC5</td>
</tr>
<tr>
<td>Lig4</td>
<td>Ligase IV, DNA, ATP-dependent</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair pathway</td>
</tr>
<tr>
<td>Mre11</td>
<td>MRE11 meiotic recombination 11 homolog A</td>
</tr>
<tr>
<td>MRN</td>
<td>Mre11-Rad50-Nbn complex</td>
</tr>
<tr>
<td>MT</td>
<td>Tail moment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NBN</td>
<td>Nibrin (formerly NBS1)</td>
</tr>
<tr>
<td>NBQX</td>
<td>2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair pathway</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining repair pathway</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>PARP-1</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphatidylinositol-3 (PI-3) kinase-related kinases</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>SQ/TQ</td>
<td>Serine (S) or threonine (T) residues, followed by glutamine (Q) residues</td>
</tr>
<tr>
<td>SSA</td>
<td>Single-strand annealing repair pathway</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-strand break</td>
</tr>
<tr>
<td>SSBR</td>
<td>Single-strand break repair</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TTD</td>
<td>Trichothiodystrophy</td>
</tr>
<tr>
<td>UV(B, C)</td>
<td>Ultraviolet (B, C)</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable (diversity) joining (immune receptor gene segments)</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blotting</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor (a.k.a. Cernunnos)</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma Pigmentosum</td>
</tr>
<tr>
<td>XRCC</td>
<td>X-ray repair complementing defective repair in Chinese hamster cells</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>Phosphorylated H2AX</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer (micron)</td>
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INTRODUCTION
Work in the field of DNA damage and repair has by and large been the province of cancer research and relatively little attention has been given to this topic within Neuroscience. DNA double strand break (DSB) repair in normal mature neurons remains largely unexamined, particularly following neuronal excitation, despite compelling indirect evidence of its importance. This evidence comes from animal models as well as from human pathologies, such as Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington Disease (HD), and Amyotrophic Lateral Sclerosis (ALS), whose onset is believed to be associated with an accumulation of DNA damage or with defects in DNA damage repair (Rolig and McKinnon, 2000; Barzilai, 2007; Rao, 2007; Brasnjevic et al., 2008; Brooks et al., 2008; Coppedè and Migliore, 2009; 2010; Jeppesen et al., 2011). Furthermore, various neurodegenerative diseases, such as Ataxia Telangiectasia (A-T), Nijmegen Breakage Syndrome (NBS), Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS), among others, are directly related to defects in proteins that mediate different mechanisms of response and repair to DNA damage (see Table 1, p. 42).

Due to its role as carrier of the genetic information needed for the proper development and functioning of all living organisms, the preservation of DNA is of paramount importance. This macromolecule is under the permanent attack of both exogenous sources (e.g., ionizing radiation – IR) and endogenous sources (e.g., reactive oxygen and nitrogen species – ROS/RNS) that can damage or alter its sugar backbone and nucleobases in numerous ways (Lindahl, 1993). It is believed that human DNA receives tens or even hundreds of thousands of DNA insults or ‘hits’ per day, and that rats might receive 10 times more than that (Ames and Shigenaga, 1992; Ames and
Shigenaga 1993; Lindahl, 1993; Rich et al., 2000; Slupphaug et al., 2003; Giglia-Mari et al., 2011 and see Table 2, p. 47), with the vast majority of the damage affecting individual bases and causing single strands breaks (SSBs). DNA damage, if left unrepaired, can lead to mutations and disease. Fortunately, evolution has endowed cells with numerous efficient mechanisms to deal with different types of DNA damage, therefore ensuring the continuity of accurate genetic information across generations. Due to the complementary nature of the DNA double helix, damage to single strands and bases can be repaired using the information from the intact strand through efficient mechanisms, such as Base Excision Repair (BER – Fishel et al., 2007). Conversely, DSBs caused by a stalled replication fork or by the occurrence of SSBs in close proximity (Woodbine et al., 2011) are among the most lethal types of DNA damage (Hoeijmakers, 2001; van Gent et al., 2001; Jackson, 2002; Valerie and Povirk, 2003; Helleday et al., 2007). While DSBs represent a small fraction of the daily DNA insults (see Table 2, p. 47), their lack of a guiding template makes them particularly noxious, as they can lead to the development of genomic rearrangements and cancer. The gravity of DSBs is underscored by the finding that in some organisms, the presence of a single, unrepaired DSB suffices to cause cell death (Bennett et al., 1993; 1996). DNA DSB damage gives rise to an intricate and complex series of cellular events, called the DNA Damage Response (DDR), which grants the cell the necessary time to assess and repair its DNA (Zhou and Elledge, 2000). DDR may involve the activation of a cell cycle checkpoint, commencement of transcriptional programs, execution of DNA repair, or if the damage is severe, initiation of apoptosis (Zhou and Elledge, 2000). One of the
challenges of studying DDR is that it is not a single, linear pathway, but rather a complex, multidirectional and simultaneous set of events that is better described as different classes of proteins at different levels, acting as sensors or signalers, transducers and effectors (Zhou and Elledge, 2000). This complexity makes it challenging to establish a clear sequential timeline, as DDR involves numerous proteins that act at different times and places along the repair pathway, sometimes even performing multiple roles (Fig. 1, p. 49). The work described in this thesis focuses on a particular outcome of the DDR: the DSB repair mechanism, particularly through the Non-Homologous End Joining (NHEJ) pathway, with a special emphasis on whatever information is available for neurons. While this narrower view makes it possible to follow a simpler, more sequential set of events, as well as to outline a general timeline of the events following DNA damage, it is important to remember that DNA repair proteins often perform other tasks, and that other events and pathways are taking place, sometimes in parallel, that feed from and contribute to the repair of DNA DSBs.

Sources of DNA damage

Damage to the DNA molecule can originate from exogenous and endogenous sources. Examples for external sources of DNA damage include the ultraviolet (UV) and higher ionizing frequencies of the electromagnetic radiation spectrum, such as X-rays and gamma rays, beta particles (high-speed electrons), alpha particles (Helium nuclei) neutrons, protons, and other heavy ions, as well as from toxins and chemicals (e.g., topoisomerase inhibitors and radiomimetic drugs), heat and some viruses (Roulston et al.,
1999; Ohnishi and Ohnishi, 2004; Rao, 2007). Space radiation can be a significant threat, however, the Earth’s atmosphere can block the ionizing effects of cosmic X-rays, gamma rays and UVC radiation (Goodsell, 2001; Ohnishi and Ohnishi, 2004). Solar UVB radiation can damage DNA, creating pyrimidine dimers (Goodsell, 2001); however, this mostly poses a threat for skin cells. Under normal conditions the skull and the blood brain barrier can act as effective barriers against exogenous sources, except with therapeutic uses of irradiation for brain tumors and of certain drugs (Rao, 1993; Brooks, 2002). Therefore, it is reasonable to assume that in earth-bound individuals, most of the neuronal DNA damage arises largely from endogenous sources.

Endogenous DNA damage arises mainly from ROS and RNS produced by normal cellular metabolic processes as well as pathological stimuli and other agents, and replication stress that causes DNA replication forks to progress slowly or stall (De Bont and Larebeke, 2004; Burhans and Weinberger, 2007). Mature neurons are post-mitotic and thus are not affected by damage caused due to DNA replication errors. Therefore, the main source of DNA damage in mature neurons stems from the effects of endogenous ROS, RNS and free radicals, as well as from the state of oxidative stress, caused when these molecules surpass the cellular antioxidant capacity (Kohen and Nyska, 2002).

**Oxygen, reactive species and oxidative stress**

It is an interesting paradox that oxygen (O\textsubscript{2}) plays such an essential role for aerobic organisms, yet it is inherently dangerous to our existence (Davies, 1995). Due to its high electronegativity (second only to fluorine), O\textsubscript{2} is used by the cell to drive
electrons through the mitochondrial electron transport chain (ETC). During this process of cellular respiration, also called oxidative phosphorylation, the acceptor O₂ molecule is reduced to H₂O by the addition of four electrons, and the proton gradient generated by the electronic movement across the ETC generates the potential energy needed to drive the ATP synthase and generate adenosine triphosphate (ATP). Our reliance on aerobic respiration can be plainly demonstrated by the cell and organismic death that rapidly follows after the process is halted with cyanide or carbon monoxide. However, exposure to O₂ concentrations higher than those of atmospheric levels (~21%) can also lead to injury and death (Halliwell, 1994; Mach et al., 2011). The noxious effects of O₂ result from the reactive species formed within the cell. While the production of ROS can be achieved intentionally, such as in the generation of superoxide (O₂⁻) via NADPH oxidase in neutrophils (Babior, 1999), or from the enzymatic synthesis of nitric oxide (NO•) from L-Arginine (Groves and Wang, 2000; and see Fig. 2A, p. 50), most cellular ROS is produced uncontrollably by the mitochondria. As shown in Fig. 2B (p. 50), a four electron concerted reduction of O₂ to water takes place within the enzyme cytochrome oxidase (complex IV) of the ETC. While mitochondrial ROS can also be produced by the Krebs cycle (Tretter and Adam-Vizi, 2004), the majority of these species arise from the partial reduction of O₂ and electron leakage of electrons from the ETC (mainly complexes I and III) (Reiter et al., 2001; Adam-Vizi, 2005 and see Fig. 2C, p. 50). It is estimated that up to 5% of the O₂ processed by cells during respiration ends up as ROS (Reiter et al., 2001).
Additionally, a non-enzymatic reduction pathway outside of the mitochondria, whereby O₂ is successively reduced one electron at a time, via inadvertent reactions with other reduced electron carriers, leads to the formation of the reactive intermediates O₂•⁻, hydrogen peroxide (H₂O₂) and the hydroxyl radical (•OH) (Davies, 1995).

The addition of one electron to O₂ gives rise to O₂•⁻, and as mentioned, considerable amounts of this radical anion are unintentionally produced from mitochondrial leakage. Superoxide, however, is not considered highly reactive, relative to other species. Furthermore, endogenous enzymatic activity of cytosolic and mitochondrial superoxide dismutases (SODs) – CuZnSOD and MnSOD, respectively – that can reduce O₂•⁻ to H₂O₂, as well as spontaneous dismutation, prevent this molecule from being too damaging to the cell.

Hydrogen peroxide is mainly the product of O₂•⁻ dismutation, however it can also be produced by microsomal cytochrome P450 and from the redox cycling of compounds (Reiter et al., 2001), and like O₂•⁻, is a weak oxidizing agent without major reactivity in vivo. H₂O₂ can react with some thiols and thus inactivate enzymes containing an essential thiol (Reiter et al., 2001). As H₂O₂ is relatively stable, it has time to diffuse across membranes. Due to its permeability and migratory capacities, H₂O₂ is the most likely candidate to cause DNA damage, by using DNA-bound metals as catalysts to produce the highly toxic •OH radical, via the Fenton reaction: Fe(II) + H₂O₂ → Fe(III) + OH⁻ + •OH (Halliwell, 1994). Fortunately, cellular H₂O₂ levels are held in check by the activity of the enzyme glutathione peroxidase (GPx) that uses the tripeptide glutathione (GSH) to reduce H₂O₂ to H₂O. As GSH becomes oxidized to glutathione disulfide (GSSG), the
enzyme glutathione reductase (GSR) can reduce GSSG back to GSH, thus resetting the cycle (Fig. 2A, p. 50).

The hydroxyl radical is the most harmful ROS (Da Silva, et al., 2010), able to react with almost any molecule (e.g., proteins, carbohydrates, DNA, and lipids) at diffusion-limited rates (Halliwell, 1994). Due to its high reactivity, •OH is extremely short-lived, (half life ~1x10^{-9} s, at 37°C), and can only diffuse ~5-10 molecular diameters before reacting with another molecule (Reiter, et al., 2001). Therefore the noxious effects of •OH are limited to the site of generation. When •OH is generated adjacent to DNA, it will attack both the purine and pyrimidine bases producing base modifications, and the deoxyribose sugar, giving rise to strand breaks (Lindahl, 1993).

While the effects of •OH are possibly always detrimental for the cell, other less reactive free radicals may often be useful in vivo. As mentioned, some radicals are produced metabolically in living organisms. For example, the free radical NO• can be synthesized from the amino acid L-arginine by endothelial cells, phagocytes, neurons, and other cell types (Ignarro, 1990; Halliwell, 1994). Nitric oxide can act as a signaling molecule, mediating vasodilation (Ignarro, 1990), and as a neuromodulator and possible neurotransmitter (Snyder, 1992; Rand and Li, 1995; Vincent, 2010). Furthermore, NO• is used for cellular defense; macrophages produce NO• as part of the immune response (Halliwell, 1994).

As mentioned, the cell possesses endogenous defenses against reactive species, however they barely counterbalance the ongoing oxidative and nitrosative attacks of
radicals (Halliwell, 1994). When cellular countermeasures are overcome by ROS and RNS activity, the cell enters a state of oxidative stress.

**Oxidative Stress in the Brain**

While all aerobic cells can suffer from oxidative stress, the mammalian brain is considered to be especially sensitive to oxidative damage. This is due to a number of factors: (i) *The high degree of O$_2$ consumption and high metabolism.* In adults, the brain accounts for <2% of total body weight, yet it consumes ~20% of the total O$_2$, thus processing large amounts of O$_2$ per unit tissue mass. The disproportionate use of O$_2$ and glucose in the brain is due to the large amounts of ATP generated (~4x10$^{12}$ ATP molecules / min) needed to maintain neuronal homeostasis, membrane potential, neurotransmitter release, and other cellular activities (Friedman, 2011). Brain metabolism generates ROS, such as O$_2$•$^-$ and H$_2$O$_2$, and because during periods of higher brain activity, O$_2$ and glucose requirements increase to generate more ATP, more reactive species are also generated (Halliwell, 2006). (ii) *The use of excitatory neurotransmitters, such as glutamate (Glu).* Under normal conditions, extracellular Glu amounts are low (in the order of a few µM), yet constant or disproportionate binding of Glu to ionotropic glutamate receptors (iGluRs) causes excessive and prolonged increases in intracellular Ca$^{2+}$. An increase in Ca$^{2+}$ leads to rises in NO•, via neuronal nitric oxide synthase (nNOS) activation (which in addition to exerting local oxidative damage, can diffuse to other cells in the brain), activation of phospholipase A2 and activation of calpain peptidases, causing further ROS generation (Coyle and Puttfarcken, 1993). Additionally,
rises in intracellular Ca\(^{2+}\) interfere with normal mitochondrial function, causing the formation of mitochondrial O\(_2\)•\(^-\) (Coyle and Puttfarcken, 1993; Halliwell, 2006). An increase in O\(_2\)•\(^-\) leads to the formation of peroxynitrite (ONOO\(^-\)), upon combining with NO• (Beckman and Koppenol, 1996 and Fig. 2A, p. 50). Like H\(_2\)O\(_2\), ONOO\(^-\) is more stable and can diffuse into the nucleus and damage DNA (Burney et al., 1999; Pacher et al., 2007). Additionally, protein oxidation by ONOO\(^-\) and other reactive species can decrease glutamate uptake by glial cells, cause damage to glutamate transporters (thus slowing the clearance of glutamate), and inactivate glutamine synthetase, preventing conversion of glutamate to glutamine and leading to an increase in extracellular glutamate levels (Halliwell, 2006). In this manner, oxidative stress can damage cells and promote the creation of additional reactive species, thus generating a “vicious cycle.”

(iii) There are high levels of iron in the brain. An adult brain contains ~60 mg of non-heme iron, as well as proteins that contain iron, including cytochromes, ferritin, aconitases, non-heme iron proteins in the mitochondrial ETC, and tyrosine and tryptophan hydroxylases (Halliwell, 2006). Iron is more prevalent in certain brain areas, such as the basal ganglia (substantia nigra, caudate nucleus, putamen, and globus pallidus). While most of the iron in the brain is bound to ferritin, hemosiderin and transferrin, numerous insults can lead to the release of iron ions, enabling them to catalyze ROS synthesis. As the cerebrospinal fluid (CSF) has no iron binding capacity, released iron (and copper) can remain for long periods of time acting as catalysts (Halliwell, 2006). (iv) Neurons are rich in oxidizable lipids. There are high levels of polyunsaturated fatty acids (PUFAs) that can be easily oxidized. These lipid
peroxidation products can be deleterious to neurons. A product of lipid peroxidation, 4-Hydroxynonenal (4-HNE), can cause a rise in intracellular Ca$^{2+}$ and cell death via apoptosis or necrosis, based on its concentration (Liu, et al., 2000; Kruman and Mattson, 2002). *(v) Antioxidant defenses in the brain are low.* Despite its high O$_2$ consumption and ATP generation, the brain has significantly lower antioxidant capacity relative to other organs. Compared to the liver, for example, the brain has 10% of the levels of catalase (an enzyme able to decompose H$_2$O$_2$ into O$_2$ and H$_2$O), GPx and vitamin E (Uttara et al., 2009; Friedman, 2011). Furthermore, ascorbic acid (vitamin C), present at high levels in both gray and white matter, can have pro-oxidant activity as well its antioxidant activity (Friedman, 2011). The lack of appropriate endogenous antioxidant in the brain can be seen by the alleviating effect that exogenous antioxidant treatment has on oxidative stress following brain injury (Letechipía-Vallejo et. al., 2001; Mishima et al., 2003; Onem et al., 2006), indicating that neurons are not well equipped for handling high ROS levels. *(vi) Most mature neurons do not divide and must endure for the lifespan of the organism.* The lack of cell division and therefore of DNA replication, means that the neuron is unable to use some of the repair mechanisms that mitotic cells can use to correct DNA damage (see below), leading to a slow accumulation of DNA damage over the years.

**Melatonin as an antioxidant for ROS prevention in the brain**

In addition to its known roles in circadian rhythm regulation, the naturally occurring neurohormone melatonin (N-acetyl-5-methoxytryptamine) has been shown to
be a powerful antioxidant (Tan et. al., 1993, Reiter et al., 2000; 2001; 2007a; Carpentieri et al., 2012). This indole readily crosses the blood brain barrier and is easily absorbed by cells and organelles (Reiter et al., 2007b; 2008). It has been extensively documented that in addition to the direct antioxidant effects of melatonin, such as being able to neutralize many reactive species (such as •OH, O$_{2}^{•−}$, H$_{2}$O$_{2}$, ONOO$^{−}$, and the peroxyl radical), this indole also possesses indirect antioxidant faculties, whereby it can both stimulate gene expression and increase the activity of cellular antioxidant enzymes (such as SOD and GPx), as well as stabilizing mitochondria (Leon et al., 2004; Reiter et al., 2007a).

Due to the low antioxidant capacity of the brain, the ability of melatonin in decreasing low level DSBs formation could prove a powerful neuroprotective therapy in preventing the deleterious effects of faulty DNA DSB repair in neurons. Melatonin is considered to be well-tolerated both by humans and non-humans, and numerous in vivo and in vitro studies exist, where chronic administration of high melatonin doses are given (often for 2 or 3 years), either for basic research, or as part of a treatment against AD, PD, ALS and HD (see (Pandi-Perumal et al., 2012) and references therein). One particular study by Weishaupt and colleagues examined the antioxidant and neuroprotective effects of melatonin administration in ALS. Researchers administered melatonin to cultured motor neurons, to a transgenic mouse model for ALS, and to 31 human patients with sporadic ALS (Weishaupt et al., 2006). In humans, high doses of melatonin (5 mg/kg), were given rectally to 31 ALS patients for up to 2 years. Mice received even higher melatonin doses, ranging from ~88 mg/kg, during 8 to 15 weeks of age, to ~57 mg/kg, between 16 and 20 weeks of age, with no adverse effects. For the in vitro experiments,
melatonin levels reached 50 µM (Weishaupt et al., 2006). While the authors did not elaborate on their choice for *in vitro* doses, the discrepancies between mice and human doses are attributable to the fact that mice can metabolize compounds at least ten times faster than humans (Weishaupt et al., 2006). Interestingly, the choice for rectal melatonin application has the advantage of largely avoiding first-pass liver metabolism, where during a single passage through the liver, most of the melatonin (up to 90%) is cleared (Reiter, 1991). In addition, the half-life of blood melatonin is around 10-40 min (Gibbs and Vriend, 1981; Reiter, 1991), so by providing a rectal delivery, the authors ensured a higher melatonin bioavailability. Literature regarding the bioavailability for melatonin and its metabolites cites measurements from plasma, urine and cerebrospinal fluid (CSF) (Cheung et al., 2006; Tan et al., 2010; Galano et al., 2011). As most of the time measurements arise from these *in vivo* aqueous environments, melatonin amounts are expressed in units of mass over volume (often pg/ml) (Waldhauser et al., 1984; Cheung et al., 2006). In humans and most mammals, the majority of melatonin is secreted in a circadian fashion, with levels that peak during darkness. Pinealocytes from the pineal gland release melatonin into the blood capillaries of the vascular system, where it is mostly bound to albumin. Due to its amphiphilicity, it is likely that melatonin rapidly enters CSF and other bodily fluids, and indeed melatonin has been found in every bodily fluid in which it has been tested (Reiter, 1991). Melatonin can reach the CSF indirectly, after circulating in the blood, targeting the brain ventricles via the choroid plexus (Reiter, 1991); however, it appears that the largest amount of melatonin in CSF is a product of its direct release into the third ventricle via the pineal recess of the pineal gland (Tan et al.,
2010). Until recently, it was thought that the physiological levels of blood melatonin, which can range from a few pg/ml during the day to more than 50 pg/ml at night (depending on age), can be an indicator of the levels throughout the body (Tan et al., 2010). However, these values do not reflect the measured concentrations of melatonin from different tissues or other body fluids, and sometimes these estimates greatly miscalculate in vivo melatonin levels. Data from gut melatonin levels indicate that this organ produces several hundred-fold more melatonin than the pineal gland (Tan et al., 2010). Other measurements from bone marrow, bile, amniotic fluid, and CSF, from humans and other animals, also point to discrepancies with plasma melatonin estimates (Tan et al., 1999a; 1999b; 2010). While data from in vitro experiments exist, its availability is less than that of the in vivo data from humans and whole animal models. Particularly, in vitro data for rat melatonin levels vary greatly, with studies ranging from the picomolar to the molar levels, and across different cell types (Peters et al., 2005; Yoo and Jeung, 2010). For rat brains, melatonin levels may vary even with the region investigated, even in subcellular organelles, making the physiological melatonin levels in the rat brain unknown (Dr. Russell Reiter, personal communication).

