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Analysis of tau protein phosphorylation in adult NMRI male mice following low dose neonatal irradiation

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Abstract

Everyone on this planet is daily exposed to some kind of radiation, which we cannot escape from. Most of the time the doses are very low and not harmful and if so, the body has many defense mechanisms to repair the damage. But there are some stages in life when we (mammals) are more sensitive to exposure of radiation. A critical time period has been shown to occur under the neonatal brain development. Changes during this time period may lead to different malfunctions. This study has investigated adult NMRI male mice, irradiated neonatally (on postnatal day 10) to a single dose of gamma radiation. The phosphorylation of the microtubule associated protein (MAPs), tau, was analyzed in adult (6 month old) NMRI male mice. Although no significant changes were seen, the level of phosphorylated tau was higher in the cerebral cortex of mice neonatally exposed to gamma radiation.

Introduction

There are many agents in our environment that can be hazardous and toxic to human. Among the most vulnerable demographic groups are children. A minor disruption during a developmental period can cause major alterations that can alter the future development. One of our most essential and important organ is the brain. The most vulnerable period is the brain growth spurt (BGS) (Dobbings and Sands, 1978) which is the period when the brain has its highest developmental activity and its different "areas" are developed, during different time periods. The characteristic of the BGS is notified by the brain's rapid weight gain, which includes the myelination of the axonal outgrowth. Other characterizing factors of the BGS are the high increase of neurotransmitters and maturing of the axons, dendrites and the establishment of connections between the neurons (Davison and Dobbing, 1968).

The time for the BGS differs from species to species, which can be useful when deciding which laboratory animal that should be used in toxicity studies for evaluating effects on the development of the brain. In many toxicity studies, humans are unethical to use. It is also difficult to do studies on humans due to their long developmental time (Dobbings and Sands, 1978). Rodents, on the other hand, are well known and often used laboratory animals, mainly for their short life span and easy handling. They are also relatively similar to humans in the brain development (Dobbings and Sands, 1978). However, there are differences between humans and rodents regarding timing of the brain development. Humans have their BGS perinatally (around birth, starting around third trimester and continues up to about 2-years of age) and rodents neonatally (after birth, up to four weeks) (Davison and Dobbings, 1968). The NMRI mice have their BGS peak around postnatal day (PND) 10 (Davison and Dobbings, 1968). One advantage of conducting experiments on animals that have a postnatal BGS, is that they can be directly exposed to a toxicant (substance or radiation).

The nature is on a daily basis exposed to radiation. Radiation comes in many different forms, from manmade like radio waves, X-rays, microwaves to the natural radiations such as light, heat and cosmic radiation. Radiation is mainly categorized into two different groups; non-ionizing- (radio waves, light, heat etc) and ionizing radiation (IR) (X-rays, cosmic radiation, atomic activity). For radiation to be called IR, it must be ≥30eV/ionization and give rise to at least 3 ionizations or have an energy > 100 eV (Chopping *et al.,* 2002).

DNA is a critical target of IR. The effects on DNA can either be acute or indirect. The indirect effects are mostly caused by free radicals, generated from the IR on molecules, most commonly water molecules. The effects can result in DNA strand brake, single- (SSB) and double-strand brake (DSB). SSB are more common and for the most not a major problem for the cell to repair, but DSB on the

other hand can give more critical lesions. There are several defense mechanisms to prevent any further damage to the cell, most of them found under the cell cycle, protecting the DNA. If none of the repair systems work, the inflicted damage can lead to the development of cancer or trigger the cell to go into apoptosis.

Although IR is hazardous it can also be used for good things and it is widely used in medicine for therapy *e.g* injection of radioactive substances to kill cancer cells or as a screening tool *e.g* locate cancer cells or the X-ray for locating fractures or growing tumors. The use of CT–scans (computerized tomography-scan) is increasing for diagnostic purposes. CT-scans functions as an X-ray but divides the scans in many thin slices, which will give a more accurate image and also give 3D-images.