**ROS and DNA DSB damage**

As mentioned earlier, reactive species can damage the DNA macromolecule, attacking purine and pyrimidine bases, as well as the sugar backbone (Fig. 3A, p. 53). As seen in Table 2 (p. 47), tens of thousands of base-damage events and strand breaks occur daily for DNA. For the nucleobases, guanine is the most readily oxidized as it has the
lowest reduction potential (1.29 V) followed by adenine (1.56 V), cytosine and thymine (~1.6 -1.7 V) (Steenken and Jovanovic, 1997; and a personal communication with Dr. Miral Dizdaroglu, 2012). Thus, purines oxidize more easily than pyrimidines. This is evident by the greater number of depurination events relative to the instances where pyrimidines are lost of pyrimidine events (Table 2, p. 47). Additionally, oxidative damage to the nucleobases yields numerous products, such as O6-methylguanine, 8-oxoguanine and thymine glycol, among others. As this thesis deals with strand breaks, base damage products are not explored and the reader interested in oxidative base modifications is directed to excellent reviews (Lindahl, 1993; Slupphaug et al., 2003; Evans et al., 2004; Dizdaroglu, 2012; Dizdaroglu and Jaruga, 2012).

Mechanistically, the creation of strand breaks via oxidation occurs following the abstraction of a hydrogen atom from the sugar moiety (Fig. 3B, p. 53). Radical attacks can occur on any of the 2′-deoxyribose carbons, however the ease of hydrogen abstractions correlates with the specific C-H bond strength, and the steric accessibility to the 2-deoxyribose within DNA (Miaskiewicz and Osman, 1994). Hydrogen atoms at positions 4′ and 5′ are more exposed to solvent and thus more accessible to abstraction by reactive species than the other H atoms (Dizdaroglu and Jaruga, 2012). The radical produced at carbon 4′ appears to be the major product generated by hydrogen abstraction from the 2′-deoxyribose in DNA (Miaskiewicz and Osman, 1994). In addition to strand breaks, damage to the sugar in DNA can lead to abasic sites and numerous other products (Dizdaroglu and Jaruga, 2012 and see Fig. 3C, p. 53). When simultaneous breaks occur on two complementary stands of the DNA at locations that are sufficiently close to one
another that base-pairing hydrogen bonds and chromatin structure are insufficient to keep the two DNA ends juxtaposed, DNA DSBs are generated (Jackson, 2002).

**Countering DNA Damage with DNA Repair**

As shown in Table 1 (p. 42), numerous neurodegenerative disorders related to DNA damage are also related to deficiencies in DNA repair elements. DNA repair appears to decline with aging and following injuries that cause neurodegeneration (Ren and Peña de Ortiz, 2002; Intano et al., 2003; Krishna et al., 2005; Rao, 2007), linking deficient DNA repair mechanisms with cell death. The significance of functioning DNA-repair pathways in neuronal survival is emphasized by the irregular neuronal development and vulnerability to injury observed in DNA repair-deficient animal models (Culmsee et al., 2001; Laposa and Cleaver, 2001; Meira et al., 2001; Vemuri et al., 2001) and humans (Roglic and McKinnon, 2000; Brooks, 2002; Rao, 2007; Jeppesen et al., 2011), pointing to a balance between the DNA damage and compensatory repair processes that could impact neuronal survival following injury.

As shown in Table 2 (p. 47), there are different damage events that can occur to DNA bases and backbone. Fortunately, numerous specific DNA repair mechanisms have evolved to deal with such threats (see Table 2, p. 47). There are three main repair mechanisms that deal with the repair of single stranded DNA damage: Base Excision Repair (BER), Nucleotide Excision Repair (NER) and Mismatch Repair (MMR), and two main mechanisms that repair double strand breaks: Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ). Besides these well defined repair pathways,
there are other forms of DNA repair that involve the direct reversal of lesions caused by alkylation and some UV-induced damage that leads to base modifications (Direct Reversal - see Table 2, p. 47), and a group of mechanisms present in replicating cells that allows them to survive some replication blocking lesions (DNA Damage Tolerance). There is evidence that Direct Reversal takes place in neurons and glia (Fishel et al., 2007), however, questions remain regarding the significance of this repair process in neurons and its contribution to neurodegeneration. Direct reversal and DNA Damage Tolerance will not be discussed here and the reader is directed to (Fishel et al., 2007; Eker et al., 2009) or (Andersen et al., 2008), respectively, for more information.

This dissertation focuses on DSB repair, and particularly, on the Non-Homologous End Joining repair mechanism of DSBs, which is the main DSB repair mechanism in mature neurons (Jeppesen et al., 2011). However, the mechanisms for repair of DNA are not discrete, but rather function along a continuum with a large degree of cross-talk and redundancy among them. Therefore, a brief discussion regarding the main repair pathways is presented (BER, NER, MMR and HR) followed by a more detailed account of NHEJ.

**Single Strand Break and Base Repair Pathways – BER, NER & MMR**

**Base Excision Repair – BER**  
Base Excision Repair (BER) is the major repair pathway for ROS-induced DNA damage (Rao, 2007; Maynard et al., 2009; Jeppesen et al., 2011), and it is involved in the largest number of events (see Table 2, p. 47). BER can repair base damage and
modifications caused by oxidation, alkylation or deamination, and is also able to repair apurinic/apirimidinic sites (also called abasic or AP sites) and DNA single strand breaks (SSBs) (Maynard et al., 2009, Robertson et al., 2009). As the majority of the DNA damage in neurons is endogenous ROS damage, BER is the main neuronal DNA repair pathway (Fishel et al., 2007). In addition, as SSBs can give rise to DSBs, the BER pathway plays also a key role in the prevention of DSBs. For the repair of alkylated or oxidated bases, the BER mechanism removes the modified nitrogenous base, creating an abasic site, which is then processed by endonucleases, a repair polymerase and ligase, using the complementary strand as a template, and ultimately leading to the complete restoration of the original DNA sequence (Fig. 4, p. 55). To this end, BER utilizes specialized enzymes, called DNA glycosylases, which can precisely recognize and act upon a particular group of lesions, based on their structure. These enzymes catalize the excision of damaged bases by severing the N-glycosyl bond linking the base to the deoxyribose-phosphate sugar backbone. There are numerous glycosylases, for a variety of damaged substrates, and the interested reader is directed to (Fromme et al., 2004; Altieri, et al., 2008; Robertson et al., 2009) for more information. Glycosylases can be divided into two classes according to their catalytic mechanism: monofunctional and bifunctional. Monofunctional glycosylases catalyze the single-step removal of the damaged base, creating an AP site, while leaving the sugar-phosphate backbone intact. An example of this class of glycosilase is Uracil-DNA glycosylase (UNG). UNG removes uracil bases from DNA, which were created from cytosine deamination or accidental dUMP incorporation. Bifunctional glycosylases can excise the unwanted base,
and they also possess endonuclease activity, and thus are able to sever the backbone, creating a single strand break, that is either 3’, or 3’ and 5’ to the site of damage (Fromme et al., 2004). An example of a bifunctional glycosylase is 8-Oxoguanine DNA glycosylase (OGG1). OGG-1 can remove 8-oxo-7,8-dihydroguanine (8-OxoG), and sever the DNA backbone (see Fig. 4, p. 55). 8-OxoG is one of the most common oxidative lesions to guanine, which can result in a G→T and A→C substitution mutation, leading to cancer and other pathologies (Cheng et al., 1992; Maynard et al., 2009; Kanvah et al., 2010). Research suggests that OGG-1 might play a key role in disease prevention, as it is actively involved in the repair of oxidative damage in the brain (Lin et al., 2000; Fishel et al., 2007). There is evidence that oxidative stress occurs in AD and that the neurotoxicity of amyloid beta peptides might derive from their generation of free radicals (Huang et al., 2004; Leutner et al., 2005; Olinski et al., 2007), and increased 8-oxodG levels have been found in DNA isolated from brain tissues and in leukocytes of AD patients (Migliore et al., 2005). A major decrease in OGG-1 levels has been found in AD patients, relative to controls, pointing to an increase in oxidative stress and a diminished capacity to repair oxidative DNA damage in the pathogenesis of this disease (Olinski et al., 2007). Also, a significant increase in the activity of this glycosylase after ~90 min of ischemia and ~30 min of reperfusion in mice brain suggests that OGG-1 might function in reducing oxidative gene damage in the brain after ischemia-reperfusion (Lin et al., 2000). In humans, Fukae and colleagues observed high levels of the mitochondrial isoform of this glycosylase in the substantia nigra of PD patients, as well as in the pontine nuclei of patients with progressive supranuclear palsy (PSP) and
corticobasal degeneration (CBD). For PSP and CBD, OGG-1 activity was inversely proportional to cell death. The researchers postulated that upregulation of OGG-1 can help the neuronal mitochondria cope with the oxidative damage to mtDNA by boosting BER (Fukae et al., 2007). For monofunctional glycosylases, following the glycosylase reaction, AP endonucleases such as Ape1/Ref-1 (APE1) cleaves 5’ of the AP site, leaving behind a terminal 5’-deoxyribose-phosphate (dRP) abasic residue and creating an SSB, as in the case of bifunctional glycosylases (see Fig. 4, p. 55). SSBs are then processed by short-patch BER, in which a single nucleotide is incorporated, or long-patch BER, in which ~2-12 nucleotides are incorporated (Caldecott, 2008). The choice between short-patch BER and long-patch BER is still under investigation, but it is presumed to be determined by the initial type of damage, protein interactions, the state of cell cycle, and the amount of cellular ATP present (Fortini and Dogliotti, 2007; Robertson et al., 2009). These processes employ factors also involved in DNA replication, such as DNA polymerases δ/ε/β, proliferating cell nuclear antigen (PCNA), Flap endonuclease (Fen1) and DNA ligases I and III. Lastly, DNA polymerases fill the gap and DNA ligase seals any nicks, completing the repair process.

For the repair of directly created SSBs, as the product of endogenous ROS attack on the sugar backbone, the enzyme poly (ADP-ribose) polymerase 1 (PARP-1) binds to the broken DNA strand, creating long polymers of ADP-ribose on itself and other proteins, a process termed poly(ADP-ribosyl)ation. This polymerization results in the rapid relocation of repair proteins such as XRCC1 to the site of the lesion. The final addition of new nucleotides by polymerases and ligation process follows a similar
pathway as described above (Fortini and Dogliotti, 2007; Caldecott, 2008; Maynard et al., 2009; and see Fig. 4, p. 55).

As mentioned above, the importance of BER in protecting neurons against oxidative damage can be appreciated by the evidence linking deficiencies to the components along this pathway to a rise in cell death and neurodegenerative disorders. Work by Fishel and colleagues found that a reduction in Ape1 levels significantly increased neurotoxicity of primary neurons in vitro after exposure to H2O2-mediated oxidative damage, as measured by cell viability assays, caspase 3 activity and γ-H2AX foci. Conversely, researchers also noted that in vitro overexpression of Ape1 in neurons using adenovirus appears to have a neuroprotective effect (Fishel et al., 2007). In humans, mutations in specific repair proteins have been linked with Spinocerebellar Ataxia with Axonal Neuropathy (SCAN1) and Ataxia with Oculomotor Apraxia - Type 1 (AOA1) (see Table 1, p. 42, and references therein). Furthermore, the etiology of major neurodegenerative diseases, such as AD, PD and ALS, HD and Down syndrome, appears to be linked to an increase in DNA damage caused by oxidative stress (Fishel et al., 2007; Rao, 2007; Jeppesen et al., 2011), thus underscoring the role of BER as a main mechanism in the fight against disease. Readers interested in the role of BER in neurodegeneration are encouraged to review (Fishel et al., 2007; Rao, 2007; Jeppesen et al., 2011).

**Nucleotide Excision Repair – NER**

Nucleotide excision repair (NER) is a versatile DNA repair pathway that can handle a wide range of structurally unrelated DNA lesions. Unlike BER, which uses
specific glycosylases to detect particular lesions, NER acts on the removal of lesions that physically distort the DNA double helix, such as erroneous base pairing or physical adducts that hinder DNA duplication and transcription (Nouspikel, 2008). Common examples of these lesions are the cyclobutane pyrimidine dimers and 6-4 photoproducts, induced by ultraviolet (UV) light, as well as chemically induced bulky adducts (Costa et al., 2003). NER is strongly conserved throughout evolution; however, enzymatic differences exist between prokaryotes and eukaryotes. There are two sub-pathways for NER: global genomic repair (GG-NER, or GGR) and transcription-coupled repair (TC-NER, or TCR). GG-NER repairs DNA damage across the whole genome, in a manner independent of transcription, whereas TC-NER specifically removes lesions in areas that are actively undergoing transcription (Altieri, et al., 2008). The basic process of GG-NER involves DNA distortion recognition by a complex composed of XPC (for Xeroderma Pigmentosum, group C), hHR23B and Cen2 that binds to DNA and recruits other NER factors, such as the transcription factor II H (TFIIH), a complex with multiple subunits that is also involved in gene transcription. TFIIH opens a denaturation bubble around the region containing the lesion, by the action of its helicase subunits XPB and XPD. Following denaturation, the damaged strand is identified by XPA and RPA, although TFIIH can also perform this step (Nouspikel, 2008). Subsequently, the damaged strand is cut on both sides of the lesion: XPG cuts the 3′ side and the XPF:ERCC1 heterodimer cuts on the 5′ side, creating an oligonucleotide ~30 bp long that includes the lesion site. Lastly, the gap is filled by DNA polymerases, Pol δ or Pol ε, using the undamaged strand as a template, and ligated by either ligase I or III.
In the case of TC-NER, the exact molecular details have not been fully elucidated (Nouspikel, 2008); however, it is believed that RNA polymerase II serves as the lesion sensor while scanning the transcription strand to synthesize mRNA (Altieri, et al., 2008; Nouspikel, 2008). The presence of a bulky adduct on the transcribed strand stalls the polymerase that recruits several additional proteins, exclusive to TC-NER, such as CSA (for Cockayne syndrome A), CSB, XAB2 and TFIIS. Additional steps are similar to those of GC-NER: formation of the denaturation bubble and lesion demarcation by XPA, RPA and TFIIH, excision of damaged strand, gap filling by DNA polymerase, and sealing by a ligase (Altieri, et al., 2008). Studies show that NER activity is significantly attenuated in terminally differentiated cells, such as macrophages and keratinocytes and neurons at the global genome level, but maintained in expressed genes (Nouspikel and Hanawalt, 2000; Nouspikel, 2007; 2008).

Nouspikel and colleagues have proposed a third specialized sub-pathway that has been observed in neurons and macrophages, called transcription domain-associated repair (DAR) (Nouspikel, 2008). DAR employs factors used in GG-NER, particularly XPC, and unlike TC-NER, which operates on the transcribed strand but not on the non-transcribed strand, DAR operates on both strands of active genes, including regions that are not reached by RNA polymerase II, thus complementing TC-NER (Nouspikel, 2008). Because it can also detect other, non-bulky lesions, NER can act as a backup repair mechanism for BER and Mismatch Repair (Kondo et al., 2010). Defects in NER components give rise to various disorders, such as Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD) and Cockayne syndrome (CS), which also display
neurolopathological features (see Table 1, p. 42 and references therein), and has also been implicated in AD, PD and ALS (Laposa and Cleaver, 2001), possibly due to its BER backup role. The interested reader is directed to (Nouspikel, 2008) for more information on the impact of NER on neurological diseases.

**Mismatch Repair – MMR**

Mismatch repair (MMR) is a conserved mechanism that involves the recognition and repair of an incorrectly paired nucleobase, followed by its excision along with a fragment of DNA from the mismatched base strand and the synthesis of a new strand with the correct sequence. MMR is active during DNA replication when incorrect base pairing, due to erroneous incorporation and subsequent improper proofreading by DNA polymerase, can lead to permanent mutations in future daughter strands. In addition to erroneous base polymerization, mismatches can occur due to nucleotide damage and because of spontaneous deamination of 5-methylcytosine to thymine (G→T mismatch) or cytosine to uracil (G→U mismatch). MMR can also correct extra-helical nucleotide loops (called also insertion deletion loops, IDLs) caused by the spontaneous “slippage” between primer and template strands during DNA replication (Fishel et al., 2007; Altieri, et al., 2008). In humans, the MSH2:MSH6 protein heterodimer complexes recognize base pair mismatches and initiate repair by recruiting additional proteins, such as PCNA, EXO1, RPA, Polymerases \( \delta \) and \( \varepsilon \), DNA ligase, and others, that facilitate the excision, polymerization and ligation of the correct DNA sequence (Marti et al., 2002; Hsieh and Yamane, 2008).

Although post-mitotic neurons do not undergo DNA replication, elements of
MMR are present (Brooks et al., 1996, Marietta et al., 1998, Belloni et al., 1999; Fishel et al., 2007). Most likely, MMR recognizes mismatches arising from spontaneous deamination base modifications or refractory errors left by the BER pathway, such as DNA Pol β (Fishel et al., 2007). Adult rat neurons possess repair activity for correcting G→T and G→U mismatches (Brooks et al., 1996), and display detectable levels of MMR proteins (Marietta et al., 1998). Using immunohistochemistry, Belloni and colleagues observed high levels of MSH2 in the nucleus of neurons of the hippocampus, entorhinal cortex and in the cerebellar granular layer. Researchers also detected a dose-dependent increase of MSH2 immunoreactivity in the hippocampal neurons of the CA3/CA4 fields using kainate-induced seizures (Belloni et al., 1999). Research shows that MMR also functions in stabilizing CTG and CAG DNA repeats; expansion of these repeats is believed to contribute to several human diseases including HD (Fishel, 2007). Therefore, it is likely that neurons are able to use MMR to repair DNA mismatches, and that defects in this repair system might lead to increased neurotoxicity.

**Double Strand Break and Repair Pathways**

DSBs are the most lethal type of damage, and a single unrepaired DSB might lead to cell death (Bennett et al., 1993; 1996). As previously described, the common theme of the excision pathways described above is the repair of lesions to one strand by using the opposite (undamaged) strand as an accurate template. The toxicity of DSBs stems from the fact that, for the most part, damage to both strands leaves the repair machinery without the proper template to reproduce the undamaged genetic sequence. Furthermore,
since DSB ends are also prone to degradation, this type of damage leads to additional loss of information. The presence of DSBs puts the genome at risk of gross genomic rearrangement, which can lead to serious mutations. Due to the severity of DSBs, dedicated cellular repair pathways have evolved to minimize the harmful impact of this lesion.

There are two main pathways that repair DSBs: Homologous Recombination (HR) and Non-homologous End Joining (NHEJ). HR requires a sister chromatid to act as a template for accurate repair, and is thus confined to late S/G2 stages of the cell cycle (San Filippo et al., 2008). NHEJ is active throughout the entire cell cycle, however it is most prevalent during G1 and early S phases (Lieber, 2008), and is therefore the main repair mechanism in non-dividing neurons (Jeppesen et al., 2011). Whereas some researchers also consider microhomology mediated end joining (MMEJ) and single-strand annealing (SSA) as discreet DSB repair (DSBR) pathways (Vilenchik and Knudson, 2003; McVey and Lee, 2008), these pathways can be classified as “sub-pathways” to NHEJ and HR repair systems, based on mechanism and proteins involved. For this thesis, I will focus on explaining NHEJ, as it is the repair pathway involved in mature neurons. HR will be briefly described, since it is less relevant for non-dividing cells. While non-NHEJ pathways are less relevant to terminally differentiated (G0) neurons, a study of these pathways is important in gaining an appreciation for the complexity of events taking place in the cell during DSB repair. This complexity is further emphasized by the fact that many of the repair proteins can participate in more than one pathway and often have redundant roles - an interrelation that is also seen across
pathways, as well as sub-pathways, able to “step in” and compensate for defective mechanisms. In reality, it is very likely that all repair pathways (BER, NER, MMR, HR, NHEJ, etc.) are not isolated and interact along a continuum with ever-fluctuating boundaries (as with most biological events), controlled by cell type, protein levels and cell cycle stage.

**Early Events following DSB Damage – \( \gamma \)-H2AX, ATM and MRN**

There is great overlap in the initial detection and signaling events following DSBs. One of the earliest events to happen after a DSB is the phosphorylation of histone H2AX (named \( \gamma \)-H2AX) across thousands of bases near the lesion site. H2AX phosphorylation occurs within minutes following DSBs by members of the phosphatidylinositol 3-kinase-related kinases (PIKKs); mainly ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK); however, the ataxia telangiectasia and Rad3-related protein (ATR) can also phosphorylate H2AX following stalled replication forks and UV-mediated damage (Stiff et al., 2004; Bonner et al., 2008; Yuan et al., 2010). DSB recognition occurs via the MRN complex (Mre11:Rad50:Nbn) independent of \( \gamma \)-H2AX (Yuan and Chen, 2010). The Mre11 nuclease recognizes the DSB and recruits Rad50 and Nbn (formerly called Nbs1), forming a complex that, together with a second MRN complex, tether the DSB ends together (Rupnik et al., 2008; 2010; Stracker and Petrini, 2011). Nbn recruits ATM, which in addition to phosphorylating H2AX, can phosphorylate a plethora of downstream targets mediating not only DSB repair but also cell cycle arrest. While null mutations to any one of the
MRN proteins is lethal, hypomorphic mutations to Mre11, Rad50 or Nbn lead to disorders with neurodegenerative characteristics, such as A-T like disorder (ATLD), Nijmegen breakage syndrome-like disorder (NBSLD) and Nijmegen breakage syndrome (NBS), respectively, and mutations to ATM give rise to Ataxia Telangiectasia (A-T, see Table 1, p. 42).

H2AX phosphorylation leads to the aggregation of numerous repair related factors, such as MDC1 and 53BP1, as well as MRN and ATM that can subsequently phosphorylate more histones, thus creating a positive feedback loop that amplifies the damage signal (Bonner et al., 2008). Although repair proteins can bind DSBs independently of γ-H2AX, this histone stabilizes these proteins and it is therefore necessary for their retention at the lesion site (Celeste et al., 2003). The choice of DSB repair mechanism is a complex process that is mediated by the interaction between MRN, Ku70/80, DNA-PK and other proteins, as well as by chromatin state and the cell cycle (Shrivastav et al., 2008; Chapman, et al., 2012). In mature neurons, repair is carried out via NHEJ.

**Homologous Recombination (HR)**

Homologous Recombination (HR) is one of the two main mechanisms for the repair of DSBs. HR is largely restricted to late S and G2 phases of the cell cycle, during which it uses the clearly defined undamaged sister chromatid as a template to guide the repair of the DSB-damaged strand. This mechanistic “extrapolation” of the single strand repair mechanism allows HR to be very accurate and has lead to this repair pathway being commonly referred to as “error-free” (Cui and Lieber, 2009; Nagy and Soutoglou,
2009; Bohgaki et al., 2010). However, this is probably not entirely accurate, since a subpathway (Single Strand Annealing – SSA) can lead to genomic loss (San Filippo et al., 2008; Krejci et al., 2012). In addition to DSBs, HR is also employed to repair crosslinks in DNA (highly dangerous lesions that prevent replication and lead to cell death if unrepaired), mediate allelic exchange during meiotic chromosomal crossover, and ensures karyotypic stability by mediating the proper segregation of homologous chromosome pairs (San Filippo et al., 2008). Since mature neurons are arrested in G1, it is likely that they do not employ HR. Furthermore, given HR’s complexity, presenting a complete mechanistic picture of this pathway is beyond the scope of this work. Therefore, a basic recount of this pathway is given, and the reader interested in a detailed account of HR is directed to (San Filippo et al., 2008; Krejci et al., 2012) for comprehensive reviews. Upon occurrence of a DSB, the ends are resected, forming 3’ single-strand DNA (ssDNA) overhangs. HR repair proteins (such as RPA and Rad51) bind these overhangs and mediate the strand invasion of the homologous sections in the sister chromatid. After a successful homology search, strand invasion occurs and DNA synthesis can proceed using the homologous sequence as an accurate template. Various pathways can then ensue leading to crossover or non-crossover of genetic material between chromatids. The reaction is completed by gap-filling DNA synthesis and ligation.

**Non Homologous End Joining - NHEJ**

The non-homologous end joining repair pathway (NHEJ) is referred to as “non-homologous,” because broken DNA ends are directly ligated with minimal or no
homologous template, as opposed to HR, in which the presence of a sister chromatid with a homologous sequence is a *bona fide* template for accurate repair. NHEJ evolved in both prokaryotes and eukaryotes to incorporate the large degree of mechanistic flexibility needed to tackle the random combinations of base and sugar damage present at DNA DSB ends (Gu and Lieber, 2008). This repair pathway was initially discovered in mammalian cells, and it was thought to be only present in eukaryotes - a theory reinforced by the finding that the most studied prokaryotic organism, *Escherichia coli* (*E. coli*), cannot rejoin linear plasmids or ends without cohesive overhangs (Dudásová et al., 2004; Gu and Lieber, 2008; Lieber, 2010). However, further discoveries of homologous mammalian NHEJ proteins present in various bacteria (including *E. coli*) led to the realization that NEHJ is present in all domains of life, including prokaryotes and viruses (Aravind and Koonin, 2001; Dudásová et al., 2004; Pitcher et al., 2007; Gu and Lieber, 2008). It is postulated that NHEJ is conserved in prokaryotes because it can efficiently repair DSBs during periods of long inactivity, when there is a lack of rapid proliferation, for example, during periods of sporulation (Pitcher et al., 2007). Conversely, in HR, active cell division leads to the synthesis of new DNA and the rearrangement of chromatin, allowing the duplicate genome to act as a template donor due to the close proximity of the sister chromatid, and it is the main repair mechanism for actively replicating bacteria, like *E. coli* (San Filippo et al., 2008; Chayot et al., 2010). NHEJ is prevalent throughout all domains of life, yet there are some differences between prokaryotic and eukaryotic NHEJ (see Table 3, p. 48). In prokaryotes, NHEJ DNA repair is performed mainly by two polypeptides, Ku and LigD, that carry out the functions of
end processing, joining and ligation, (Lieber, 2010; Nair et al., 2010; Wright et al., 2010). As Table 3 (p. 48) shows, LigD can perform various steps in the repair pathway. In contrast, NHEJ in eukaryotic organisms makes use of different proteins to perform each step of DSB repair. NHEJ does not rely on a homologous DNA template to guide repair (i.e. as in the case of HR), and thus it is the primary method of DNA repair when one chromosomal copy is present (Lieber, 2010) such as during G0 or G1. NHEJ is present during the entirety of the cell cycle, and it is considered to be the main eukaryotic DSB repair pathway during the G1 phase of the cell cycle (Takata et al., 1998; Rothkamm et al., 2003), as well as the main repair pathway in mature (G0) neurons, because only one copy of each chromosome is present in the cell during these phases (Vyjayanti and Rao, 2006; Jeppesen et al., 2011). Despite its prevalence during these points in the cell cycle, NHEJ is an error-prone repair system (Kuhfittig-Kulle et al., 2007; Sharma, 2007; Lieber, 2010).

Although NHEJ repairs DNA DSBs produced by endogenous and exogenous damaging agents, it also plays a critical role in those instances where DSBs are intended, such as in antigen receptor gene generation, via V(D)J (or somatic) recombination in B or T cells, as well as class switch recombination (CSR), which takes place in B cells following V(D)J recombination (Dudley et al., 2005; Stavnezer et al., 2008). During V(D)J recombination, DSBs are induced by RAG endonucleases in a site-specific manner, presenting hairpin sealed coding ends, and 3’-OH phosphorylated blunt ends, which are joined via NHEJ to form coding and signal joints (Dudley et al., 2005). V(D)J recombination and CSR are essential processes for the immune system to initiate and
adjust its defense against diseases, and it illustrates why disorders involving NHEJ proteins convey organismal immunodeficiency, as well as the radiosensitivity or propensity to DNA damage, caused by a lack of efficient repair. There are several human disorders specifically involving NHEJ components, such as LIG4 syndrome, Artemis-SCID and XLF/Cernunnos-SCID (Kerzendorfer and O'Driscoll, 2009), as well as animal models for SCID (Severe Combined Immunodeficiency), with deficiencies to DNA-PKcs (DNA-PK, catalytic subunit – Culmsee et al., 2001) and Ku (Li et al., 2007). As this thesis deals with neuronal DNA damage and repair in normal cells, the immunological and pathological implications of NHEJ deficiencies are not explored beyond the previous brief description, and the reader is invited to consult the various publications regarding these topics (see Kerzendorfer and O'Driscoll, 2009, and references therein).

The basic mechanism for NHEJ is illustrated in Figure 5 (p. 57). While ample information is available on the shape of DNA DSB ends, this knowledge is constrained by the random interactions of ROS with DNA that can generate unpredicted damaged ends configurations. Therefore, an important aspect of NHEJ is the degree of enzymatic flexibility it possesses in order to process and join these various substrates. Nevertheless, much of the progress in understanding the general mechanism for NHEJ was achieved by studying V(D)J recombination in somatic cells, (Lieber, 2010), and HO endonuclease-induced DSB, in Saccharomyces cerevisiae (Moore and Haber, 1996) where relevant enzymatic activity produces repetitive starting substrates. As with other repair processes, NHEJ utilizes nucleases, polymerases and ligases, to resect, add new base pairs, and restore the phosphodiester bonds, respectively. Due to the existence of homologous
proteins (see Table 3, p. 48), mechanistic similarities across organisms can be inferred. However, I will focus here on mammalian NHEJ and the reader interested in yeast and prokaryotic NHEJ is encouraged to refer to excellent reviews (Lewis and Resnick, 2000; Shuman and Glickman, 2007; Pitcher et al., 2007).

DSB damage ends are believed to be first recognized by Ku, based on its nuclear abundance and high affinity for DNA ends ($K_d \sim 2 \text{ nM}$) (Blier et al., 1993; Lieber et al., 2010). Studies with HeLa cells estimate that around 400,000 Ku and 50,000-100,000 DNA-PKcs molecules are present in the nucleus, therefore, these molecules are always in close proximity to any occurring DSB and are able to quickly recognize them simply by diffusion (Labhart, 1999; Lieber et al., 2010; Lieber, 2010; Lieber, 2011). While DNA-PKcs can recognize and bind to DNA ends directly (Hammarsten and Chu, 1998), the formation of the Ku70:Ku80:DNA-PKcs heterotrimeric complex, also known as the DNA-PK holoenzyme, or simply DNA-PK (Smith and Jackson, 1999; Sartorius et al., 2000; Neal et al., 2011), stabilizes and increases the binding affinity of DNA-PKcs to DSB ends by about 100-fold (Yaneva et al., 1997; Hammarsten and Chu, 1998; West et al., 1998; Lu et al., 2007). The Ku70/Ku80 heterodimer forms a ring around broken DNA double stranded ends (Walker et al., 2001) regardless the presence of 3’ or 5’ overhangs, blunt ends or other terminal configurations (Downs and Jackson, 2004), and serves as a docking station, recruiting DNA-PKcs (and forming DNA-PK) as well as nucleases, polymerases and ligases. Since there are two broken ends, two DNA-PK molecules (one at each end) serve to juxtapose and protect the DNA ends while they undergo repair (Neal et al., 2011). The recruitment of repair factors to each Ku:DNA
complex appears to occur in a non-linear fashion, independently at either end of the DSB lesion (Ma et al., 2004; Lieber, 2008), briefly halting or ending, based on the degrees of annealing and ligation of both strands. Thus, while the “algorithmic” progression of NHEJ calls for removal of damaged bases, resection of single strands, polymerization and ligation, in reality the process does not follow this trend: Strands might be newly synthesized, only to be later oncleaved by nucleases, etc. and this process can continue in random iterations until its completion (Lieber, 2010). The degree of processing or enzyme recruitment is also dictated by the initial substrate, such as the dispensability for Ku when ends share regions of 4 bp microhomology (Gu et al., 2007) or for the enzymatic activity of Artemis:DNAPKcs (Lieber, 2010). While these are all versatile mechanistic strategies, they nevertheless give rise to a number of end products that differ from the initial DNA structure, even after the same initial template (Lieber, 2010) and therefore make this repair mechanism error-prone (see Fig. 5, p. 57).

**DNA-PKcs**

DNA-PKcs (known also as DNPK1 and p460) is the catalytic subunit that together with the regulatory subunit, Ku, forms a ~610 kDa (kilodalton) holoenzyme, named DNA-PK. DNA-PKcs was discovered independently by different labs in the mid to late 1980’s (Smith and Jackson, 1999) and is encoded by the *PRKDC* gene. At around 470 kDa and 4128 aa (aminoacids), this catalytic subunit is the largest kinase known to date and the only one to be activated by binding to DNA ends (Lieber, 2010; Neal and Meek, 2011; Pawelczak et al., 2011). Based on its sequence, DNA-PKcs belongs to the PIKK superfamily of kinases, together with ATM, ATR, mTOR, SMG-1, and TRRAP
Like other PIKKs, DNAPKcs phosphorylates SQ/TQ motifs but not lipids (Hartley et al., 1995; Smith and Jackson, 1999; Mahaney et al., 2009; Pawelczak et al., 2011). Like other PIKKs this kinase possess a FAT domain, a kinase domain (PI3K) and a FAT-C domain. Its FAT and FAT-C domains are believed to be involved in kinase activity, however, the FAT-C domain might also be involved in protein-protein interactions (Lempiäinen and Halazonetis, 2009). Additional studies of its three-dimensional structure, using cryo-electron microscopy, reveal that DNS-PKcs possesses a larger central channel that can accommodate a double strand DNA (dsDNA) molecule, and a smaller channel that could allow passing of ssDNA molecules. This architecture enables the molecule to perform both end processing, as well as repair pathway selection. (Williams et al., 2008).

As mentioned before, while DNA-PKcs can bind to DNA ends directly, the presence of Ku increases its affinity to DNA-PKcs by 100-fold (Yaneva et al., 1997; West et al., 1998). DNA-PKcs binds Ku via the last 12 aa on extreme C-terminus of the Ku80 protein (Gell and Jackson, 1999) and causes it to displace inwards about one revolution, placing itself at the end (Neal and Meek, 2011). One DNA-PK molecule at each end form a stable bridge, that serves to align and prevent further end degradation, while promoting their fusion (synapsis) and the recruiting of repair factors (Neal and Meek, 2011). DNA-PK autophosphorylation at specific residues in a location known as the “ABCDE cluster” causes the dissociation of the DNA-PK complex, granting other enzymes physical access to the DNA strands for processing and repair (Merkle et al., 2002; Ding et al., 2003; Cui et al., 2005; Goodarzi et al., 2006).
DNA-PK is an integral part of NHEJ, as its abrogation causes cells to become increasingly sensitive to DSBs and hinders V(D)J recombination (Collis et al., 2005; Neal and Meek, 2011); however its exact role in NHEJ remains to be elucidated. While DNA-PK has been shown to phosphorylate core NHEJ components, such as Ku70, Ku 80, Artemis, XLF and Lig4, the involvement of the kinase is essential (Neal and Meek, 2011). The kinase activity of DNA-PK on these substrates has been studied in vitro (Smith and Jackson, 1999; Collis et al., 2005), and so far the only in vivo substrate for DNA-PK appears to be itself (Meek et al., 2008; Dobbs et al., 2010). DNA-PKcs has more than 40 in vitro and in vivo phosphorylation sites (Dobbs et al., 2010; Neal and Meek, 2011; Neal et al., 2011) arranged mostly in residue clusters that mediate important aspects of its NHEJ functionality, such as end processing, enzymatic activity, and dissociation, as well as regulating the choice for repair pathway along the cell cycle. In addition to its multiple autophosphorylation sites and its involvement in NHEJ, this large kinase interacts with other cellular processes, like telomere maintenance, gene transcription and apoptosis (Lakin and Jackson, 1999; Collis et al., 2005). Readers interested in DNA-PK phosphorylation sites are directed to an excellent recent review (Dobbs et al., 2010).

It is not known whether DNA-PKcs remains bound to DNA during the whole NHEJ repair process, but its phosphorylation increases the access of other proteins to the damaged ends, suggesting its dissociation from DNA (Lieber, 2010). Furthermore, while its exact physiological role is still investigated, it appears that DNA-PKcs is essential for interacting with other NHEJ elements, such as the nuclease Artemis, the XRCC4:Lig4
ligase complex and XLF (Ma et al., 2002; Goodarzi et al., 2006; Meek et al., 2008; Mahaney et al., 2009).
Focus of this work – DNA DSB Damage and Repair in the Brain following iGLuR activation, and protection against DSB by melatonin

As mentioned above, as most mature neurons are in a state of terminal differentiation (G0) they must endure for decades without proliferation and they do not possess the capacity to thoroughly assess and accurately repair the genomic DSB as well as cycling cells via HR, instead relying on NHEJ to repair DSB lesions (Rothkamm et al., 2003; Sharma, 2007). NHEJ quickly processes and reattaches broken DNA ends, but leads to the introduction of errors into the genome that arise from the removal and processing of damaged DNA ends during lesion repair (Lieber, 2008; Cui and Lieber, 2009; Lieber, 2010). Therefore, normal repair of DNA DSBs in post-mitotic neurons is problematic, as it introduces mutations into the cellular genome that may lead to an accumulation of errors over the life of the neuron.

A prevalent source for the endogenous ROS/RNS mediation of neuronal DNA DSB damage is the activation of iGluRs (Crowe et al., 2006; Crowe and Kondratyev, 2009; Crowe et al., 2011). Excessive iGluR activation following neurological insults, such as ischemia, seizures and traumatic brain injury (Palmer et al., 1993; Chan, 2001; Nishizawa, 2001; Patel, 2004) causes an increase in intracellular Ca$^{2+}$, leading to mitochondria destabilization and the generation of noxious ROS/RNS and oxygen derived species (Imlay and Linn, 1988; Didier et al., 1996; Ermak and Davies, 2002; Patel, 2004; Brennan et al., 2009; Wang and Michaelis, 2010).

The oxidative and nitrosative effect of these species damages proteins, lipids and DNA (Lindahl, 1993; Cooke et al., 2003; Brookes et al., 2004; Szydlowska and
Tymianski, 2010; Dizdaroglu, 2012), and can even lead to cell death - a process termed excitotoxicity (Olney, 1969). The deleterious effects of neuronal excitotoxicity due to excessive iGluR activity have been extensively documented (Olney, 1969; Manev et al., 1989; Olney, 2003; Szydlowska and Tymianski, 2010). However, work from our laboratory (Crowe et al., 2006; Crowe and Kondratyev, 2009; Crowe et al., 2011) has shown that mature cortical neuronal cultures sustain DSB damage even when exposed to sublethal doses of glutamate agonists, as well as in vivo following short duration of seizure activity, which does not result in neuronal death. In these studies, DSB damage was assessed by an increase in the number of \( \gamma \)-H2AX foci, a method widely used as an indicator of DNA DSBs formation. (Rogakou et al., 1998; 1999; Paull et al., 2000; Bonner et al., 2008; Nakamura et al., 2010).

Nevertheless, there is still some controversy regarding the use of \( \gamma \)-H2AX, as studies suggest that \( \gamma \)-H2AX foci might form in the absence of DSBs (Pospelova et al., 2009; Ziegler-Birling et al., 2009; de Feraudy et al., 2010). Furthermore, as the appearance of foci is indicative of DSB damage onset, it is also still a matter of debate whether the disappearance of \( \gamma \)-H2AX foci by itself can serve as a bona fide measurement of DNA DSB repair (Bouquet et al., 2006; Cleaver et al., 2011; Revet et al., 2011). In particular, the dynamics of \( \gamma \)-H2AX foci disappearance has not been clearly established, and it is not known whether \( \gamma \)-H2AX dephosphorylates directly in response to DNA repair or due to other chromatin structural reorganization events (Svetlova et al., 2010). \( \gamma \)-H2AX has been used to monitor repair (Löbrich et al., 2010); however, since H2A.X is present only every 5-10 nucleosomal octamers (Bonner et al., 2008; Cui and
Lieber, 2009), some DSB lesions, particularly those repaired focally by NHEJ, might go undetected by the phosphorylation of this histone (Cui and Lieber, 2009).

In the present work, DSB damage and repair will be assessed with a more direct method, which is implemented for the first time in mature rat cortical neurons in culture. Additionally, the antioxidant properties of melatonin are examined, as a possible mechanism to prevent DSB damage and the accumulation of genomic errors from ROS-mediated DSBs, as a consequence of a faulty repair mechanism in neurons. Lastly, the role of two important proteins involved in DNA DSB repair is examined in the context of neuronal viability.

**Hypotheses**

The first part of this work will test the hypothesis that **mature neurons will repair DSBs following DNA damage caused by non-lethal doses of iGluR agonists.** For this purpose, I examine both DSB damage and repair following ROS-mediated DNA DSB damage in post-mitotic neurons as a result of iGluR activation by non-lethal doses of selective glutamate agonists N-Methyl-D-Aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), using Single Cell Gel Electrophoresis (Ostling and Johanson, 1984) as a quantitative method of measurement independent of γ-H2AX. This assay (also known as the Comet Assay) is a sensitive method to detect single or double stranded DNA damage, under alkaline or neutral pH conditions, respectively, and has been widely used to detect DNA damage since the mid 1980s, mainly in non-neuronal cells (Olive et al., 2001; Olive, 2002; Sutherland and
The second part of this work will test the hypothesis that preventing ROS generation will protect neurons from DSB damage, following iGluR activation. Due to their post-mitotic characteristic, the DNA repair mechanism in adult neurons is inherently error prone. Consequently, prevention of DSB onset should be a logical approach to minimize the deleterious effects associated with DNA damage. Since the iGluR mediation of DNA damage is caused by an increase of Ca\(^{2+}\), that leads to the generation of ROS, I also examine the effects of cellular Ca\(^{2+}\) chelation and the protective action of the naturally occurring neurohormone melatonin (N-acetyl-5-methoxytryptamine), which possesses strong antioxidant faculties (Reiter et al., 2000; 2001; 2007a; Carpentieri et al., 2012) in protecting neurons against DSBs by sub-lethal doses of glutamate agonists.

The last part of this thesis considers the role of ATM and DNA-PKcs in mature rat neurons, examining the hypothesis that inhibition of ATM and DNA-PKcs, two key DNA damage repair proteins, will lead to cell death following “physiological” iGluR activation. As mentioned, ATM is a main kinase involved in cell cycle regulation as well as orchestrating many important elements for the response to DSBs, and DNA-PKcs plays a key role in NHEJ repair. There is a large degree of redundancy and catalytic overlap between these two kinases (Gapud et al., 2011; Gapud and Sleckman, 2011) and, while animal models deficient in each protein exist (Fulop and Phillips, 1990; Barlow, et al., 1996; Blunt et al., 1996; Jeggo et al., 1998; Vemuri et al., 2001; Mashimo et al., 2012), they are viable, pointing to compensatory mechanisms that
allow the cells to survive. These mechanisms likely evolve slowly during conditions of chronic protein deficiency; however, they might not be able to offset the damage induced by the sudden loss of function of either of these kinases. Therefore, for the last part of this thesis, I begin to study whether the effects of a sudden abrogation of protein function (of DNA-PK and/or ATM) impacts cell viability under normally non-lethal levels of iGluR activation.
### Table 1 - Neuropathologies related to defects in the DNA Damage Response and DNA repair.