There are a lot of acute effects (table 1) from IR that have been recorded and reported, like the cases of Chernobyl and Hiroshima. The lethal dose for 50% of a human population (LD_{50}) is around 4 *gray* (Gy). Gy is a measure of the amount of absorbed dose of the radiation, which is defined as 1 joule of energy deposited in 1 kilogram of mass. The old unit used is rad (radiation absorbed dose), 1 Gy = 100 rad (WHO, 2012).

Table 1. Acute effects that might occur after exposure to high doses of radiation (U.S Department of Health and Human Services)

Effects	**Absorbance (Gy)**
Sterility	**1-5**
Hair loss	**3-4**
***Bone marrow syndrome* (total loss of bone marrow stem cells)**	**3-4**
Skin effect	**6**
Open wounds	**10**
***Gastro-internal syndrome* (total loss of gastro internal stem cells)**	**10-15**

Most studies on effects from IR are preformed after high doses of exposure. However, Hall *et al.* (2004) showed in a Swedish study that low doses of IR in human newborns and infants (< 18 month of age) can lead to decreased cognitive ability in adulthood. The subjects had been treated for *haemangioma* (a kind of benign tumor of the endothelial cells in the blood vessels) with IR.

Also, recent (Eriksson *et al.,* 2010) and ongoing (Buratovic *et al.*, 2012) research at the Department of environmental toxicology, Uppsala University, Sweden, shows that a single low dose of gamma-radiation during a critical period of the brain development can affect the cognitive ability, in both sexes of adult NMRI mice. The low neonatal dose of 0.5 Gy caused a significantly altered spontaneous behavior in a novel home environment of 2 and 4 months old NMRI mice. An even lower dose of 0.35 Gy also, to some extent, resulted in altered adult spontaneous behavior, but not as pronounced as in the formerly mentioned one.

In the previous study, mentioned above (Buratovic *et al.*, 2012), it has also been shown that the irradiation caused an increased level of the total tau protein at the age of 6 month. It has also been shown, in other studies, that neurofibrillary tangles (NFT) can correlate with decreasing cognitive ability in both human (Ikonomovic *et al.,* 2004) and mice (Grundke-Iqbal, 1986). The Alzheimer's disease is one of those examples where the NFT is an indicator.

The tau proteins are mostly present in the axons in the central nervous system (CNS), but have also been shown to be present in the oligodendroglia, astrocytes and also in the peripheral nervous system (PNS), but at a lower concentration.

Microtubules are one key component in the cytoskeleton, which functions as a stabilizer of the cell and as a transport way for many substances (for example many neurotransmitters in neurons). It mainly consists of two subunits, alpha and beta tubulin and are assembled by microtubule associated proteins (MAPs). The tau protein and its different isoforms are one type of MAPs (MAPT) and are believed to be essential for the tubulin to bind into the microtubule (Witman *et al.,* 1976; Karen 1991). Tau was first discovered in 1975 by an American science team as a co-purifying with tubulin (Kirschner *et al.* 1975) and has been widely studied since then. Tau interacts with its microtubule-binding domains (MBD), which constitute a positively charged and a negatively charged domain, with the alpha and beta tubulin.

The tau-proteins have several binding domains. In the human brain there are six different isoforms resulting from alternative splicing. Three of the isoforms have three binding domains and the other three have four. Isoforms with four binding domains are more stable. The isoforms are developmentally regulated and differs between CNS and PNS (Brandt, 1996).