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Affected gene (Protein)</th>
<th>Function(s)</th>
<th>Associated DNA Repair Pathway(s)</th>
<th>Neurological features</th>
<th>Affected Brain Area</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ataxia telangiectasia (A-T)</td>
<td>ATM (ATM)</td>
<td>DDR Kinase</td>
<td>DSBR (NHEJ, HR)</td>
<td>Cerebellar Ataxia, progressive neurodegeneration.</td>
<td>Cerebellum; extrapyramidal system</td>
<td>(Savitsky et al., 1995; Frappart and McKinnon, 2006)</td>
</tr>
<tr>
<td>Nijmegen breakage syndrome (NBS)</td>
<td>NBN (Nibrin) c</td>
<td>DNA damage sensor, DDR signal transducer, DDR effector, DDR Modulator</td>
<td>DSBR (NHEJ, HR)</td>
<td>Microcephaly, mental retardation.</td>
<td>Cerebral cortex</td>
<td>(Frappart and McKinnon, 2006; Brooks et al., 2008)</td>
</tr>
<tr>
<td>A-T like disorder (ATLD)</td>
<td>MRE11A (MRE11)</td>
<td>Nuclease</td>
<td>DSBR (NHEJ, HR)</td>
<td>Similar to A-T d, but with later onset / slower progression.</td>
<td>Cerebellum</td>
<td>(Frappart and McKinnon, 2006)</td>
</tr>
<tr>
<td>Nijmegen breakage syndrome-like disorder (NBSLD)</td>
<td>RAD50 (RAD50)</td>
<td>Tethering unit of the MRN complex</td>
<td>DSBR (NHEJ, HR)</td>
<td>Microcephaly, mental retardation</td>
<td>Whole brain</td>
<td>(Waltes et al., 2009)</td>
</tr>
<tr>
<td>Spinocerebellar ataxia with axonal neuropathy (SCAN1)</td>
<td>TDP1 (TDP1)</td>
<td>Phosphodiesterase (removal of 3'-PG)</td>
<td>NHEJ, BER</td>
<td>Cerebellar atrophy.</td>
<td>Spinocerebellar pathway</td>
<td>(Inamdar et al., 2002; Brooks et al., 2008; Lebedeva et al., 2011)</td>
</tr>
<tr>
<td>Syndrome</td>
<td>Affected gene (Protein)</td>
<td>Function(s)</td>
<td>Associated DNA Repair Pathway(s)</td>
<td>Neurological features</td>
<td>Affected Brain Area</td>
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<tr>
<td>Ataxia with oculomotor apraxia - Type 1 (AOA1)</td>
<td><em>APTX</em> (Aprataxin)</td>
<td>Hydrolase</td>
<td>NHEJ, BER, SSBR</td>
<td>Early onset ataxia, brain atrophy, cerebellar neurodegeneration, axonal sensorimotor neuropathy.</td>
<td>Cerebellum; nigrostriatal pathway</td>
<td>(Rass et al., 2007; Brooks et al., 2008)</td>
</tr>
<tr>
<td>Ataxia with oculomotor apraxia - Type 2 (AOA2)</td>
<td><em>SETX</em> (Senataxin)</td>
<td>Helicase e</td>
<td>SSBR (BER?), DSBR (NHEJ?)</td>
<td>Brain atrophy, axonal sensorimotor neuropathy, cerebellar atrophy, mild cognitive impairment.</td>
<td>Cerebellum, spinal cord</td>
<td>(Brooks et al., 2008; Jeppesen et al., 2011; Yüce and West, 2013)</td>
</tr>
<tr>
<td>Xeroderma pigmentosum (XP)</td>
<td><em>XP (A-G), XPV</em> (Various)</td>
<td>Various</td>
<td>NER</td>
<td>Progressive neurodegeneration. f</td>
<td>Cerebrum</td>
<td>(Hanawalt and Ford, 2001; Hoeijmakers, 2009)</td>
</tr>
<tr>
<td>Trichothiodystrophy (TTD)</td>
<td><em>XP (B and D), TTD-A</em> (Various)</td>
<td>Various</td>
<td>NER</td>
<td>Mental retardation.</td>
<td>Cerebral cortex</td>
<td>(Hanawalt and Ford, 2001; Hoeijmakers, 2009)</td>
</tr>
<tr>
<td>Cockayne syndrome (CS)</td>
<td><em>CS (A and B), XP(B,D and G)</em> (Various)</td>
<td>Various</td>
<td>NER</td>
<td>Mental retardation. Progressive neurodegeneration, retinopathy.</td>
<td>Cerebral cortex</td>
<td>(Hanawalt and Ford, 2001; Hoeijmakers, 2009)</td>
</tr>
<tr>
<td>Syndrome</td>
<td>Affected gene (Protein)</td>
<td>Function(s)</td>
<td>Associated DNA Repair Pathway(s)</td>
<td>Neurological features</td>
<td>Affected Brain Area</td>
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<tr>
<td>Fanconi anemia (FA)</td>
<td><em>BRCA2, FANC group genes</em> (Various)</td>
<td>Various</td>
<td>HR, NHEJ(?)^h, NER</td>
<td>Microcephaly, ventricular defects.</td>
<td>Brain and spinal cord</td>
<td>(Rorig and McKinnon, 2000; McKinnon, 2009; D'Andrea, 2010)</td>
</tr>
<tr>
<td>Werner syndrome (WS)</td>
<td><em>WRN</em> (WRN)</td>
<td>Helicase, nuclease</td>
<td>DSBR (NHEJ, HR), BER</td>
<td>Progressive neurodegeneration.</td>
<td>Whole brain</td>
<td>(Rorig and McKinnon, 2000; Chen et al., 2003; Das et al., 2007; Singh et al., 2012)</td>
</tr>
<tr>
<td>Bloom syndrome (Bs)</td>
<td><em>BLM</em> (BLM)</td>
<td>Helicase, nuclease</td>
<td>HR, NHEJ</td>
<td>Mental retardation.</td>
<td>Whole Brain</td>
<td>(Rorig and McKinnon, 2000; Chen et al., 2003; Singh et al., 2009)</td>
</tr>
<tr>
<td>Rothmund-Thomson syndrome (RTS)</td>
<td><em>RECQL4</em> (RecQ4)</td>
<td>Helicase, hydrolase</td>
<td>DSBR, SSBR</td>
<td>Cerebral atrophy, mental retardation</td>
<td>Whole Brain</td>
<td>(Larizza et al., 2010; Singh et al., 2012)</td>
</tr>
<tr>
<td>Seckel syndrome</td>
<td><em>ATR</em> (ATR)</td>
<td>DDR kinase</td>
<td>DSBR</td>
<td>Microcephaly, craniofacial abnormalities, mental retardation.</td>
<td>Cerebral cortex</td>
<td>(Brooks et al., 2008; Kerzendorfer and O'Driscoll, 2009; McKinnon, 2009)</td>
</tr>
<tr>
<td>Ligase 4 Syndrome</td>
<td><em>LIG4</em> (LIG4)</td>
<td>Ligase</td>
<td>NHEJ</td>
<td>Microcephaly, craniofacial abnormalities.</td>
<td>Cerebral cortex</td>
<td>(Brooks et al., 2008; Kerzendorfer and O'Driscoll, 2009)</td>
</tr>
<tr>
<td>Syndrome</td>
<td>Affected gene (Protein)</td>
<td>Function(s)</td>
<td>Associated DNA Repair Pathway(s)</td>
<td>Neurological features</td>
<td>Affected Brain Area</td>
<td>References</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------</td>
<td>-------------------------------------------</td>
<td>----------------------------------</td>
<td>-----------------------</td>
<td>---------------------</td>
<td>--------------------------------------------------------------</td>
</tr>
<tr>
<td>XLF/Cernunnos-SCID</td>
<td><em>NHEJ1</em> (XLF / Cernunnos)</td>
<td>Member of Lig4 complex</td>
<td>NHEJ</td>
<td>Microcephaly.</td>
<td>Cerebral cortex</td>
<td>(Brooks et al., 2008; Kerzendorfer and O'Driscoll, 2009; McKinnon, 2009)</td>
</tr>
<tr>
<td>Cerebro-Oculo-Facio-Skeletal Syndrome (COFS)</td>
<td><em>XP (D,G), CSB, ERCC1</em> (Various)</td>
<td>Various</td>
<td>NER</td>
<td>Peripheral neuropathy, demyelination, microcephaly neurodegeneration.</td>
<td>Whole Brain</td>
<td>(Del Bigio et al., 1997; Brooks et al., 2008)</td>
</tr>
<tr>
<td>Primary Microcephaly (PM)</td>
<td><em>MCPH1</em> (Microcephalin)</td>
<td>DDR protein, chromatin structure</td>
<td>DSBR (?)</td>
<td>Microcephaly</td>
<td>Whole Brain</td>
<td>(Trimborn et al., 2004; Xu et al., 2004)</td>
</tr>
</tbody>
</table>

- In some cases, pathway effect is presumed due to protein interaction.
- Overview of features, sometimes defects vary.
- Formerly NBS1.
- No telangiectasia is observed.
- Role still unknown.
- Not present in all cases.
- Only some genes for the FA phenotype have been isolated.
- FA proteins are thought to be involved in the replication fork complex.
- DDR, DNA Damage Response; DSBR, Double Strand Break Repair; NHEJ, Non-Homologous End Joining; HR, Homologous Recombination; SSBR, Single Strand Break Repair; BER, Base Excision Repair; NER, Nucleotide Excision Repair; 3'-PG, 3'-phosphoglycolate.

(Adapted from Rolig and McKinnon, 2000; Hanawalt and Ford, 2001; Brooks et al., 2008; McKinnon, 2009)
**Table 2** – Daily occurrences of nuclear DNA damage in eukaryotic cells and associated repair mechanisms. *

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Type of Damage</th>
<th>Events / cell / day</th>
<th>Associated repair mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-strand break</td>
<td>Backbone gap</td>
<td>&gt;50,000</td>
<td>BER</td>
</tr>
<tr>
<td>Depurination</td>
<td>Loss of Adenine/Guanine</td>
<td>~10,000</td>
<td>BER, NER**</td>
</tr>
<tr>
<td>Depyrimidination</td>
<td>Loss of Cytosine/Thymine</td>
<td>~600</td>
<td>BER, NER**</td>
</tr>
<tr>
<td>Oxidative Lesions</td>
<td>Multiple</td>
<td>~2000</td>
<td>Varies</td>
</tr>
<tr>
<td>O&lt;sup&gt;6&lt;/sup&gt;-methylguanine</td>
<td>Base modification (G)</td>
<td>&gt;3,000</td>
<td>Direct Reversal, MMR**, NER**</td>
</tr>
<tr>
<td>Hydroxymethyluracil</td>
<td>Base modification (T)</td>
<td>~600</td>
<td>BER</td>
</tr>
<tr>
<td>Thymine glycol</td>
<td>Base modification (T)</td>
<td>~250</td>
<td>BER</td>
</tr>
<tr>
<td>Cytosine deamination (Formation of Uracil)</td>
<td>Base modification (C)</td>
<td>~300</td>
<td>BER, MMR(?)</td>
</tr>
<tr>
<td>8-oxo-Guanine</td>
<td>Base modification (G)</td>
<td>~200</td>
<td>BER</td>
</tr>
<tr>
<td>Glucose-6-phosphate adduct</td>
<td>Adduct</td>
<td>~5</td>
<td>NER (?)</td>
</tr>
<tr>
<td>Interstrand cross-link</td>
<td>Cross-link</td>
<td>~10</td>
<td>NER, HR</td>
</tr>
<tr>
<td>Double-strand break</td>
<td>Backbone gap</td>
<td>~10-50</td>
<td>NHEJ, HR</td>
</tr>
</tbody>
</table>

* Table is not comprehensive. Examples represent main sources of damage. ** Backup pathway. BER, Base Excision Repair; NER, Nucleotide Excision Repair; MMR, Mismatch Repair; HR, Homologous Recombination; NHEJ, Non-Homologous End Joining. (Adapted from Lindahl, 1993; Brooks, et al., 1996; Dianov et al., 2000; Vilenchik and Knudson, 2003; Gruver et al., 2005; Grillari et al., 2007; Eker et al., 2009; Kondo et al., 2010; Lieber, 2010; a personal communication with Dr. Yossi Shiloh, 2011; Hashimoto et. al, 2012.)
Table 3 – Prokaryotic and eukaryotic NHEJ enzymes

<table>
<thead>
<tr>
<th>Functional component</th>
<th>Prokaryotes</th>
<th>Eukaryotes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td><strong>Polymerase</strong></td>
<td>LigD (POL domain)</td>
<td>Pol4</td>
</tr>
<tr>
<td><strong>Nuclease</strong></td>
<td>LigD (PE domain)</td>
<td>MRX, FEN-1</td>
</tr>
<tr>
<td><strong>Kinase/ phosphatase</strong></td>
<td>LigD (PE domain)</td>
<td>Tpp1 and others</td>
</tr>
<tr>
<td><strong>Ligase</strong></td>
<td>LigD (LIG domain)</td>
<td>Nej1:Lif1:Dnl4</td>
</tr>
</tbody>
</table>

\(^a\) Involved in V(D)J recombination. Adapted from (Lieber, 2010; Wright, 2010)
Figure 1 – The DNA damage response (DDR). The cellular response to DSB damage is a complex set of cellular responses that seek to restore genomic integrity. These mechanisms are better understood as employing sensor or signaling proteins that initiate the response, mediated by transducer proteins that activate or inhibit effector proteins giving rise to multiple outcomes; one of them being the repair of DNA, but also leading to cell death if the damage is too severe. The figure illustrates the fact that processes can occur simultaneously, that are not necessarily unidirectional and that certain proteins can have more than one role.
Figure 2 (previous page) – Generation of cellular ROS. A) General scheme for generation of cellular ROS and free radicals from molecular oxygen (O\textsubscript{2}), and the relation of nitric oxide (NO•) to the process. This figure exemplifies what is referred to as the “oxygen paradox”: aerobic organisms depend on O\textsubscript{2} for survival, as it is the basis of their metabolism and is required for the generation of energy (ATP) in mitochondria, however cellular and metabolic process generate numerous reactive species from O\textsubscript{2}. Shown also, is the combination of species to form new ones, such as superoxide (O\textsubscript{2}•-) with NO•, to form peroxynitrite (ONOO⁻). The cell does possess endogenous defenses (shown in blue): the enzymes superoxide dismutase (SOD) and glutathione peroxidase (Gpx) can help combat O\textsubscript{2}•- and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), respectively. B) The univalent pathway for the four-electron reduction of molecular O\textsubscript{2}, ultimately generating two water molecules. These reactions normally occur within the enzyme cytochrome oxidase (complex IV) of the electron transport chain (ETC), however one-electron transfers to O\textsubscript{2} can also occur outside of it, via inadvertent reactions with other reduced electron carriers, resulting in partially reduced and reactive species. As seen in panel A, H\textsubscript{2}O\textsubscript{2} can also produced by the enzymatic or spontaneous dismutation of O\textsubscript{2}•-, and the hydroxyl radical (•OH) can be generated by the reaction of iron with H\textsubscript{2}O\textsubscript{2} (Fenton reaction). C) Superoxide can leak from mitochondrial ETC complexes I and III. This molecule can be dismutated by the mitochondrial Manganese SOD (MnSOD), or by the cytosolic Copper Zinc SOD (CuZnSOD). Dismutation of O\textsubscript{2}•- creates H\textsubscript{2}O\textsubscript{2}, a more stable molecule. However, while H\textsubscript{2}O\textsubscript{2} is less reactive, it can travel farther and readily cross membranes such as the nuclear membrane where, via Fenton chemistry, create the highly reactive
•OH and damage DNA. OH⁻, hydroxide; HO₂*, hydroperoxyl radical; GSH; reduced glutathione; GSSG, oxidized glutathione (glutathione disulfide); GSR; Glutathione reductase; ONOOH, peroxynitrous acid.
Oxidative Damage

Sugar-phosphate backbone

Oxidative Damage

Phosphoglycolate

Base Propenal

2,5-dideoxypentos-4-ulose
(as an end group)

2,3-dideoxypentos-4-ulose
(as an end group)

2-deoxytetradialdose
(as an end group)

3'-phosphoglycolate
(as end group)

5'-aldehyde
(as end group)
Figure 3 (previous page) – Oxidative damage to DNA. A) ROS and free radicals can attack the DNA macromolecule at the nucleobases and at the sugar phosphate backbone generating numerous oxidized products. B) Representative mechanism leading to a DNA strand break: a radical attacks the C2 of the sugar moiety, abstracting a proton, and leading to a strand break as well as the separation of the base from the molecule. Products of this specific reaction are a 3’-phosphoglycolate end-group and a base propenal. C) Structures of the major oxidatively-induced end products of the 2′-deoxyribose moiety of DNA (from Dizdaroglu and Jaruga, 2012).
Figure 4 (previous page) – Base Excision Repair (BER). BER repairs damaged bases by generating single strand breaks (SSBs) through monofunctional or bifunctional (with lyase activity) glycosylases. SSBs can then be processed by either short-patch BER (where a single nucleotide is incorporated) or long-patch BER (~2-12 nucleotides incorporated), based on the type of damage, protein interactions, cell cycle state and ATP levels. An alternative pathway repairs SSBs that arise directly, mainly as the product of endogenous ROS attack on the sugar backbone. The enzyme poly(ADP-ribose) polymerase 1 (PARP-1) recognizes an SSB, and upon binding to the broken DNA strand, creates long polymers of ADP-ribose on itself and other proteins — poly(ADP-ribosyl)ation. This polymerization results in the rapid relocation of repair proteins, such as XRCC1, to the site of the lesion. Filled diamond, damaged or non-conventional bases (e.g., uracil); AP, apurinic/apyrimidinic site; filled circle, 5′-deoxyribose-phosphate (dRP) abasic residue; thick lines, incorporated nucleotides; P, phosphate; UA, unsaturated aldehyde; PG, 3′-phosphoglycolate (from Maynard et al., 2009).
Figure 5 – General mechanism of NHEJ. DSB damage can be inflicted by endogenous or exogenous factors. Broken ends are recognized by the Ku heterodimer that bridges the juxtaposed ends and mediates the recruitment of subsequent repair components, such as nucleases, polymerases and ligases. In addition to having multifunctional characteristics, the repair proteins can repeatedly bind to, and process either end independently, conveying the large degree of flexibility needed to deal with different initial DSB scenarios. However, processing and joining of DSBs can give rise to diverse end products that differ from the initial (pre-lesion) DNA strand. See text for additional details. Adapted from Lieber (2010).
MATERIALS AND METHODS
All experimental protocols involving animals were performed in compliance with standards set by the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC), and approved by the Georgetown University Animal Care and Use Committee (GUACUC).

Cortical Neurons

Cortical neuronal cultures were obtained from rat embryonic cortices as described previously (Movsesyan et al., 2004; Crowe et al., 2006). In brief, cortices from embryonic day 17-18 (E17-E18) Sprague-Dawley rat embryos (Charles River, Wilmington, MA) were dissected and cleaned from their meninges and blood vessels in Krebs-Ringers bicarbonate buffer containing 0.3% bovine serum albumin (BSA, Sigma-Aldrich Aldrich, St. Louis, MO). All cortices were then minced and dissociated in the same buffer with 1800 U/ml trypsin (Sigma-Aldrich) at 37°C for 20 min. Following the addition of 200 U/ml DNase I (Sigma-Aldrich) and 3600 U/ml soybean trypsin inhibitor (Sigma-Aldrich) to the suspension, tissues were triturred through a 5 ml pipet. The supernatant was then gently centrifuged through a 4% BSA layer. The cell pellet was resuspended in Neurobasal Medium (NBM - Life Technologies, Carlsbad, CA), supplemented with 1% antibiotic-antimycotic solution (Life Technologies), 25 μM Sodium Glutamate, 0.5 mM L-Glutamine, and 2% B-27 Supplement (Life Technologies). Cells were seeded at a density of 5x10⁵ cells/ml onto 6- or 24-well tissue culture plates (Corning Inc., Corning, NY) pre-coated with Poly-D-lysine (70-150 kD, Sigma-Aldrich) at 1.0 mg/ml in 0.1M borate buffer, prepared by adding 1.24 g H₃BO₃ and 1.9 g sodium
tetraborate (both from Sigma-Aldrich) in 400 ml ddH$_2$O, pH 8.5; or at 7.5x10$^4$ cells/ml on 12 mm Poly-D-lysine pre-coated glass coverslips (Fisher Scientific, Pittsburgh, PA, USA) in 24-well plates (Corning), and placed in a humidified incubator at 37ºC and 5% CO$_2$. On day 4 in vitro, and every 3 days after that (DIV4, 7 and 11), NBM media without B-27 supplement and sodium glutamate, to avoid selection of neurons resistant to glutamate, was added to the cultures in a 1:3 proportion, until DIV14, when experiments were conducted. This cell culture technique yields almost pure (91 ± 5% of cells were neuron specific enolase-positive) cortical neuronal cultures, and has been used extensively in our laboratory for many years (Yakovlev et al., 2001). Cell culture purity was confirmed by staining with Iba1 (microglia), GFAP (astrocytes) and Class III β-Tubulin (neurons) (see Immunocytochemistry methods, for antibody amounts).

Pregnant H2AX mutant mice dams were a kind gift from Drs. Andre Nussenzweig and Bill Bonner at the National Institutes of Health (NIH – Bethesda, MD). E16 mouse pup cortical neurons were processed in a similar fashion as rats, and using the same reagents, however, due to genotypic differences, each brain was processed individually and kept in a separate reservoir. After genotyping, cells were identified and the media was changed in a similar manner as that described for rat pups. Experiments were performed at DIV14, as well.

Astrocytes

Astrocytes were prepared from the cerebral cortex of 1–2 day-old SD rats according to an established protocol (Avdoshina et al, 2010). In brief, the cortex was
dissected and cleaned from the meninges. The tissue was mechanically dissociated by through a 5 ml serological pipette. Cells were seeded on poly-L-lysine pre-coated tissue culture flasks (Corning) in Dulbecco's Modified Eagle Medium (DMEM)/F12 containing 10% fetal bovine serum (FBS), 2% antibiotic–antimycotic, and grown at 37°C in a 5% CO₂ / 95% air atmosphere. The culture medium was replaced twice a week. Starting on DIV6, the flasks were continually shaken at 200 rpm for 4 days. Cells were then trypsinized for 5 min, collected and centrifuged at 400×g for 8 min. The pellet was re-suspended in DMEM/F12 containing 10% FBS, 2% antibiotic-antimycotic and plated in cell culture flasks. Cells were grown until cultures reached ~80% confluency.

**PC12 Cells**

Rat PC12 adrenal cells were a kind gift from Dr. Christopher Albanese in the Department of Oncology, Georgetown University. Cells were cultured onto cell culture flasks in DMEM with high glucose, L-glutamine, and sodium pyruvate (Life Technologies), supplemented with 10% FBS (Life Technologies), 1% Penicillin/Streptomycin (Life Technologies), 44 mM NaHCO₃, and 25 mM HEPES, pH to 7.3 with NaOH. Cells were maintained in a humidified incubator at 37°C in a 5% CO₂ atmosphere and passaged 1:20 with Ca²⁺- and Mg²⁺-free Dulbecco's Phosphate-Buffered Saline (DPBS Life Technologies) weekly. Cells were subcultured onto Poly-D-lysine-coated glass coverslips for experiments.
Ionizing Radiation (IR)

Cultures were exposed to different amounts of gamma radiation (see Results) using Georgetown University’s J. L. Shepard Mark I $^{137}$Cs irradiator (San Fernando, CA). Following IR treatments, cells were incubated at 37ºC in a 5% CO$_2$ atmosphere for varying times as indicated.

Drugs

Cells were pulse-treated with N-Methyl-D-aspartate (NMDA) (Sigma-Aldrich) dissolved in DPBS; or 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) dissolved in 1% dimethyl sulfoxide (DMSO – both from Sigma-Aldrich) in DPBS and incubated, at 37ºC with 5% CO$_2$, for varying doses and times (see Results). Glutamate (Sigma-Aldrich) was dissolved in DMSO and given as described in the results section. NMDA and AMPA treatments were stopped with their respective antagonists MK801 (20 µM) or NBQX (20 µM - both from Sigma-Aldrich) to minimize cell death. AMPA experiments were performed in the presence of MK801 (20 µM), to block indirect NMDA receptor activation. Drug treatments were consistent with previous laboratory work and were established to cause minimal neuronal death (Crowe et al., 2006). The calcium chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis(acetoxyethyl ester) BAPTA-AM (Sigma-Aldrich) was prepared in DMSO and diluted in culture medium (final concentration of DMSO in cultures was < 0.1%). Pre-treatment with N-acetyl-5-methoxytryptamine (melatonin - Sigma-Aldrich) was done by incubating the cells at varying doses (see Results) for 30 min before treatments.
Melatonin was dissolved in DMSO and further diluted in DPBS, so that DMSO was at ≤1%. The kinase inhibitors KU60019 and NU7441 (Tocris Bioscience, Minneapolis, MN) used to block the actions of ATM and DNA-PKcs, respectively, were diluted in DMSO and further diluted with cell culture media, and added to the cultures 1 h prior to treatments, as indicated (final concentration of DMSO in cultures was < 0.1%). Control groups were treated accordingly with equal volumes of their respective vehicles.