Phosphorylation of the tau protein can create NFT (Grundke-Iqbal *et al.,* 1986; Kosik *et al.,* 1986). Tau proteins can be phosphorylated in several different ways, for example by the protein kinase, PNK (Kawamata *et al.,* 1998). Phosphorylation occurs for the most part on the amino-acids, serine and threonine (Grundke-Iqbal *et al.,* 1986; Baudier *et al.,* 1987), but it has also been shown to be common on the amino-acid tyrosine (Williamson *et al.,* 2002). The tau protein is made out from a single gene by alternative splicing and can be posttranslationally modified (PTM) in several ways, like tyrosine phosphorylation (Lee *et al.,* 2004), acetylation (Cohen *et al.,* 2011 and Min *et al.,* 2010), cross-linking by transglutaminase (Wilhelmus *et al.,* 2009), glycation (Ledesma *et al.,* 1994), isomerization (Miyasaka *et al.,* 2005b), nitration (Reyes *et al.,* 2008), sumoylation (Dorval and Fraser, 2006), O-GlcNAcylation (Arnold *et al.,* 1996) and ubiquitination (Cripps *et al.,* 2006).

Despite the many years of studying tau, its function is still not fully understood.

The present study was undertaken to establish an assay to analyze phosphorylated tau and how a low single dose of gamma radiation, given to neonatal mice, would affect the phosphorylated tau levels at adulthood.

Materials and methods

Animals and exposure

Pregnant NMRI mice were purchased from B&K, Sollentuna, Sweden, put in separate cages and checked twice daily, (08.00 and 18.00 h) for birth. The housing room had a constant temperature of 22°C and a 12/12-hour cycle of light and dark. The animals were supplied, *ad libitum* with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water. The size of the litters was adjusted to 10-12 pups, within the first 48 hours after birth. By the age of 4 weeks, the litters were separated and males kept with sibling in groups of 3-7 individuals and raised in a room for only male mice.

The exposure was preformed according to Eriksson *et al.* 2007. At postnatal day (PND) 10, the mice were irradiated (The Svedberg Laboratory, Uppsala University) with a single dose of gamma-radiation of 0.5 Gy from a source ^{60}Co. The mice were irradiated in plastic dishes with the surface dose-rate around 0.070 Gy/min. The dose-rate is homogenous within ± 3% over the dish area (10 cm diameter). The dose-rate was obtained by relative measurements using an ionization chamber (Markus chamber type 23343, PTW-Freiburg). The dose-rate at the irradiation position was measured by comparing the

dose-rate at this position with the dose-rate at a position of a known dose-rate (obtained previously by absolute measurements of the dose). The control animals were put in the radiation equipment for the same amount of time but without the exposure of the IR.

At 6 month of age the male mice were sacrificed by cervical dislocation. Cerebral cortex was dissected out on an ice cold glass-plate, immediately frozen in liquid nitrogen and stored at -80°C until use.

For development of assay and analyses of P-value

The cerebral cortex was homogenized in a RIPA cell lysis buffer (Assay Design) (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1% sodium deoxycholate) with 0.5% protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). The homogenates were then centrifuged in 14.000 x g for 10 minutes in 4°C. For the quantification of the total protein content in the homogenates, bicinchoninic acid protein assay reagent (Pierce) method was used. The homogenates were stored at -80°C until use.

The Western blot was preformed as follows; the samples were diluted 1:1 with Leammli Sample buffer (Bio-Rad) with 5% beta-mercaptoethanol and then sonicated and then boiled for 10 minutes in 95°C before running on Tris-HEPES-SDS 10% acrylamide (Thermo Scientific) gel with the suitable buffer, Tris-HEPES-SDS Running buffer (Thermo Scientific) using a Mini-Protean® II Cell (Bio-Rad). Gel electrophoresis was carried out by initial 15 minutes at 100 V followed by 60 minutes at 125 V, washed with Transfer Buffer containing 20% methanol (48 mM Tris-Base, 39 mM glycine, 0.00375% SDS, Bjerrum and Schafer-Nielson) and then transferred to a 0.20µm nitrocellulose membrane (Bio-Rad) using a Semi-dry transfer apparatus (Bio-Rad). The membrane was first blocked in Tris-Buffered Saline (TBS) (0.9 % NaCl, 42.1mM Tris-HCl, 7.5mM Tris-base) containing 0.03% Tween-20 and 5% milk (Tween-Milk/blocking buffer) for 60 minutes and incubated over night at 4°C with the primary antibody. The type of antibody that was used was a monoclonal antibody, specific for paired helical filaments (PHF), AT100 (MN1060, Thermo Scientific). AT100 recognizes PHF-tau phosphorylated at Ser212 and Thr214, with no recognition of normal fetal or adult biopsy tissue in human samples.