**Neutral Comet Assay - NCA**

The CometAssay® kit (Trevigen Inc., Gaithersburg, MD) and protocol were used with some modifications. Cells in 60-mm plates were washed of their media, rinsed with ice cold DPBS (Ca²⁺ / Mg²⁺ free), gently scraped and transferred to a centrifuge tube where they were pelleted. Pellets were washed in ice-cold DPBS and cells were resuspended at 1 x 10⁵ cells/ml in ice-cold DPBS. Cells were combined with molten low-melting-point agarose at 37°C (LMAgarose - Trevigen) at a ratio of 1:10 (v/v), and 50 µl of the cells/LMAgarose mixture was spread onto special slides (CometSlides - Trevigen). After cooling at 4°C for 10 min to allow LMAgarose to solidify, slides were placed in lysis buffer (Trevigen) overnight. Following lysis, cells were washed with 1X TBE buffer (Cellgro Mediatech Inc., Manassas, VA) and placed in an electrophoresis chamber (CometAssay™ tank - Trevigen) with TBE buffer. Electrophoresis was done at 1 V/cm for 30 min at 4°C. After electrophoresis, slides were washed twice with ddH₂O for 10 min and dehydrated with 70% EtOH (Sigma-Aldrich) for 5 min. Slides were placed in a dry oven at 45°C until dry (~15 min). Subsequently, cells were stained with SYBR-
Green (Trevigen) for 10 min, air-dried and stored in the dark with desiccating material until imaging.

**Immunocytochemistry - ICC**

Cells grown on poly-D-lysine coated coverslips in 24-well plates were rinsed 3 times in ice-cold DPBS, then fixed in ice-cold 4% formaldehyde for 20 min at 4°C. After fixation, the coverslips were rinsed 3 times in ice-cold DPBS and the cells were permeabilized in methanol for 10 min at -20°C. Cells were rinsed again 3 times in ice-cold DPBS and incubated in blocking solution, consisting of 0.05% Triton X-100 and 5% normal goat serum (Sigma-Aldrich) in DPBS, for 2 h at 4°C. Primary antibodies were added to the cultures and the cells were incubated for 24-48 h at 4°C. Primary antibodies were as follows: mouse monoclonal anti-phospho-H2AX (Upstate Biotechnology), 1:500; rabbit polyclonal anti-GFAP (Abcam, Cambridge, MA), 1:1000; mouse monoclonal anti-Iba1 (Abcam), 1:1000; rabbit monoclonal anti-class III β-tubulin (Millipore, Billerica, MA), 1:250; mouse monoclonal anti-Rad51 (Abcam), 1:250; mouse monoclonal anti-ATM (Abcam), mouse monoclonal anti-ATM (pSer1981) (Abcam), 1:250; mouse monoclonal anti-DNAPKcs (Abcam), 1:500; rabbit polyclonal anti-DNA-PKcs (pThr2609) (Acris Antibodies, San Diego, CA), 1:500. Antibodies were diluted in blocking solution. Primary antibodies were omitted as a negative control. Coverslips were washed 3 times in DPBS for 5 min, and then incubated for 1.5 h at room temperature with secondary antibodies (Alexa Fluor 488 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 568 goat anti-rabbit,
Alexa Fluor 633 goat anti-mouse or Alexa Fluor 633 goat anti-rabbit) depending on the host, all from Life Technologies, at 1:500 in Blocking Solution. Cells were then washed 3 times in DPBS for 5 min, counterstained with DAPI to visualize nuclei (1:10,000 - Life Technologies) for 5 min, and washed 3 more times in DPBS followed by 3 times in ddH₂O water. Coverslips were mounted onto glass slides using Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences).

Epifluorescent Microscopy

NCA slides were imaged using a Nikon E600 upright fluorescent microscope with a 10X, 0.3NA objective (Nikon Inc., Melville, NY) and equipped with a Nuance digital camera (CRi, Hopkinton MA).

Confocal Microscopy

Images were captured using an Olympus FV300 Scanning Laser Confocal Microscope (Olympus, Center Valley, PA) with a 60X, 1.6NA, oil-immersion lens with a 2X digital zoom. The laser intensity and gain were optimized and kept constant among all experimental groups. Images were captured sequentially for each fluorescent label to minimize bleed-through between the channels.

Multiphoton Microscopy

Samples were imaged with a 63X, 1.3NA, oil-immersion lens on a Zeiss 510LSM/META/NLO live imaging, multiphoton microscope (Carl Zeiss, Jena
Germany), equipped with a Chameleon XR titanium-sapphire tunable laser (Coherent Inc., Santa Clara, CA).

**Image Analysis**

For NCA, acquired TIFF images were converted to 8-bit bitmap images with ImageJ (NIH). Images were spatially calibrated using a known distance from an acquired stage micrometer image, and scored using the CometScore™ software (TriTek Corp., Sumerduck, VA). Tail Moment \( M_T = \text{tail length in } \mu \text{m} \times \% \text{ of tail DNA/100} \) was used as an indicator of DNA damage and repair (Ashby et al., 1995), where \( M_T \) is directly proportional to damage. DNA repair was assessed by measuring \( M_T \) decrease, following the highest damage time-point. Comets that showed apoptotic morphology, with a drop-shaped tail that is separated from the head (Wada et al., 2003), were excluded (<5% of imaged cells). For γ-H2AX foci, quantification was performed using the publicly available software FociCounter (Jucha et al., 2010). Foci were counted from DAPI-stained neuronal nuclei, in which the entire nucleus was visible and free of apoptotic features. Optical density threshold and measurement parameters were determined by visual inspection after comparing them to manual counts, performed as described in Crowe et al. (2006), and these settings were kept constant between treatment groups. Treatment with 100 µM H\(_2\)O\(_2\) was used as a positive control for DSB induction and untreated cells were used for estimating basal foci levels. Images were taken from randomly chosen fields from coverslips of sister cultures.
ROS Measurement

Primary cortical neurons were plated at a density of 5 x 10^5 cells / well onto glass bottom 24-well black plates (VWR International, Radnor, PA), as described above. Cells were washed once with pre-warmed (37°C) Hank’s Balanced Salt Solution (HBSS – Life Technologies) and incubated with the cell-permeable dye 2’,7’-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich) in HBSS (Life Technologies) at a concentration of 7.5 µg/ml for 45 min at 37°C in the dark. DCFDA is a cell permeable fluorogenic dye capable of measuring hydroxyl, peroxyl and other intracellular ROS activity. After diffusing into the cells, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into the fluorescent form 2’, 7’ – dichlorofluorescin (DCF). An increase in fluorescence is proportional to ROS levels (Eruslanov and Kusmartsev, 2010). This makes DCFDA a quick and reliable method to assess ROS in the cellular milieu. After incubation, DCFDA treated cells were washed twice with warm HBSS and treated with an NMDA-pulse wash (20 µM) for 15 min in HBSS, stopped with MK801 (20 µM). Pre-treatment with melatonin was done by incubating cells with 1 mM of the indole in cell culture media for 30 min, prior to the loading of the dye. Cells were washed twice with warm HBSS, and incubated for an additional 10 min in 37°C HBSS in the dark before measurements. H₂O₂ (100 µM) was used as a positive control for ROS. DCFDA fluorescence intensities were measured from triplicates, using a Bio-Rad Fluoromak™ plate reader (Hercules, CA) with excitation set at 485 nm and emission at 538 nm; gain 20. An increase in DCFDA relative fluorescence units (RFUs) is proportional to ROS increase.
**Lactate Dehydrogenase (LDH) cell viability assay**

Since following glutamate receptor activation, neurons can die via necrotic or apoptotic pathways (Nicotera et al., 1999), LDH release was used to measure cell death. (Decker and Lohmann-Matthes, 1988). LDH release was assessed using the CytoTox-96® non-radioactive cytotoxicity assay kit (Promega, Madison, WI) according to protocol provided by the manufacturer. Absorbance was measured at 490 nm, using a VersaMax™ microplate reader (Molecular Devices, Sunnyvale, CA). Control LDH levels were measured in cultures that had not received treatment, and against maximum LDH release achieved by cell lysis.

**Mitochondria membrane potential assessment with JC-1**

The dye 5,5′,6,6′-tetrachloro1,1′,3,3′tetramethylbenzimidazolylcarbocyanine-iodide (JC-1) is a cationic molecule that accumulates in mitochondria (Smiley et al., 1991; Eide and McMurray, 2005; Chazotte, 2011). This permeable dye is widely used in studies that examine mitochondrial health and apoptosis. At low concentrations JC-1 exists as a monomer that yields green fluorescence, in a manner similar to fluorescein. At higher concentrations, JC-1 forms J-aggregates that exhibit a broad excitation spectrum and an emission maximum at ~590 nm (Smiley et al., 1991). Healthy mitochondria display a higher red/green ratio and conversely, mitochondrial depolarization can be assessed by a decrease in the red/green fluorescence intensity ratio. For neuronal labeling with JC-1, cell culture medium was aspirated from neurons grown on glass bottom multi-well plates (MatTek Corporation, Ashland, MA) and replaced with
JC-1 (Life Technologies) initially dissolved in DMSO at 1 µg/ml in DMSO and diluted to a 1 µg/ml working dilution in cell culture medium at 37°C. Cells were incubated with the JC-1 labeling solution for 20 min at 37°C in a cell culture incubator. The JC-1 labeling solution was aspirated, and cells were rinsed three times in cell culture medium without JC-1. Cells were imaged in the multiphoton microscope with excitation 488 nm and emissions 515/545 and 575/625 nm.

**Lentivirus Preparation**

Lentiviral vectors were generated with the help of Dr. Seung T. Lim (Department of Neuroscience, Georgetown University) following a method described elsewhere (Tiscornia, et al., 2006). Four sequences corresponding to rat DNAPKcs, were obtained from OriGene Technologies (Rockville, MD) and cloned into the pGreenPuro™ expression lentivector (System Bioscience), using the vendor’s instructions. Each template sequence consisted of two complementary oligonucleotides – the top strand and a complementary bottom strand. The pGreenPuro™ vector is designed to express a single-stranded small hairpin RNA (shRNA) sequence with a fold-back stem-loop structure (or “hairpin”) from an RNA polymerase III H1 promoter (Abbas-Terki, 2002; Qin, 2003; Wiznerowicz, 2003). The expression vector was linearized using EcoRI/BamHI (New England BioLabs, Ipswich, MA) and shRNA template sequences were designed to directionally insert between the BamHI and EcoRI nucleotide overhangs. Template sequences for top and bottom strand oligos were annealed using a thermocycler and ligated into the linearized expression vector using T4 DNA ligase (New
England BioLabs) overnight at room temperature. Ligation was confirmed by electrophoresis using a 1% agarose gel. Transformation of the ligation products was done using DH5-α competent E. Coli cells (kindly provided by Dr. Lim), by combining them in eppendorf tubes in ice, and heat-shocking the cells at 37°C for 45 s. LB broth (Sigma-Aldrich) without antibiotics was added to each tube, and the cells were incubated at 37°C in a shaking incubator for 1 h. Cells were seeded onto LB agar plates with 50 µg/ml ampicillin (Sigma-Aldrich) and incubated overnight at 37°C. Subsequently, 10 well-separated colonies were randomly picked up from each plate, and each clone was grown in 100 µl of LB broth with 50 µg/ml ampicillin at 37°C for 6 h with shaking. To check for clones with the target shRNA template, samples from each clone were sent for overnight sequencing at a commercial vendor (Genewiz Inc., Germantown, MD) following the vendor’s instructions, while the rest of the samples were kept at 4°C. Upon obtaining of a successful sequence, the clone was amplified by overnight incubation in 200 ml of LB broth with ampicillin, at 37°C with shaking, and a maxipreparation of plasmid DNA was done using a commercial kit (Qiagen, Venlo, The Netherlands) following the vendor’s instructions. DNA concentration and purity was measured using a spectrophotometer (Thermo Scientific, Waltham, MA). A negative control lentivector, expressing a scrambled sequence (provided by Dr. Lim), as well as 3 additional lentiviral packaging plasmids, pPACKH1-GAG, pPACKH1-REV and pVSV-G containing the necessary HIV viral proteins and the envelope glycoprotein were constructed in a similar fashion as the expression lentivector. The pPACKH1-GAG plasmid contains the genes for structural proteins (gag), reverse transcriptase and integrase (pol), and envelope (env)
proteins needed for lentiviral production. The pPACKH1-REV plasmid contains the regulatory protein (rev) needed for HIV replication. pVSV-G expresses the envelope glycoprotein of the vesicular stomatitis virus (VSV-G), that mediates viral entry into the target cell (Burns et al., 1993). Pseudoviral particles were assembled using the calcium phosphate method as described elsewhere (Kingston et. al., 2003) and transfected onto 293FT cells (Life Technologies) plated on T-75 flasks (Fisher Scientific) with DMEM / 10% FBS (Life Technologies) and incubated overnight at 37°C / 3% CO₂. Transfection efficiency (>90%) was checked with a fluorescent microscope, as the lentivector also expresses an EGFP reporter. Old media was removed and replaced with fresh DMEM / 10% FBS. Cells were placed back in the incubator, overnight at 37°C in 10% CO₂. Media containing the secreted lentiviral particles were pooled from the flasks, fresh medium was added and the flasks returned to the incubator overnight. As the collected suspensions contained infectious lentiviral particles, supernatants were kept at 4°C in a flask with secondary containment. Subsequently, media was collected again in the same fashion and pooled with the first harvest media. Virus containing media were cleared of debris by filtration through a 0.45-µm filter (Fisher Scientific). Viral particles were concentrated by ultracentrifugation (70,000×g for 2 h at 20°C), using sterile conical tubes (Beckman Coulter Inc., Brea, CA), and subsequently purified by ultracentrifugation (50,000×g for 2 h at 20°C) through a sucrose layer (20% sucrose in HBSS – Life Technologies). Viral particles were resuspended onto HBSS, aliquotted, and kept at -80°C until use. Viral titration was done by adding 20 µl of each serial dilution (1, 1:10, 1:100, 1:1000 and 1:10,000) onto poly-l-lysine coated plates, containing 1x10⁵ neurons.
(DIV14) and calculating the percentage of GFP-positive cells 24 h post-infection. Dilutions yielding >70% GFP positive cells were used. Gene knockdown was assessed 72 h post-infection, by western blotting of infected neuronal lysates, probed against DNA-PKcs (see following section on Immunoblotting). Out of the four DNA-PKcs sequences, one sequence (5’-GGAGAAGACAAACCAGTCTGCTGCTAACCATT-3’) was successful in producing an effective knockdown of DNA-PKcs in rat neuronal cultures.

Immunoblotting

Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer supplemented with CompleteMini® protease inhibitor and PhosSTOP® phosphatase inhibitor tablets (Roche Applied Science, Penzburg, Germany). Polyacrylamide gel electrophoresis using 4-12% bis-tris gels (Life Technologies) was performed for 90 min at 150V, held constant. Nupage transfer buffer (Life Technologies – 1x containing 10% methanol) was prechilled for at least 1 h before use. Proteins were transferred onto a 0.2 micron nitrocellulose membrane (Thermo, Rockford, IL) at 4°C for 25h at 30V constant using a Bio-Rad mini PROTEAN II cell. Membranes were blocked in 5% nonfat dry milk buffer, and incubated overnight at 4°C with primary antibodies for: ATM (pSer1981) (1:500, Abcam), total ATM (1:1000, Cell Signaling, Beverly, MA), DNA-PKcs (1:500 Abcam). As a loading control, all membranes were re-probed with β–actin (1:10000, Sigma-Aldrich) primary antibody for ≥1 h at room temperature. Membranes were subsequently incubated as appropriate, in mouse or rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz Biotechnology, Dallas TX) and
proteins were subsequently detected using HyGLO™ Quick Spray Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific, Metuchen, NJ), prior to film exposure and development.

Genotyping

Small tissue samples (~3 µm²) were collected from each mouse pup, and from the dam (a known heterozygous), and placed in separate eppendorf tubes with 500 µl digestion buffer, consisting of 100 mM Tris-HCl pH, 8.0 (Promega, Madison, WI); 200 mM NaCl, 5 mM EDTA pH 8.0; 0.2% SDS (all from Sigma); and 4 µg Proteinase K (Sigma), and incubated overnight (800 rpm, 55ºC) in a thermomixer (Eppendorf, Hauppauge, NY). Samples were then centrifuged at 16,000×g for 10 min and 450 µl of the supernatant was transferred to a new tube and mixed 1:1 isopropanol (Fisher Scientific) and the tube was carefully inverted 5-6 times. Samples were centrifuged at 16,000×g for 5 min, and the supernatant was discarded, being careful not to compromise the DNA precipitate; 200 µl of 70% ethanol (Fisher Scientific) were added and each tube was carefully inverted 5-6 times. Samples were centrifuged at 16,000×g for 5 min and the supernatant were carefully removed. The remaining pellet were dried in a warm chamber (37ºC) for 20 min, suspended in 100 µl Tris-EDTA Buffer (Sigma Aldrich), vortexed for 1 min and placed at 4ºC overnight. The following primers were custom ordered from Life Technologies: 5'-CTCTTCTACCTCGTACACCATGTCCG-3' (forward H2AX), 5'-CGAAGTGTCGTCCTGCACGACGCGAGC-3' (reverse H2AX) and 5'-GTCACGTCCTGCACGACGCGAGC-3' (reverse upstream neo sequence). For PCR,
two separate reactions are needed: (fwd / rev, and fwd/ rev up neo seq), in order to determine wildtype (WT) and knockout (KO) genotypes, respectively. Each reaction tube contained 12.5 µl GoTaq® Green Master Mix (Promega), 5 µl primer mix, 1 µl DNA and 6.5 µl dH₂O. Following amplification, samples were ran at 90V for 1h through a 1% agarose gel (Life Technologies), with 10% SYBR® Safe (Life Technologies) in TAE buffer. Gels were imaged using a UV transilluminator system (UVP LLC, Upland, CA) with a CCD camera (Hamamatsu Photonics, Middlesex, NJ).

**Statistical Analysis**

Results are presented as mean ± SEM. Means were compared using one-way ANOVA with Tukey’s post-hoc test. Analyses were performed using the statistical software GraphPad Prism (version 5 - GraphPad Software, San Diego, California). Statistical significance was assigned at the p<0.05 level.
RESULTS
1. EXPERIMENTAL OPTIMIZATION AND CHARACTERIZATION OF THE MODEL SYSTEM

1.1. iGluR agonist pulse treatments are sublethal to neurons.

In order to verify the non-lethal nature of iGluR agonist treatments, as well as to show that DSBs were not attributable to an increase in cell death, lactate dehydrogenase release (LDH) levels were measured in DIV14 cortical neuronal cultures, following pulse-treatment with iGluR agonists. Thirty-minute pulse treatments with NMDA (up to 50 µM) and AMPA (up to 25 µM) were stopped with 20 µM of MK801 and NBQX, respectively, and cells were returned to the incubator (37°C, 5% CO$_2$) for 24 h, when LDH assay was performed, as described in methods. Negative controls were treated with the same volume of vehicle. Maximum LDH release was established by measuring LDH levels from cell lysates. LDH levels from NMDA and AMPA treatments were not different from vehicle (see Fig. 6, p. 103). These results suggest that treatments under these conditions are not cytotoxic for DIV14 neuronal cells. Conversely the results also ensure that DSB formation is not caused by ongoing cell death. These results are consistent with previous laboratory findings (Crowe et al., 2006).

1.2. The NCA is a sensitive assay to monitor DNA DSB damage.

In order to create a baseline to assess the sensitivity of the experimental setup, DIV14 rat cortical neurons were exposed to γ-IR – a known inducer of DSBs (Frankenberg-Schwager, 1990). Cells were exposed to 1, 2, 5, 10 or 15 Gy, or sham irradiated (0 Gy), as described in the methods section, and allowed to recover for 30 min
prior to being processed for NCA (see material and methods). As seen in Figure 7 (p. 104), dose response experiments showed that the NCA detects DSBs produced by as low as 1 Gy (Fig. 7, p. 104). Tail moment ($M_T$) analysis showed a clear dose-dependent trend with increasing levels of IR that were significantly different from control (sham irradiated) even at 1 Gy ($P<0.001$, $n=60$ cells). These experiments demonstrate that the NCA is a highly sensitive assay to quantify DSB damage in neurons, able to detect DSBs at levels that are below the detection thresholds of other assays, such as pulsed field gel electrophoresis (Gobbel et al., 1998).

1.3. iGluR-mediated DSB damage is specific to neurons.

Immunocytochemistry (ICC) staining was conducted in order to characterize experimental cell culture populations and to ensure that the majority of cells are neurons. DIV14 rat cortical cells were labeled with antibodies against established markers for neurons (Class III β-tubulin; Don et al., 2004), astrocytes (Glial fibrillary acidic protein – GFAP; Middeldorp and Hol, 2011), and microglia (Ionized calcium-binding adapter molecule 1 – Iba1; Ito et al., 1998), and examined under fluorescence microscopy, as described in methods. As shown in Figure 8 (p. 105), DIV14 neuronal cultures are constituted in large majority by neurons, with very little levels of non-neuronal contamination (<5% glial cells). This culture method, which produces a highly pure yield of neurons, is well established in our laboratory, and the data are consistent with previous laboratory findings (Crowe et al., 2006).

Glutamate induced DSB lesion is receptor mediated, and while these receptors are considered specific to neurons, some studies have shown that NMDA receptors can be
expressed in rodent and human glial cells (Conti et al., 1997; Lee et al., 2010). In one of these studies, researchers using *in-situ* hybridization histochemistry and immunocytochemistry, found that 4% of GFAP-positive cells (a marker for astrocytes), expressed low mRNA levels for the NR1 subunit of the NMDA receptor (Conti et al., 1997). Thus, in order to assess that the NMDA-mediated DSB damage was specific to neurons, and also to ensure that non-neuronal cells (still present in small numbers) would not substantially contribute to the observed effects, NCA experiments were conducted using 30-min pulse washes with NMDA and AMPA (50 µM and 25 µM, respectively; stopped with 20 µM of MK801 and NBQX, respectively) in pure astrocytic cell cultures to determine if they would indeed be a confounding factor to the DSB damage analysis. Astrocytes were subjected to NMDA and AMPA treatments and examined for DSB formation using NCA and relative to IR – a positive control for cellular DSBs. As seen in Figure 9 (p. 106), rat astrocytes showed no increase in NCA $M_T$, relative to controls or to each other, at 1 h and 4 h following iGluR agonist pulse-treatment. In contrast, IR, which indiscriminately causes DSBs to all cells, elicited high levels of DSBs in astrocytes, as measured by $M_T$ increase (Fig. 9, p. 106). These results indicate that non-neuronal cells, even if present in culture in low numbers, would not confound neuronal DSB quantification, when treated with NMDA and AMPA.

1.4. NMDA-induced DSB damage is receptor mediated and correlates with NMDA receptor maturation.

As described in the methods section and in concordance with previous work by the laboratory, experiments were performed on cultures *in vitro* at 14 days as functional
iGluR receptors are fully expressed at this time (Crowe et al., 2006). Additionally, studies by Mizuta and colleagues showed that mice cortical neurons are affected by excitotoxicity in vitro from DIV11 onwards (Mizuta et al., 1998). In order to test that the levels of DSBs induced by sublethal NMDA treatment correlate with mature receptor expression, neuronal cultures were subjected to sublethal NMDA treatments (15 µM) at DIV4 and DIV14. Cells were treated as described, and collected for NCA at 30 min post-treatment. DIV4 and DIV14 $M_T$ values were compared to those of untreated sister cultures, and against IR-treated sister cultures (5 Gy). As seen in Figure 10 (p. 107), DIV4 NMDA-treated cells were not different from controls, and both groups differed from IR ($p<0.0001, n = 70$ cells / group). By two weeks, both NMDA and IR groups elicited a significant response, relative to controls ($p<0.0001, n = 70$ cells / group). At DIV14, NMDA $M_T$ was comparable to that induced by 5 Gy IR (Fig. 10, p. 107). To verify that DSBs are NMDA receptor mediated, DIV14 cells were pulse-treated as before with NMDA (15 µM), with and without the presence of the NMDA antagonist MK-801. For the MK-801 group, mature, DIV14 neuronal cells were pre-treated with 20 µM of the antagonist for 15 min, prior to pulse treatments and compared to regular pulsed treated cells, as described. Control cells were treated with the same amount of vehicle. Cells were monitored for $M_T$ at 15 min, 30 min, 1h, 4h and 6h post-treatment. NMDA-induced DSBs peaked around 1 h post-treatment and decreased slowly, returning to control levels by 6h. In contrast, NCA $M_T$ levels of MK-801 pre-treated cells did not differ from control cells (Fig. 11, p. 108).
1.5.AMPA iGluR-mediated DSB damage is comparable to Glutamate

Following NMDA experiments, the role of AMPA and glutamate in generating neuronal DSB damage was examined. While NMDA receptors are permeable to Ca\(^{2+}\), the permeability of AMPA to divalent cations, such as Ca\(^{2+}\) and Zn\(^{2+}\) is mediated by the GluR2 subunit. AMPA receptors lacking the GluR2 subunit allow for the passage of Ca\(^{2+}\) (Seeburg and Hartner, 2003; Liu and Zukin, 2007); however, as GluR2 subunits are almost always modified via RNA editing, the presence of the edited GluR2 subunit makes it energetically unfavorable for Ca\(^{2+}\) to pass through the channel (Seeburg et al., 2001; Seeburg and Hartner, 2003). Nevertheless, certain neurons in the striatum and cortex can under-edit the GluR2 site, rendering them more susceptible to the passage of Ca\(^{2+}\) and excitotoxicity (Kim et al., 2001). Furthermore, under certain stressful conditions, such as ischemia and seizures, as well as under neuropathological conditions, cells can down-regulate GluR2 expression (Liu and Zukin, 2007). Additionally, since NMDA and AMPA are specific agonists to iGluR receptors, I wanted to compare their response to glutamate, which will indiscriminately target ionotropic and metabotropic glutamate receptors, when released into the synapse.

DIV14 neurons were treated with AMPA or with two concentrations of glutamate (15 µM and 50 µM) to follow the same design used for NMDA: low and high sublethal amounts. For AMPA treated cells, 25 µM AMPA treatments were done in the presence of MK-801, in order to prevent damage by secondary glutamate release (Crowe et al., 2006), and with either a pretreatment of 20 µM NBQX or not (Fig. 12, p. 109). Controls received vehicle treatments (DMSO at the same concentration as the drugs: <1% v/v). Cells were analyzed for NCA at 15 min, 30 min, 1h and 4h post-treatment. As seen in
Figure 12 (p. 109), AMPA-treated cells had high levels of DSB damage, which were prevented in the presence of the specific AMPA antagonist, as evidenced by $M_T$ levels. Additionally, 15 $\mu$M and 50 $\mu$M glutamate treatments elicited a response around the same magnitude than AMPA treatments (Fig 12, p. 106)

### 1.6. Conclusion

Taken together, these experiments show that DSBs caused by iGluR activation are non-lethal to neuronal cells (Fig. 6, p. 103), and that the use of NCA as a $\gamma$-H2AX independent method to assess DSB damage is very sensitive (Fig. 7, p. 104). Furthermore, the effect is specific to neurons in the model used for these studies: DIV14 cultures that are >95% neuronal (Fig. 8, p. 105). Residual glial cells (<5%) do not confound measurements of neuronal DSBs caused by iGluR activation (Fig. 9, p. 106). NCA data confirmed that the damage is receptor mediated and iGluR mediated: 1) due to the lack of $M_T$ increase (indicating DSB induction) following NMDA pulse treatments prior to receptor maturation, around 11 days *in vitro*, while still responding to IR treatments (Fig. 10, p. 107), and 2) following treatments with the specific antagonists MK-801 and NBQX, which completely blocked DSB onset by NMDA and AMPA respectively (as seen in Figs. 11 and 12, pp. 108-109). For all treated cells the peak of DSB damage appeared to be ~30-60 min post-treatment and slowly declined afterwards, as monitored by 4h and 6h post-treatment. In addition, treatment with glutamate also elicited a response. As NMDA and AMPA are not endogenous substances produced in the brain, the data with glutamate show that *in vitro* glutamatergic activity is able to generate similar levels of DSB, and makes a compelling case for the conditions occurring
in vivo in the brain.
2. NMDA- AND AMPA-MEDIATED DNA DAMAGE AND REPAIR USING NCA

Unlike \( \gamma \)-H2AX foci, NCA allows for quantification of DSB repair. Thus, to characterize DSB damage and repair following NMDA and AMPA, the time course of DSB formation and disappearance was examined using NCA.

2.1. NCA analysis of iGluR-mediated DSB damage

Eight-hour time course experiments using pulse treatments with sublethal doses of NMDA and AMPA were conducted to examine their effects on DNA DSB damage. Cells were given 30 min pulses of either 25 \( \mu \)M AMPA or 15 \( \mu \)M NMDA (stopped with either 20 \( \mu \)M NBQX or MK801, respectively), or vehicle treatments (DMSO at the same concentration as the drugs: <1% v/v), and analyzed for NCA. As before, AMPA treatments were done in the presence of MK-801, in order to prevent damage by secondary glutamate release. As shown in Figure 13 (p. 110), both treatment groups showed a similar pattern of increasing DSB damage that peaks at ~1h post-treatment. At peak damage levels, NMDA-treated cells were almost 10 times more damaged, and AMPA-treated cells were ~13 times more damaged, relative to respective vehicle-treated control (Fig. 13, p. 110). Shown on top of the bars, are representative images of single cells with varying lengths of \( M_T \) at specified time points (Fig. 13, p. 110).

2.2. NCA analysis of DSB repair

Subsequently, the repair of NMDA and AMPA DNA damage was analyzed by
using the NCA to assess the disappearance of DSBs, as measured by a decrease in \( M_T \), following the peak damage levels described above. As shown in Figure 13 (p. 110), DNA DSBs repair shows that by 6 h post-treatment, \( M_T \) for NMDA and AMPA treated cells decreased to ~ 5 times that of respective controls. By 8 h post-treatment, AMPA-treated cells showed a return to control levels (p > 0.05), and NMDA-treated cells were also greatly repaired but still showed slightly higher damage levels relative to controls (p < 0.05, Fig. 13, p. 110). This residual damage is consistent with higher levels of \( \gamma \)-H2AX reported by the lab previously (Crowe et al., 2006).

2.3. Adult neurons repair DSBs via NHEJ, not HR

As mentioned earlier, NHEJ is considered to be the DSB repair pathway for mature neurons, since sister chromatids are not present, thus making HR repair an unlikely mechanism (Vyjayanti and Rao, 2006). However, clear evidence of NHEJ in neurons is lacking. Our laboratory had shown co-localization of Mre-11 protein with sites of \( \gamma \)-H2AX foci after low IR levels, and provided the first evidence of neuronal DSB repair following sublethal insults (Crowe et al., 2006). However, Mre-11 is not a specific indicator of DSB repair but (as part of the MRN protein complex) can have multiple DDR roles besides DNA repair, such as telomere maintenance, cell-cycle checkpoint signaling, meiotic recombination and responses to stalled replication forks (Zha et al., 2009; Rupnik et al., 2010).

In order to gain definitive evidence as to the specific DSB repair pathway, which cannot be necessarily deduced by examining Mre11, the use of proteins that are specific to each of the two major DSB repair pathways was considered: DNA-PKcs for NHEJ and...
Rad51 for HR. As mentioned earlier, DNA-PKcs is the main kinase for NHEJ and Rad51 plays a key role in DNA DSB repair by HR, where it coordinates homology search and strand invasion of the sister chromatid (San Filippo et al., 2008; Lieber, 2010). For these experiments, DIV14 neurons were immunolabeled with antibodies against γ-H2AX and either Rad51, or DNA-PKcs (pThr2609), 30 min after sublethal NMDA treatment, as described in the methods section. Additionally, since the HR repair pathway is active in mitotic cells, rat PC12 cells were also labeled for Rad51, 30 min after being treated with 100 µM of H2O2 (see methods) in order to induce DSBs, thus serving as a positive control for HR. Cells were imaged using multi-photon fluorescence microscopy as described in the methods. Figure 14 (p. 111), shows representative images from the immunocytochemical experiments. The first column from the left (panels 14A, 14E and 14I) shows cellular nuclei, as indicated by the staining of the blue fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) (p. 109). The second column from the left (14B, 14F and 14J) indicates the sites of DSB damage, as reflected by γ-H2AX foci in green. The third column from the left indicates pathway selection, as indicated by the staining for DNA-PKcs (14C) or Rad51 (14G and 14K, p. 109). Lastly, the rightmost column (14D, 14H and 14L) is a composite merge of their respective rows. The top and middle rows show mature neuronal cells, and the bottom row depicts undifferentiated rat PC12 cells, used as a positive control for the HR pathway (p. 111). As seen in Figure 14 (p. 111), mature neurons stained for DNA-PKcs show clear nuclear localization (Fig. 14C) as well as co-localization with γ-H2AX foci (Figs. 14B and 14D). However, neurons stained with the HR-specific protein Rad51 stained in a perinuclear pattern (Fig. 14G), indicating cytosolic Rad51 localization, rather than nuclear, where γ-H2AX foci are visible (Figs.
14F and 14H). Conversely, mitotic PC12 cells did show nuclear focal co-localization between Rad51 and γ-H2AX, as well as some extra-nuclear Rad51 staining (Figs. 14K and 14L).

These results confirm that the DSB repair pathway in mature neurons is indeed performed by NHEJ, as it is commonly contended, and demonstrate that HR is not engaged in the DSB repair process, due to the lack of Rad51 in the neuronal nucleus, following NMDA-mediated insults.

2.4. A deficiency in DSB detection increases DSB damage in adult neurons

Previous studies have shown that H2AX−/− mice display growth retardation and immune deficiency (Celeste et al., 2002). Therefore, the effects on neuronal DSB damage and repair following sublethal NMDA doses due to the lack of this histone variant were examined. Cortical neuronal mouse cultures were obtained from H2AX+/+ mice, H2AX+/− mice and controls, as described in the methods. Animals were subjected to 15 min pulse-treatments with 15 µM NMDA. Cells were assayed for NCA at different intervals post-treatment, to determine the degree of damage and repair. As shown in Figure 15 (p. 113), H2AX+/+ and H2AX+/− cells had moderate DSB levels (p<0.05), which peaked around 15 min post-treatment. Conversely, H2AX−/− neurons had a slower progression, but sustained significantly more DSBs, which peaked dramatically at ~1h (p<0.001), in a manner similar to that of the rat neuronal cells (Fig. 13, p. 107). DSB repair did not seem to be affected by the lack of this histone, based on the NCA, and by 6h post-treatment, all 3 groups had returned to control levels (Fig. 15, p. 113). These results indicate that a lack of H2AX renders neuronal cells susceptible to NMDA-
mediated DSB damage, relative to those with either one copy of the gene or WT control mice.

2.5. Conclusion

The experiments described above demonstrate that following pulse-treatment with sublethal doses of iGluR agonists (NMDA and AMPA) DNA DSB damage peaks around ~1h post-treatment and is subsequently repaired, returning to almost baseline levels at ~8h (Fig. 12, p. 109) post-treatment, as measured by the NCA. These results are consistent with previously published results from our laboratory, using γ-H2AX and for the first time provide quantification of DSB repair. As for the pathway employed to repair DSBs, these experiments provide evidence that mature neurons employ NHEJ, but not HR, confirming what is commonly posited (Fig. 14, p. 111). This is also in line with previous laboratory findings, where Mre11 was found to form foci at γ-H2AX sites. However, Mre-11 data are not conclusive proof of pathway specificity due to the plethora of roles the MRN complex has. Data from experiments that use pathway-specific proteins (such as DNA-PKcs for NHEJ and Rad51 for HR), as well as PC12 cells (that can serve as a positive control for DSBs by HR), shows that neurons do not employ HR when repairing DSBs following sublethal doses of NMDA, but rather rely on NHEJ, which was an expected outcome. The data also shows that H2A.X helps decrease the level of DSB damage in mouse cortical neurons following NMDA, as evidence by the effects of NMDA on WT, heterozygous and KO mice for H2AX.

The use of NCA to assay DSB repair in mature, DIV14 neurons, represents the first time this technique is implemented, to the best of our knowledge. The findings are in line with reported literature findings that make use of γ-H2AX foci to assess DNA
damage and repair, and indicate that NCA is a powerful tool to assess neuronal DNA damage. Current results confirm that γ-H2AX foci are a powerful marker to measure DNA damage, and that their disappearance can also indicate ongoing repair, as published by our laboratory in 2006 by Crowe et al. Furthermore, we have not found instances in the literature where researchers examined DSB repair pathway in mature rat neurons, particularly by testing whether HR components can also take part in DSB repair. A study by Rao and colleagues was the first to report that isolated cortical neurons possess NHEJ activity (Vyjayanti and Rao, 2006). However, the authors of this study did not investigate whether HR can also be involved in DSB repair, but rather assumed that NHEJ would be the sole repair mechanism in mature neurons (Vyjayanti and Rao, 2006). To the best of my knowledge, this is the first instance where NHEJ and HR were examined based on foci co-localization, and the first examination of where Rad51 is localized in mature rat neurons during DSB damage. Lastly, the use of neurons from of H2AX−/− mouse to examine DSB damage and repair via the NCA is also novel (Fig. 15, p. 113). The data shows that the absence of γ-H2AX compromises the ability of neurons to resist DSB damage. These experiments are in line with literature findings that indicate that H2AX is necessary for recruitment of DSB-associated proteins (Celeste et al., 2002). However, the current data do not point to repair deficiencies in mature neurons. It is possible that in this case, γ-H2AX deficient neurons could do without many of the proteins that the histone serves to recruit, particularly as it pertains to factors involved in cell-cycle regulation. Furthermore, as it has been posited before, it is possible that for very specific, quickly repaired DSB lesions, γ-H2AX might not be needed (Cui and Lieber, 2009).
3. NEURONAL PROTECTION FROM NMDA-MEDIATED OXIDATIVE STRESS AND DSB DAMAGE.

3.1. Introduction

Oxidative stress is the main endogenous source of DNA damage and poses a particular threat to neurons with their high levels of transcriptional activity. During transcription, chromatin remodeling causes DNA molecules to uncoil and become loosened from the protection of the nucleosome, in order to allow interaction with the transcriptional machinery. Therefore, gene expression events expose essential genomic regions to the nuclear milieu. During oxidative stress, the transcribed DNA regions are exposed to a nuclear environment with high levels of ROS that quickly oxidize the macromolecule and lead to DSBs. As previously proposed, due to its error-prone nature, it is very likely that the repair of DSBs via NHEJ, leads to an aggregation of genomic errors. The non-dividing nature of neurons mandates that their DNA must be well maintained throughout their lifetime if they are to carry appropriate gene expression. However, the combination of DNA damage and faulty repair, coupled with the lack of an accurate DSB repair mechanism (HR), interferes with DNA upkeep and it might be the cause for the onset of neuropathologies (Brooks et al., 2008). Even as frank cell death is absent, we show here that insults that do not immediately interfere with cell viability can cause DSB breaks. Furthermore, even though the DNA is repaired, as shown here, a faulty repair system would not prevent the DNA from accumulating errors. Therefore in order to counteract the ROS mediated DSB damage that arises from iGluR activation, it
is more desirable to prevent DSB damage than to rely on its repair. There are two apparent approaches to achieve this: 1) to mitigate the Ca\(^{2+}\)-mediated response that arises from NMDA activation, and can lead to ROS production, and 2) to make use of an antioxidant such as melatonin (see Introduction: Melatonin as an antioxidant for ROS prevention) as a method to prevent / minimize intracellular ROS generation.

3.2. Calcium chelation does not diminish DSB formation in DIV14 neurons

Bohr and colleagues reported that SSB damage could be prevented by calcium chelation with BAPTA (Yang et al., 2010). In the current work, it was investigated whether BAPTA is equally protective against DSB damage. It is essential to obtain this information since SSBs, unlike DSBs, are readily and accurately repaired and do not have long-lasting consequences for neuronal functioning. Thus, the experimental conditions used by Bohr and colleagues were replicated in order to assess whether BAPTA can minimize DSBs in neurons, as measured by the NCA. Cells were pulse-treated with 20 µM NMDA for 10 min (stopped with 20 µM MK801) with or without a BAPTA pre-treatment (10 µM, 30 min prior to NMDA application), and assayed for NCA at similar time points as the ones indicated by Bohr and colleagues. However, as in previous experiments, neuronal cells were DIV14 instead of DIV8-10, as done by the authors (Yang et al., 2010). While Bohr and colleagues showed that BAPTA treatments blocked SSB DNA damage in DIV10 cells, the data presented here show that DSB levels as assayed by the NCA, did not differ from NMDA treated cells (Fig. 16, p. 114). NMDA with or without BAPTA pre-treatment followed a similar pattern to that shown in
previous experiments, peaking at 1h and returning towards control levels afterwards. While a pretreatment with BAPTA showed a trend towards fewer DSBs at control levels, this was not statistically significant. Further, BAPTA-pretreated cells showed a higher number of DSBs by 30 min relative to NMDA-treated cells (p<0.05). By 24 h NMDA neurons had returned to control levels, however BAPTA cells were still different from controls (p<0.01 – Fig. 16, p. 114). These results indicate that a decrease in intracellular Ca\(^{2+}\) does not diminish DSB levels in DIV14 neurons, as measured by the NCA. Moreover, the data indicate that cells with lower Ca\(^{2+}\) levels might not be as efficient in repairing DSBs, as seen by the higher levels of DSB 24 h after treatment (Fig. 16, p. 114). These results are also consistent with previous report from the laboratory (Crowe et. al, 2006), which used \(\gamma\)-H2AX to assess DSBs following BAPTA pretreatments and showed only partial protection.

3.3. Melatonin is well tolerated by neurons

As BAPTA was not efficient in preventing DSB formation, the use of melatonin as a viable strategy in decreasing DSB levels was explored. The first step was to assess potential noxious effects of different doses of melatonin on neurons. As already mentioned, accurate data on physiological melatonin levels for the rat brain remain unknown. A study by Cheung and colleagues measured melatonin levels in rat at different time points, following 5 or 15 mg/kg intravenous injections. These doses are far from the extreme cases of the ALS mice discussed before (see p. 12), and reflect more the therapeutic doses given to humans. Two min after a 15 mg/kg injection, melatonin
plasma levels reached $1 \times 10^8$ pg/ml and steadily decreased (Cheung et al., 2006). Melatonin ($C_{13}H_{16}N_2O_2$) has a molecular weight of ~232.3, therefore a level of $1 \times 10^8$ pg/ml would equate ~430 µM. While this is, by no means, an attempt to derive physiological concentrations of melatonin based on mathematical calculations, it seemed in line with some of the amounts found in the literature, that reach into the mM range (Yoo and Jeung, 2010). Therefore levels of up to 1 mM melatonin were examined.

DIV14 neurons were subjected to incremental doses of melatonin, up to 1 mM either alone, or in combination to 10- and 30-min NMDA pulse treatments (20 µM), and assayed for LDH release. Pulse treatments were stopped with 20 µM of MK801 and after 24 h, LDH assays were performed as described in the methods section. Controls were treated with the same volume of vehicle. Maximum LDH release was established by measuring LDH levels from cell lysates, and served as a positive control. As seen in Figure 17 (p. 115), melatonin doses up to 1 mM, either alone, or in combination with NMDA did not have a negative impact on cell viability (Fig. 17, p. 115). These results suggest that melatonin is well tolerated by neurons and experiments under these conditions are not cytotoxic for DIV14 neuronal cultures.

3.4. Melatonin protects neurons against NMDA-mediated DSB formation

Next, we tested the hypothesis that pre-treatment with melatonin would prevent the onset of DSB damage caused by NMDA pulse-treatments. In order to test this hypothesis, the effects of melatonin pre-treatments (30 min) at different concentrations, prior to pulse-treatments with NMDA (20 µM for 10 min, as with the BAPTA
experiments) were tested. NCA was performed at 0 min, 30 min and 1h post-NMDA treatment, and $M_T$ was analyzed as described before. These time-points were chosen because previous experiments showed that DSB levels peak at this time, and the level of damage generated (but not the repair) was of interest. As seen in Figure 18 (p. 116), melatonin dramatically attenuated DSB damage in a dose-dependent fashion. Pre-treatments with melatonin at concentrations starting at 20 µM showed significant attenuation of DSB damage as compared to NMDA treated neurons. Melatonin pre-treatments starting from 100 µM showed non-statistical differences relative to controls. At levels of 1 mM, melatonin pre-treatments prevented DSB formation (Fig. 18, p. 116).

To verify the protective effects of melatonin against DSB damage, γ-H2AX foci formation was subsequently measured, with and without melatonin pretreatment (1 mM for 30 min). As previously reported by our laboratory, DSB sites as measured by γ-H2AX foci, quickly form following non-lethal NMDA doses (Crowe et al., 2006). DIV14 Neurons were pulse-treated with 20 µM NMDA for 15 min, with or without pretreatment with 1 mM melatonin, and processed for ICC, as described in the methods. Neurons were immunolabeled with an antibody against γ-H2AX, to assess DSB damage, at 15 min, 30 min and 1 h post-treatment. Cells were imaged using multiphoton microscopy, and γ-H2AX foci were quantified as described in the methods. Foci analysis shows that pre-treatment with melatonin largely prevented the formation of DSBs, upon challenging the cells to NMDA treatments (Fig. 19, p. 117). As in previous findings from our laboratory, the phosphorylation of H2AX was rapid and average foci levels were highest, already by 15 min post-NMDA treatment. Subsequently, γ-H2AX foci
numbers decreased and by 1 h post-treatment, they were back to control levels. Melatonin treated neurons, however, were undistinguishable from control γ-H2AX levels (Fig. 19A and 19B, p. 117). Further foci analysis showed that the majority of the melatonin-pretreated cells maintained low foci numbers throughout the experiments, similar to control cells and different from cells treated with NMDA alone. The NMDA treatment alone showed only a small percentage of cells displaying few foci, and the largest portion of neurons responding with >21 foci per nucleus (Fig. 19C, p. 117). Taken together, both NCA (Fig. 18, p. 116) and γ-H2AX experiments (Fig. 19, p. 117) indicate that melatonin pre-treatment prevents DSB damage, indicating that the use of this neurohormone is an efficacious method to prevent the onset of DNA damage in adult neurons.

3.5. Melatonin prevents NMDA-induced rises in neuronal ROS levels

In order to demonstrate that the mechanism of neuroprotection against DSB damage by melatonin is based on its capacity to abrogate cellular ROS generation (the main source of DNA damage), ROS levels were measured using the ROS indicator 2′, 7′- dichlorofluorescein diacetate (DCFDA). For these assays, DIV14 neuronal cells were plated in multi-well plates and incubated with DCFDA dye prior to treatments, as described in methods. Neurons were pre-incubated with melatonin for 30 min prior to pulse treatment with sub-lethal levels of NMDA (20 μM for 15 min, stopped with 20 μM MK801). The NMDA group was treated in the same manner, but incubated with vehicle in lieu of melatonin pretreatment. Negative control cells received just the dye and
vehicle, and positive controls for ROS generation was assessed by incubating cells with 100 µM H_2O_2.

As shown in Figure 20 (p. 119), levels of relative fluorescent units (RFU – proportional to ROS levels) indicate that treatment with melatonin prior to NMDA prevented an increase in cellular ROS, resulting in ROS levels not different to those of untreated controls (Fig. 20, p. 119). Conversely, neurons with no melatonin pretreatment displayed an increase in cellular ROS levels in response to NMDA, similar in magnitude to the response to H_2O_2 in positive control cultures. These results are consistent with the powerful radical scavenging properties of melatonin.

3.6. Melatonin protects rat neuronal mitochondria from NMDA-mediated depolarization

As mentioned already, increases in intracellular Ca^{2+} and oxidative stress can destabilize mitochondria, leading to further ROS generation, DNA damage and cell death. As shown in the previous section, melatonin is effective in preventing ROS formation. Therefore it is likely that in addition to a general decrease in neuronal ROS, melatonin is also able to protect mitochondria from the noxious effects following NMDA. Since the experiments using DCFDA could not discern between the location of ROS, but rather showed overall cellular ROS levels, the effects of melatonin on mitochondrial stability following sublethal NMDA activity were evaluated. To test whether melatonin can protect mitochondria, the health of the organelle was assessed using the dye JC-1 (see Material and Methods section for technical details of this
DIV14 neurons were incubated with the dye, as described in the methods, and treated with 50 µM NMDA for 15 min. As in previous experiments, neurons were pretreated with melatonin (1 mM) for 30 min prior to NMDA treatments. Cells were imaged with multiphoton microscopy, using a single excitation of 488 nm and emissions 515/545 and 575/625 nm for unhealthy and healthy mitochondria, respectively. Gray levels of green and red channels were analyzed with ImageJ, as described in the methods, and the red/green ratios were calculated. As seen in Figure 21 (p. 120), control cells (treated with equal volumes of vehicle; >1% DMSO in media) had an average red/green ratio higher than 1, indicative of healthier, more polarized mitochondrial membranes. Melatonin treatment alone did not have any effect on mitochondrial membrane potential, relative to control (Fig. 21, p. 120). Ratiometric analysis shows that NMDA treatment greatly depolarized mitochondrial membrane, as seen by the decreased red/green ratio to below 1. Pretreatment with melatonin, prevented this depolarization and its red/green ratio were similar to the control and melatonin only treatments (Fig. 21, p 120).

3.7. Conclusion

Taken together, these experiments show that a decrease in intracellular calcium levels does not affect the level of DSB damage or the efficacy of repair, and indicate that the neurohormone melatonin is able to counteract the noxious effects of NMDA in promoting oxidative stress and DNA DSB damage in mature neurons.

BAPTA-mediated chelation of intracellular calcium had no noticeable effects (see
Fig. 16, p. 114), on DSB levels in DIV14 neurons, unlike the findings reported by Bohr et al. for its effect on SSB formation in DIV8-10 neurons (Yang et al., 2010). Conversely, the use of melatonin shows promise as a good tool not only against DSB damage, but also in reducing oxidative stress. Melatonin appears to be very well tolerated by neurons, in concentrations up to the millimolar level, and also does not potentiate any deleterious activity of non-lethal NMDA doses on cell viability (Fig. 17, p. 115). Pre-treatments with melatonin starting at 20 µM show significant attenuation of DSB damage as compared to neurons treated with NMDA alone. Doses of melatonin in the 100 µM to 1mM range prevent DSB generation, as evidenced by both the decrease in NCA Mf (Fig. 18, p. 116) as well as the absence of γ-H2AX foci in the large majority of neurons pre-treated with melatonin prior to NMDA (Figs. 19A-C, p. 115). Melatonin blocked NMDA-induced cellular ROS increase, as evidenced by the detection of fluorescent levels similar to controls, when measured by the DCFDA ROS indicator dye (Fig. 20, p. 119). Lastly, melatonin is able to target mitochondria and protect them by preventing ROS-mediated membrane depolarization after NMDA treatment (Fig. 21, p 120).

The data show that melatonin has major neuroprotective roles for mature neurons, following the administration of sublethal NMDA doses. Due to a reduction in ROS levels, melatonin can prevent much of the DSBs that are generated following NMDA receptor activation, thus diminishing the need for error-prone repair of DNA DSBs by NHEJ.
4. THE ROLE OF ATM AND DNA-PK IN NEURONAL SURVIVAL

4.1. Introduction

As previously mentioned, the PIKK kinases DNA-PK and ATM have major roles in the repair of DSBs. DNA-PK is the main kinase in the repair of DNA DSBs by NHEJ (the main repair mechanism in neurons) and ATM is a key mediator of DDR, including DSB repair, and of cell cycle regulation (Jeggo et al., 1998). There is ample overlap between these proteins (Gapud et al., 2011; Gapud and Sleckman, 2011) as seen by the backup role that DNA-PK has in ATM-deficient cells (Callén et al., 2009 and a personal communication with Dr. Yossi Shiloh), as well as by evidence showing that the absence of both proteins is lethal (Gurley and Kemp, 2001; Sekiguchi et al., 2001). Lack of a functional ATM in humans gives rise to the neurodegenerative disease AT (Rotman and Shiloh, 1998; Lavin, 2008), where patients can survive for a few decades, albeit with severe complications. In the case of DNA-PK, the lack of a functioning protein appears to be fatal for humans (Mashimo et al., 2012); however, its deficiency in rodents leads to severe combined immune deficiency (SCID) but otherwise the animals are viable (Fulop and Phillips, 1990; Blunt et al., 1996; Vemuri et al., 2001; Mashimo et al., 2012). Interestingly, while ATM-null mice have some motor dysfunction, no neurological manifestations are seen in SCID mice (Jeggo et al., 1998). Still, the viability of mouse models defective in either ATM or DNA-PK points to compensatory mechanisms that allow the cell to survive. While these mechanisms are advantageous under situations of chronic protein deficiency, they might not be able to offset the damage induced by the
sudden loss of function of either of these kinases.

Therefore, for the final part of this thesis, we decided to investigate the role that an acute selective inhibition of DNA-PKcs and ATM would have on neuronal health, following sublethal treatments with NMDA. It is possible that deficiencies in these proteins would exacerbate the sublethal insults and have an impact in neuronal survival. Since mature neurons tightly regulate their cell cycle and utilize NHEJ as their primary repair mechanism, manipulation of these proteins could further elucidate the pathways of mature neuronal DSB repair and cell cycle regulation. The hypothesis is that targeting these proteins would lead to increased toxicity and cell death, following exposure to otherwise non-lethal doses of NMDA.

4.2. DNA-PKcs knockdown via shRNA

For the targeting of DNA-PKcs, we decided to silence the expression of the PRKDC gene by using a lentiviral-mediated RNA interference (RNAi) approach. Pseudotyped lentiviral particles containing vector constructs were created, that utilize the RNA polymerase III H1 promoter to drive the expression of an shRNA sequence against DNA-PKcs, as described in the methods section. As shown in Figure 22 (p. 121), lentiviral delivery of the vector into DIV11 neurons resulted in high infection efficiency (≥90%), as assessed by fluorescent microscopy of the GFP reporter built into the vector. Densitometry analysis following western blotting revealed that one of the four sequences (5’-GGAGAAGACAACCAGTCTCTGCTAACATT-3’) produced almost a complete knockdown of DNA-PKcs in rat neuronal cultures, relative to untreated controls (>98%).
72 h post-infection of DIV11 cells (Fig. 23A, p. 123). To the best of our knowledge, this is the first instance of gene silencing of DNA-PKcs in rat neurons.

4.3. Targeting of ATM with the specific inhibitor KU60019

The ATM inhibitor KU60019 is an improved analogue of the potent selective ATM inhibitor KU55933 (Hickson et al., 2004; Golding et al., 2009). Like other PIKK inhibitors (e.g., the DNA-PK inhibitor NU7441), KU60019 prevents protein activation by acting as a competitive inhibitor and binding to the ATP active site of the kinase, preventing its phosphorylation (Hickson et al., 2004; Leahy et al., 2004). KU60019 is a potent ATM inhibitor, with an IC$_{50}$ value for ATM of 6.3 nM (around half of KU55933) and 1.7 and >10 µM for DNA-PKcs and ATR, respectively (almost 270- and 1,600-fold higher than that for ATM), as well as almost no off-target effects at 1 µM against a panel of 229 kinases (Golding et al., 2009).

Although the use of this inhibitor is well documented, we could not find instances of its use in rat cortical neurons. Therefore the effects of KU60019 treatments on ATM phosphorylation following IR in rat neurons were examined. As seen in Fig. 23B (p. 123), the use of KU60019 at 3 and 10 µM prevented the phosphorylation of ATM following IR by as much as 85-90%, as measured by densitometry analysis following immunoblotting. Figure 23A (p. 123) also shows that the use of NU7441, a highly selective and potent DNA-PK inhibitor (IC$_{50}$ values = 14 nM and >100 µM, for DNA-PK and ATM, respectively), did not affect the ATM’s phosphorylation. However, due to the lack of a reliable antibody against rat phosphorylated DNA-PKcs, the only shown
substrate specific to DNA-PKcs \textit{in vivo}, we were not able to show the converse. This is a potential limitation for the use of KU60019 in this study, because it could potentially have off-target effects and interfere with DNA-PK as well. Outside the brain, work by Chen and colleagues showed that chemical inhibition of ATM with KU55933 did not affect the activity of DNA-PK in HeLa and Chinese hamster ovary cells, at concentrations of 10 \(\mu\mathrm{M}\) (Chen et al., 2007). Therefore, 3 \(\mu\mathrm{M}\) treatments were used because they still elicit a considerable response.

\textbf{4.4. Inhibition of ATM and down regulation of DNA-PK and its effect in cell viability}

After demonstrating that KU60019-mediated inhibition of ATM and shRNA-mediated knockdown of DNA-PK were effective and specific methods to block the activity of each protein, we tested the hypothesis that targeting these proteins would cause cell death, following otherwise sublethal doses of NMDA. DIV14 neurons were subjected to pulse-treatments (15 min or 1h) of 50 \(\mu\mathrm{M}\) NMDA (stopped with 20 \(\mu\mathrm{M}\) MK801), either alone, after a 1h pre-incubation with KU60019 (3 \(\mu\mathrm{M}\)), following a 72h incubation with shRNA against DNA-PK, or a combination. Lactate dehydrogenase levels were measured 24 h after NMDA treatments and compared against maximum LDH levels, following total cell lysis. I found no statistically significant differences following inhibition of ATM and DNA-PK, either alone or in combination, relative to controls or across groups (Fig. 24, p. 124). These results suggest that DNA-PK and ATM do not mediate neurotoxicity immediately following NMDA treatments. It would be important
to investigate in follow up studies the effects of inhibition of these enzymes on the efficacy / fidelity of neuronal DSB repair, as well as long term survival patterns and functionality of neurons following repeated activation of iGluRs.

4.5. Conclusion

To the best of our knowledge, the experiments using lentiviral delivery of shRNA to against DNA-PK in rats, have not been done elsewhere and represent the first instance of gene silencing of DNA-PKcs in rat neurons. We generated a construct that was extremely efficient (>98% knockdown) in silencing DNA-PKcs expression in the rat. This can be useful when wanting to examine the effects of a lack of DNA-PKcs on cells that do not originate in KO mice. Since these mice are viable, it is plausible that a chronic lack of DNA-PKcs gives rise to compensatory mechanisms in the cell, which might not be present if inhibiting protein expression in an acute manner. As we were not able to generate effective shRNA constructs against ATM, the highly specific chemical inhibitor KU60019 was used. While KU60019 is specific for ATM (IC$_{50}$ = 6.3 nM), data regarding these compounds are derived from cell-free assays using purified protein. By testing this inhibitor within a cellular environment, it was shown that KU60019 could be used to selectively inhibit ATM. Lastly, the data did not show that inhibition of ATM or DNA-PKcs alone, or in combination, increases cell death following pulse-treatment of NMDA (50 µM). It is important to conduct additional experiments before discarding the hypothesis that targeting these proteins would lead to increased toxicity and cell death, following exposure to otherwise non-lethal doses of NMDA.
Figure 6 – iGluR pulse-treatment is non-lethal for neurons. DIV14 neuronal cytotoxicity as measured by LDH release levels, assayed 24 h following 30 min pulse-treatments with NMDA (50 µM) or AMPA (25 µM). LDH levels after NMDA and AMPA treatments did not differ from control levels. Cells were lysed to measure maximum LDH release (Mean ± SEM; *** = p<0.001, relative to vehicle – ANOVA followed by Tukey post-hoc) (n=3 experiments done in triplicate)
Figure 7 – Dose response on DIV14 cortical neurons. DSBs quantified by measuring Tail Moment (Mₜ) following different doses of ionizing radiation. Treated groups were different from control (0 Gy), and there was relatively good differentiation among groups (Mean ± SEM; ** = p<0.01; *** = p<0.001; n/s = not significant - ANOVA with Tukey) (n ≥ 65 cells / group).
**Figure 8 – Assessment of neuronal culture purity.** Representative picture showing DIV14 cell culture characterization, stained to assess neuronal purity. Neuronal cells cultured under conditions established in the laboratory prevail in culture (~95%), relative to non-neuronal cells, as shown by ICC. Channel 1 (CH1 – top left): DAPI (nuclear marker); CH2: GFAP (astrocyte marker); CH3: Iba1 (microglia marker; merged at top right with CH2, due to space limitations); CH4: Class III β-Tubulin (neuronal marker – bottom left). Bottom right: Merge image of channels 1 through 4.
Figure 9 – iGluR agonist response in pure astrocyte cell cultures. Pure astrocytic cell cultures were prepared and treated with the iGluR agonists NMDA and AMPA (50 µM and 25 µM respectively), or a vehicle for 30 min, before being processed for NCA. NCA DSBs were quantified using $M_T$ increase at 1h and 4h. Treated groups did not differ from controls or from each other. Positive control (10 Gy IR) caused significant $M_T$ increase (mean ± SEM; $^* = p<0.05; ^{***} = p<0.001$; n/s = not significant – ANOVA with Tukey; n = 30 cells/group).
Figure 10 – NMDA receptor maturation mediates DSB damage. Neuronal cultures were subjected to NMDA pulse treatment at DIV4 and DIV14 of their development. Cells showed no difference from control levels at DIV4, but were significantly different from controls by DIV14, with their $M_T$ levels being similar to those of positive controls (5Gy IR). This demonstrates that NMDA receptor maturation mediates the response to DSB in mature neurons (mean ± SEM; *** = p<0.001, relative to controls – ANOVA followed by Tukey post-hoc, n≈70 cells / group).
Figure 11 – NMDA iGluR DSB damage is receptor specific. Mature (DIV14) neuronal cells were pulse treated with 15 µM NMDA with or without pretreatment with the NMDA antagonist, MK801 (20 µM, 15 min prior to treatment). DSB damage peaked around 1h and returned to control levels by 6 h. Antagonist pre-treatment completely prevented NMDA mediated DSB damage. (Mean ± SEM; * = p<0.05; *** = p<0.001, relative to controls – ANOVA followed by Tukey post-hoc, n≈50 cells / group).
Figure 12 – AMPA iGluR-mediated DSB damage is comparable to Glutamate. Mature (DIV14) neuronal cells were treated with 25 µM AMPA with or without pretreatment with the AMPA antagonist, NBQX (20 µM, 15 min prior to treatment), or with 15 µM and 50 µM glutamate. AMPA treatments were done in the presence of MK-801, to avoid secondary glutamate release damage. NBQX prevented DSBs. Glutamate and AMPA treatments show similar DSB levels (Mean ± SEM; *** = p<0.001, relative to controls – ANOVA followed by Tukey post-hoc).
Figure 13 – DNA DSB damage and repair following iGluR agonist treatments. DIV14 neurons were subjected to 30 min pulse treatments with NMDA (15 µM) and AMPA (25 µM) and assayed for NCA at indicated times. NMDA and AMPA showed a similar trend with increased DSB damage that peaked at ~1 h post-treatment, and subsequently decreased, with AMPA levels not being statistically different than controls (p>0.05) by 8 h post-treatment. DSB levels of NMDA-treated cells decreased as well, but were still significantly different from controls by 8 h. Shown also, are representative pictures of single cell comets with their respective tail lengths at specified time points (Mean ± SEM; * = p<0.05; *** = p<0.001, relative to their respective time controls – ANOVA followed by Tukey post hoc, n ≥ 60 cells / group; combined results of 3 separate experiments.).
Figure 14 (previous page) – Mature neurons repair DNA DSBs via NHEJ but not HR. Mature neuronal cells were exposed to NMDA treatments and stained for two key proteins for the main DNA DSB repair pathways: DNA-PKcs (NHEJ) and Rad51 (HR). A-D: Neurons stained for pThr2609 DNA-PKcs (red) show nuclear co-localization (D) between γ-H2AX (green) foci (B) and DNA-PKcs foci (C). E-H: Neurons stained with the HR specific protein Rad51 (G) showed perinuclear staining, indicating that Rad51 is in the cytosol and not in the nucleus, where γ-H2AX foci are visible (H). I-L: For comparison, mitotic PC12 cells, treated with 100 µM H₂O₂ to induce DSBs, show a nuclear Rad51 pattern and focal co-localization between Rad51 (red) and γ-H2AX (green). Extranuclear Rad51 staining is also visible (K, L). Cell nuclei were stained with DAPI (blue – A, E and I). Scale bar = 10 µm.
Figure 15 – The effect of H2AX in DNA damage. Neurons were harvested from H2AX knockout (KO, -/-), heterozygous (HET, +/-), or wildtype (WT, +/+ ) DIV14 mice brains. **Figure insert:** representative images showing two separate agarose gels, following PCR, used to establish mice genotypes. For the top gel, bands for WT and HET are seen at 565bp, but are not seen for KO. For the bottom reaction, bands at 435bp are given by HET and KO, but not by WT. **Main figure:** Cells were pulse-treated with 15 µM NMDA and processed for NCA to assess DNA DSB damage. H2AX+/+ and H2AX+/- cells had moderate DSB levels that peaked around 15 min post-treatment. Neurons lacking H2AX had a slower progression, but sustained significantly more DSBs, which peaked dramatically at ~1h. By 6h post-treatment, all 3 groups had returned to control levels. (Mean ± SEM; * = p<0.05; ** = p<0.01; *** = p<0.001, relative to their respective controls, ### = p<0.001, across groups – ANOVA followed by Tukey; WT & HET = 2 pups, KO = 3 pups; ≥ 50 cells/group).
Figure 16 – The effect of BAPTA on DSB levels. DIV14 neuronal cells were exposed to pulse treatments with NMDA (20 µM, for 10 min, stopped with 20 µM of MK801) with or without pre-treatments with the Ca$^{2+}$ chelator BAPTA, and assayed for NCA at specified time points. Pre-treatment with BAPTA had almost identical effects on cells as those observed in cells treated with NMDA, and it did not diminish DSB levels, as measured by the NCA. As in previous experiments, NMDA-pulse treated cells peaked at ~1 h post-treatment. NMDA neurons returned to control levels by 24 h. BAPTA-treated neurons appeared to have a more pronounced level of damage and a slower return to baseline levels; by 24 h it was still not different from its control levels. (Mean ± SEM; ** = p<0.01, *** = p<0.001, relative to their respective controls; n/s = not significant, # = p<0.05, between groups – ANOVA followed by post-hoc Tukey; results from 3 experiments, ≥ 50 cells/group).
Figure 17 – Melatonin is well tolerated by neurons. DIV14 neuronal cytotoxicity as measured by LDH release levels, assayed 24 h following 10 and 30 min-pulse treatments with NMDA (20 µM) and increasing melatonin concentrations, or melatonin alone (1 mM). Treatment groups did not differ from control levels. Cells were lysed to measure maximum LDH release (Mean ± SEM; *** = p<0.001, relative to control – ANOVA followed by Tukey post-hoc; n=4 experiments done in duplicate).
Figure 18 – The effect of increasing doses of melatonin in DSB damage. DIV14 neurons were pulse treated for 10 min with 20 µM NMDA (stopped with 20 µM MK801) and DSBs were quantified using NCA at 0, 30 min or 1 h post-treatment, with increasing concentrations of melatonin or without melatonin, and compared to vehicle treated controls. Increasing doses of melatonin diminished DSB damage, as measured by a smaller tail moment. Pre-treatments with melatonin at concentrations starting at 20 µM showed significant attenuation of DSB damage as compared to NMDA-treated neurons. Melatonin pre-treatments starting from 100 µM showed non-statistical differences relative to controls. Treatment with 1 mM melatonin completely protected neurons against NMDA mediated DSBs. (Mean ± SEM; *** = p<0.001, relative to their respective time controls; ### = p<0.05, relative to 1 h NMDA – ANOVA followed by post-hoc Tukey; results from 1-3 experiments, 50 cells/group).
Figure 19 (previous page) – Melatonin pretreatment reduces DSB damage following NMDA (γ-H2AX foci). DIV14 cortical neurons were pulse treated with NMDA with or without pretreatment with 100μM melatonin. A) Representative images showing nuclear localization of γ-H2AX foci (in green) in untreated and NMDA treated cells, with or without melatonin. B) γ-H2AX mean foci per cell quantification shows that foci formation occurs soon after treatment and decreases by 1 h post-treatment. Melatonin pretreated neurons did not show an increase in the number of γ-H2AX foci. (Mean ± SEM; *** = p<0.001, relative to control – ANOVA followed by post hoc Tukey). C) Analysis of the percentage of cells with a certain foci range. NMDA treated cells show a shift towards more cells with a higher number of foci at 15 min, which subsides by 1 h. Melatonin pretreated cells, maintained similar bin proportions as control cells.
Figure 20 – Pre-treatment with melatonin diminishes neuronal ROS levels following NMDA. DIV14 neurons were treated with the fluorescent ROS indicator DCFDA prior to being treated with NMDA (20 µM for 15 min), with or without melatonin pretreatment (1 mM for 30 min). Levels of relative fluorescent units (RFU – proportional to ROS levels) show that treatment with melatonin prior to NMDA treatments prevents an increase in cellular ROS. Neurons with no melatonin pretreatment display an increase in ROS, which were similar to the positive control, treated with H₂O₂ (100 µM). (Mean ± SEM; * = p<0.05, *** = p<0.001, relative to control; # = p<0.05, between groups – ANOVA followed by post-hoc Tukey; results from 3 experiments in triplicate).
Figure 21 – Melatonin helps stabilize mitochondria against NMDA. DIV14 cortical neuronal cells were treated with the mitochondria membrane potential indicator JC-1. The green JC-1 monomer aggregates in healthy mitochondria producing a shift towards the red emission, thus a greater red/green ratio indicates healthier mitochondria. Cells were treated with NMDA as before, in the presence or absence of melatonin. NMDA treatment leads to a depolarization of mitochondria. Melatonin pretreatment prevents mitochondrial membrane depolarization, as evidenced by a higher red/green ratio. (Mean ± SEM; *** = p<0.001, relative to control; ## = p<0.01, between groups – ANOVA followed by post-hoc Tukey).
**Figure 22 (previous page) – Lentiviral Neuronal Infection.** Representative phase contrast, epifluorescent and merged 20X images, showing lentiviral particles expressing shRNA constructs with an EGFP reporter and a promoter driving the expression of a sequence against DNA-PKcs, (5’-GGAGAAGACAACCAGTCTCTGCTAACATT-3’) and a scrambled sequence as well as untreated cells as controls. DIV11 rat neuronal cultures were infected with DNA-PKcs, scrambled or mock infected with the same amount of vehicle, as mentioned in the methods, and imaged 72 h post-infection. High infection efficiency (≥90%) was obtained. Bar = 200 µm.
**Figure 23 – DNA-PKcs knockdown and ATM inhibition in neurons.**  

**A)** Lentiviral delivery of shRNA constructs against DNA-PKcs or a scrambled sequence was performed as described in the methods section. Immunoblotting was performed 72h post-infection as described in the methods section. Densitometry analysis revealed >98% knockdown of DNA-PKcs, relative to untreated controls in neuronal cultures using lentiviral delivery.  

**B)** DIV14 neurons were pretreated with the ATM or DNA-PKcs inhibitors KU60019 (ATMi) or NU7441 (DNAPKi – as a negative control) for 1 h and exposed to 5 Gy IR. After 15 min, cells were processed for immunoblotting as described in the methods. Densitometry analysis showed that KU60019 is very efficient in blocking ATM activation, as measured by phosphorylation of Ser1981 (~85-90% relative to positive IR control – second lane from the left).
Figure 24 – Cytotoxicity after sublethal NMDA treatments with ATM inhibition and/or DNA-PKcs knockdown in rat neuronal cells. DIV14 cortical neuronal rat cells were pulse-treated with 50 µM NMDA for 15 min or 1h (stopped with 20 µM MK801), alone or following chemical inhibition of ATM with KU60019 (ATMi), shRNA against DNA-PKcs, or both. LDH levels were read 24 h post-treatment. No statistically significant differences were found relative to controls or across groups. Cells were lysed to measure maximum LDH release. (Mean ± SEM; *** = p<0.001, relative to controls – ANOVA followed by Tukey post-hoc).
DISCUSSION AND FUTURE DIRECTIONS
DNA repair following iGluR activation measured by the NCA

In the first part of this dissertation, the role of DNA DSB repair in mature neurons subjected to non-lethal lesions of iGluR was examined, by using the NCA method to assess repair independent of γ-H2AX foci quantification. We confirmed previous work by our laboratory, by showing that the pulse-application of NMDA and AMPA (up to 50 μM and 25 μM, respectively) is non-lethal for neurons, ensuring also that DSBs shown by these lesions are not the product of ongoing cell death. Furthermore, the NCA assay was optimized for measuring DSBs in mature neurons, which to the best of my knowledge, is the first implementation of this technique in DIV14 rat cortical neurons.

Some have reported that glial cells express NMDA receptors (Conti et al., 1997; Lee et al., 2010). While I did not see increased glial DNA DSB damage at 1 h and 4 h post-NMDA and AMPA treatment, I did not measure beyond those time points. Therefore, pertinent experiments are the assessment of DNA DSB damage in glia at later time points; even using slightly higher drug concentrations. Oxidative stress damage in glial cell can lead to protein oxidation, a decrease in glutamate uptake, damage glutamate transporters, and inactivate glutamine synthetase leading to an increase in extracellular glutamate levels (Halliwell, 2006). Since oxidative stress leads to the generation of stable ROS, like ONOO− and H2O2, it is possible that, if glial damage is of later onset, the generation of new ROS from glia would inflict a “second hit” in neurons, which in turn would produce more ROS, maintaining the effects of oxidative stress long after the initial insult.
The NCA proved to be a sensitive assay for the measurement of DSB damage and repair, and it allowed us to show that iGluR-mediated DSB damage was specific to neurons and it corresponded to NMDA receptor maturation, as well as prevented by the NMDA antagonist MK801. Damage peaked ~1 h post-treatment and neurons subsequently repaired their DNA, as shown by a decrease in $M_T$, returning to baseline levels ~6-8 h after that. Interestingly, previous work in our laboratory using $\gamma$-H2AX foci had seen a rapid increase in phosphorylation peaking ~10 min and a return to baseline around 4 h (Crowe et al., 2006). While showing similar trends, this temporal shift between the two detection methods could point to a higher sensitivity for $\gamma$-H2AX as a method for detecting the onset of DSBs, relative to NCA, under these conditions. Conversely, the data indicating a later return to baseline using the NCA could indicate that repair is still undergoing and that $\gamma$-H2AX is not able to reflect that, making the NCA a more accurate method to monitor DSB repair. It has been suggested that the gradual disappearance of $\gamma$-H2AX foci could be mediated by factors independent of DSB repair, such as dephosphorylation, histone exchange, histone eviction, or nucleosome reassembly (Svetlova et al., 2007; 2010; Bao, 2011). This could potentially explain the discrepancy in the early foci disappearance relative to NCA. Due to the current experimental setup of the NCA, it is somewhat difficult to assess time-points that are very closed together (i.e. every min for the first 10-15 min), however such an experiment conducted in tandem with $\gamma$-H2AX quantification experiments, would help elucidate a sensitivity threshold for these techniques. Also, it is possible that low iGluR doses are more easily detected by $\gamma$-H2AX than by NCA, so conducting these experiments with IR, where I showed high
NCA sensitivity, could be applicable. Furthermore, interesting experiments could be planned to investigate the role of protein phosphatase 2A (PP2A) or the FACT complex. PP2A is involved in the dephosphorylation of γ-H2AX and FACT has been established as a mediator in the replacement of nucleosomal H2AX with the H2A variant (Bao, 2011). Thus, it is possible that modifying the way these proteins interact with H2AX could lead to a more prolonged presence of γ-H2AX in the nucleosome, thus relaying the DSB signal and improving repair.

**The role of HR proteins and H2AX in neurons.**

Along with demonstrating the neuronal repair of DNA DSBs, I also showed that adult neurons indeed use NHEJ but not HR to repair their DNA, as evidenced by focal co-localization between γ-H2AX and DNA-PK but not with Rad51. This is a very interesting observation, as the role of many of the HR proteins in mature neurons, among them Rad51, remains mostly unknown. My finding that neuronal Rad51 remains cytosolic, even after DSB induction, is an intriguing one. Cytosolic Rad51 localization has been reported, using ICC in HEK-293 (Bennett and Knight, 2005), as well as using subcellular fractionation and western blotting in human cancer cells (Gildemeister et al., 2009). However, in these studies the researchers focused on the shuttling dynamics into and out of the nucleus and did not speculate on the role, if any, a cytosolic Rad51 protein would play. A subsequent study by the same group, proposed Rad51 to be a mediator of mitochondrial DNA replication under oxidative stress conditions (Sage et al., 2010). It is possible that this may also be the function of neuronal Rad51, as these organelles are under a constant state of oxidative stress, and neuronal mtDNA might benefit from
additional protection, due to the high level of ROS generation in neurons. This, however, remains to be investigated. In addition, the role of other HR proteins, such as Rad52, or even the contribution of the HR repair pathway as a whole to mature neurons might merit revisiting as it remains largely unexplored. A PubMed search of “Rad52” and “neurons” returned no results (as of the writing of this work). It is indeed likely that the lack of a clearly defined sister chromatid prevents the HR machinery to carry DSB repair in post-mitotic cells. However, Single Strand Annealing (SSA), an HR sub-pathway where homologous regions in the same chromosome are used to guide the repair of the DSB could potentially be available. While SSA uses a homologous sequence to repair the original DSB, it does so with catastrophic consequences, due to the loss of the genomic information between both homologous regions. As Rad52 is a key player in SSA as well as another highly inaccurate repair sub-pathway called Break Induced Replication (BIR – San Filippo, 2008), it is important to establish its potential contributions, as well as that of other HR proteins to neuronal DSB repair. Due to their lack of replication, it is possible that neurons are “doomed” to repair their DSBs with repair mechanisms that are mutagenic by nature.

The experiments with γ-H2AX deficient mice are also promising. As previously mentioned, phosphorylation of this histone is a well-established marker for DSB detection. As H2AX has a central role in numerous DDR pathways, mediating not only repair but also cell cycle regulation and apoptosis (Bonner et al., 2008), I decided to test if a deficiency in H2AX, can have significant implications in DSB damage and repair in neurons. I demonstrated that γ-H2AX-null neurons do show higher DSB damage,
relative to heterozygous and WT cells, which is in line with previous reports of growth retardation and immune deficiency in H2AX\textsuperscript{-/-} mice, and with $\gamma$-H2AX as necessary for DSB-associated proteins recruitment (Celeste et al., 2002). However, the data do not point to repair deficiencies in mature neurons. As mentioned, it is possible that in the case of non-cycling cells, some of the proteins that $\gamma$-H2AX serves to recruit (e.g., factors involved in cell-cycle regulation) might not be required. It is also possible that $\gamma$-H2AX might not be needed for very localized lesions that are quickly repaired by NHEJ. As it is the case with many of the DDR proteins, current research does not focus on the brain or non-cycling, and is targeting primarily cancerous cells. The data shed light on a topic that can have serious implications in elucidating the roles of $\gamma$-H2AX and DSB repair in neurons, a topic that remains virtually unexplored.

While H2AX can have important roles for neurons, the fact that despite its mutation in mice, viable animals are produced and do not show particular neurological deficits, indicates that compensatory mechanisms allow the cells to make up for the chronic deficiency of H2AX. Since I have shown that a high level of gene silencing can be obtained in mature neurons, via the lentiviral delivery of shRNA against a specific target, an interesting experiment would be to use this approach to target H2AX and see if the sudden loss of this protein would have more dramatic effects in neurons. Conversely, overexpression of H2AX might improve damage recognition stage, leading to a more efficient repair of DSBs.
The role of calcium in DSB repair.

While Bohr and colleagues showed that a pretreatment with BAPTA was able to mitigate DNA SSB damage (Yang et al., 2010), in DIV8-10 neurons, as measured by the alkaline comet, I was unable to show that it has a similar effect on DSBs in mature (DIV14) neurons using the NCA, where the data showed almost no difference from untreated cells. This is somewhat counterintuitive, as SSB can give rise to DSBs and hence reducing SSB occurrence should lead to less DSBs. A possible explanation for this discrepancy could be that researchers used a concentration of BAPTA (10 µM) that is effective for DIV8-10 cells but does not cause an effect in DIV14 neurons. Indeed, previous work by our laboratory had examined role of BAPTA as well as vitamin E, in γ-H2AX foci numbers. While both BAPTA (50 µM), and vitamin E diminished the number of γ-H2AX foci for cells treated with NMDA and a weaker response was seen after AMPA treatments, no complete protection was achieved (Crowe et al., 2006). A second possibility is that, as NMDA receptors have fully matured by DIV14, NMDA treatments would likely lead to a stronger response around this time, as opposed to DIV10 (as used by Bohr and colleagues) where receptors might not mediate the response or might require a higher BAPTA dose. Additionally, my findings could also be explained by a lower sensitivity of the NCA versus γ-H2AX used by Crowe (2006). Direct comparison between DIV8-10 and DIV14 using both methods may resolve the remaining discrepancy in follow-up experiments.

An interesting caveat, however, is based on the fact that the chelation of cellular Ca$^{2+}$ with BAPTA both protects and hinders DNA damage, as Ca$^{2+}$ is a key mediator of
numerous cellular pathways, including DNA repair. Work by Smallwood and colleagues showed that increases in calmodulin (CaM) help protect macrophages against low dose hypersensitivity to radiation, by up-regulation of DNA repair pathways, among other processes (Smallwood et al., 2009). Others have also shown that proper DNA repair is accomplished by maintaining an adequate Ca$^{2+}$ concentration, and compromised by increases or decreases in Ca$^{2+}$ levels, as well as CaM inhibition and Ca$^{2+}$ chelation (Gafter et al., 1997, Ori et al. 2005). Thus, while the role of NMDA generated ROS, leading to DNA damage, is Ca$^{2+}$-mediated, it is likely that the deregulation of this metal can also lead to DNA damage. Taken together, these considerations suggest that chelating Ca$^{2+}$ may not be the optimal way to prevent DSB damage in neurons.

Neuronal protection against DSB damage by the antioxidant melatonin

As posited, since DSBs are produced by an increase in ROS, a viable approach to mitigate DNA DSBs is the use of antioxidants. Ever since Denham Harman advanced his free-radical theory of aging, in which he claims “…aging and the degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues.” (Harman, 1956), the use of antioxidants to combat the deleterious effects of oxidative stress has been an area of great interest. Antioxidant therapy has been investigated in the context of aging, as well as numerous pathologies, such as cancer (Ju et al., 2010), atherosclerosis (Steinberg, 1991) and diabetes (Pekiner et al., 2002), as well as neurodegenerative diseases and disorders, such as TBI, stroke, AD and PD (Yochum et al., 2000; Gilgun-Sherki et al., 2003; Tiwari, 2004; Hall et al., 2010). Our laboratory had previously investigated the role of vitamin E,
a powerful antioxidant molecule (Crowe et al. 2006). However, due to its lipophilicity, vitamin E might have poor permeability, which diminishes its translational value. Thus for my study, I chose to examine the efficacy of the antioxidant properties of melatonin against DSB formation. Melatonin, which is characterized by exceptionally high brain availability, can be explored in the context of its potential clinical applications to combat DSB damage in the brain. As mentioned earlier, while the more “traditional,” receptor-mediated functions of melatonin, controlling circadian rhythm regulation, sleep promotion and blood pressure are well known (Dubocovich, 2007), this pleiotropic molecule also functions as a powerful antioxidant and free radical scavenger. The antioxidant capacities of this indoleamine have been extensively documented, and appear to be in many aspects, superior to other antioxidants (Korkmaz et al., 2009). Its amphipathic nature allows it to diffuse across aqueous and lipid environments, reaching virtually every cell and subcellular compartment in the body. This indole can easily cross immunoprivileged sites, such as the blood brain barrier and the placenta, and thoroughly perfuse all cells. The amphiphilic nature of melatonin allows it to permeate freely to all subcellular regions and exert its antioxidant role, unlike other compounds such as vitamins C and E, which are only soluble in aqueous or lipid environments, respectively, confining their antioxidant capacity to specific regions, but can also be pro-oxidants (Brigelius-Flohé and Traber, 1999; Reiter et al., 1999; Rietjens et al., 2002). Furthermore, in addition to being able to scavenge almost all reactive oxygen and nitrogen species and free radicals, there is ample literature regarding the protective effects of melatonin in stimulating endogenous cellular antioxidant enzymes (such as glutathione peroxidase,
and superoxide dismutase), mitochondria (where it acts as a powerful \textit{in situ} antioxidant and can stabilize the membrane as well as individual components of the electron transport chain, increasing its efficiency), and pointing to activity being up to 60-70 times more powerful than vitamins C and E in protecting DNA (Leon et al., 2004; Anisimov et al., 2006; Escames et al., 2010; Paredes and Reiter, 2010; Srinivasan et al., 2011; Cardinali et al., 2012; Carpentieri et al., 2012; Pandi-Perumal et al., 2012). Unlike other antioxidants, mitochondria selectively take up melatonin (Srinivasan et al., 2011), where it not only scavenges mitochondrial ROS, but also stabilizes ETC complexes I and IV in a manner independent to its antioxidant properties (Leon et al., 2004; Carpentieri et al., 2012). Due to its high reduction potential, it is likely that melatonin interacts with ETC components, increasing electron flow and therefore ATP production (Leon et al., 2004). Furthermore, studies have also shown that melatonin protects mitochondrial DNA (mtDNA) from the free-radical-induced damage common to high stress conditions, such as seizures, acute ethanol administration and lipid peroxidation, both \textit{in vivo} and \textit{in vitro}. Due to its antioxidant capacity, melatonin was also able to abolish the deleterious effects caused by the amyloid protein (Leon et al., 2004).

The discovery that melatonin effectively protects neurons against ROS-mediated DSBs is one of the most promising findings of the study. I have shown that melatonin is well tolerated by neurons and that its administration prior to NMDA protects DSB damage, in a dose-dependent fashion. The mechanism of melatonin protection appears to be the rapid termination of free radicals and ROS via its antioxidant activity, as shown by a decrease in cellular ROS and mitochondrial stabilization. This is in accordance with
published work, showing that in addition to its direct, receptor independent, antioxidant capacity, melatonin is able to promote the effects of cellular antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione reductase (GRd) and the cytosolic and mitochondrial superoxide dismutases (CuZnSOD and MnSOD) both at the protein level, as well as by enhancing gene expression (Reiter et al., 2003). The ability to alter enzymatic activity likely requires an interaction between melatonin with either membrane or nuclear receptors (Reiter et al., 2003). To test the hypothesis that the antioxidant effects on neuronal ROS are mediated via molecular interactions, I conducted additional experiments using N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK), a melatonin metabolite with antioxidant qualities but low melatonin receptor affinity, and N-acetyl-2-benzyltryptamine (Luzindole), a melatonin receptor antagonist. Neurons (DIV14) were treated with Luzindole (10 µM), AFMK or melatonin (100 µM), 30 min prior to NMDA treatments as before. While no statistical significance between the experimental groups was observed, the preliminary data point towards a trend where pretreatment with AFMK or melatonin could offer greater DSB protection than pre-treatment with Luzindole (see supplemental Fig. S1, p. 138). The protection against NMDA seen following Luzindole exposure is likely attributable to its own antioxidant properties, due to its structural similarity to melatonin (Tan et al., 2001; Mathes et al., 2008). Conversely, the trend toward lower protection is likely attributable to its melatonin receptor antagonism. Unfortunately, these data do not allow me to conclusively state that melatonin protection is entirely receptor-independent. Therefore, follow-up dose response experiments with Luzindole and AFMK are needed. Additionally, experiments using melatonin receptor
agonists, such as Tasimelteon, Agomelatine or Ramelteon, or the use of melatonin receptor KO mice (Weil et al., 2006) might elucidate the mechanistic role of melatonin, its receptors and their contribution to DSB protection.

Overall, my results open the door to exciting new experiments in vivo, where a melatonin rich diet could be used to counter the effects of certain neurodegenerative disorders where ROS appears to be a key factor, such as AD, PD, ALS and AT. Furthermore, as melatonin can also exert its effects in a receptor dependent manner, it is interesting to further explore its effects in neurodegeneration, via both mechanisms. Since melatonin can easily diffuse across membranes into the nucleus and the mitochondria, it is interesting to consider whether it can also prevent DSB or other type of DNA damage directly, as opposed to quenching ROS activity in the vicinity of DNA. It is important to mention, however, that although the large majority of the literature confirms the powerful antioxidant properties of melatonin, some studies have found melatonin to be pro-oxidant under certain conditions (Anisimov et al., 2006). Therefore, a better understanding of the mechanisms of action of melatonin can greatly help in the prevention of drugs that would mediate many of the beneficial aspects of melatonin, while preventing pro-oxidant or other side effects associated with the chronic use of melatonin (i.e. complications to circadian rhythm regulation). Thus, a follow up study of the pharmacology and mechanism of action of melatonin on DSB-related machinery is required.
Targeting of ATM and DNA-PK

The data on inhibiting DNA-PK and ATM, either alone or in combination, showed no immediate effect on acute neuronal death. While these results are disappointing, they still do not prove my hypothesis wrong. Indeed, these results are from first experiments where the doses and times of NMDA treatments follow my previous experiments, and it is possible that are not representative under these new set of conditions, in which protein activity is suddenly abrogated. Therefore, as I mentioned in the results section, future experiments are needed in which different NMDA concentrations, pulse treatment duration, as well as a longer monitoring of neuronal viability should be investigated. The effects of the sudden loss of function of ATM and DNA-PK in mature neuronal cell have not been investigated, and therefore represent an exciting and major question to explore. Since ATM regulates cell-cycle arrest following DSB damage, its role in neuronal cells (with a tight cell cycle regulation already), could shed light on the activity of this kinase, beyond its known roles, benefitting not only neuroscience, but cancer research as well. In the case of DNA-PKcs, with its key role in NHEJ, its blocking following DSBs would also provide valuable information regarding the roles of other proteins or even pathways that could make up for its absence. If the blockade of both proteins does not lead to cell death, in the face of diminished DSB repair, it would suggest that DSB repair does not immediately affect neuronal survival, following physiological or supra-physiological glutamate-mediated activity. This outcome will serve as a firm foundation for concentrating future studies on long-term effects of erroneous repair on neuronal well-being and vulnerability to disease and injury.
Supplemental Figure S1 – Effects of melatonin, AFMK and Luzindole in DSB damage protection. DIV14 neurons were pretreated (30 min) with melatonin (100 µM), AFMK (a melatonin metabolite – 100 µM) or Luzindole (a melatonin receptor antagonist – 10 µM), prior to NMDA pulse treatment (20 µM for 30 min) as previously described. Cells were fixed, stained for γ-H2AX and foci were quantified as in previous experiments. No statistical significance is seen between groups, however a trend indicates that AFMK and melatonin offer greater DSB protection than Luzindole. While luzindole is a melatonin receptor antagonist, it also acts as an antioxidant, likely due to its molecular structure (see structures above bars). (Mean ± SEM; *** = p<0.001, relative to control – ANOVA followed by post-hoc Tukey; results from 1 experiment, 80 cells/group).
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