The following day the membrane adjusted to room temperature, and then washed with TBS. The membrane was incubated for 60 minutes with Tween-milk buffer containing the horseradish peroxidase-conjugated secondary antibody against mouse (mouse IgG (H+L), KPL, 1:20,000), which detects the immunoreactivity. After the incubation the membrane was washed with Tween-Wash (TBS with 0.1% Tween 20). Immunoreactive bands were detected using an enhanced chemiluminescence substrate, Super Signal West Dura (Pierce) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bands was quantified using IR-LAS 1000 Pro (Fuji Film).

Statistical analyses

A student's two-tailed t-test was used to evaluate western blot analysis.

Results

The titration of AT100 showed a linear response between 10-80 µg of the loaded protein samples (Figure 1a and 2a) and 40µg protein sample showed enough chemiluminescence to get a legible data (Figure 1b and 2b). The different concentrations of the primary antibody, AT100 1 µg/ml (figure 1) and 2.5 µg/ml (figure 2) didn't show any apparent differences from each other. Also 1 µg/ml AT100

gave less background (figure 1b) compared to 2.5 µg/ml AT100 (figure 2b) and was therefore used in further analyses.

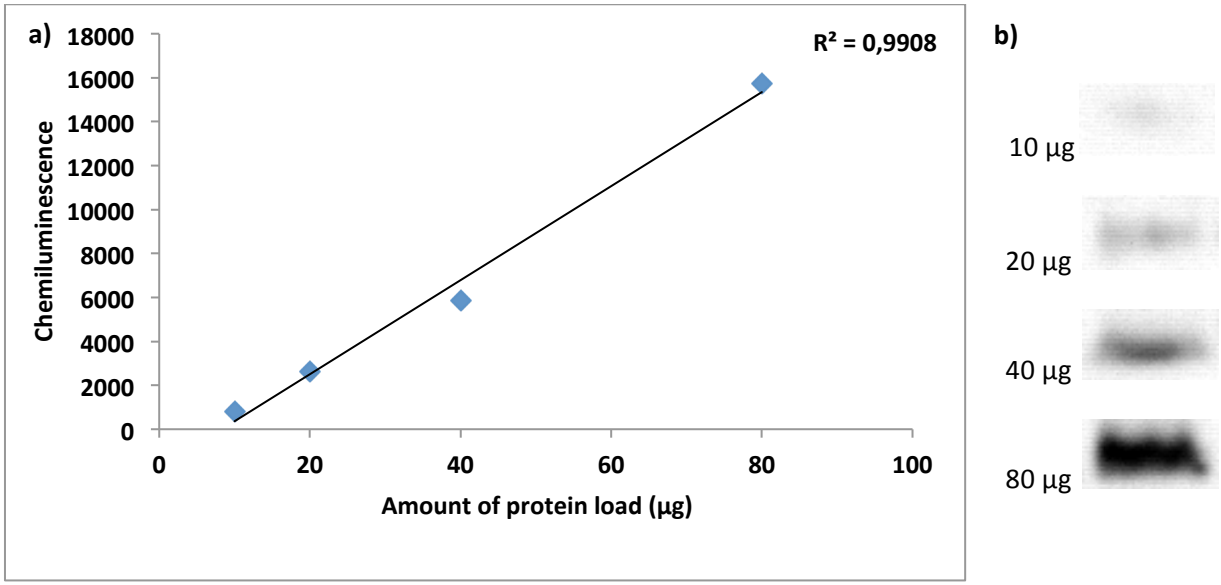

Figure 1. a) Titration from immunoblotting with 1 µg/ml of the primary antibody, AT100, on cortex homogenates from 6 month old NMRI mice irradiated on PND 10 with 0.5 Gy. **b)** The different protein loads versus the chemiluminescence.

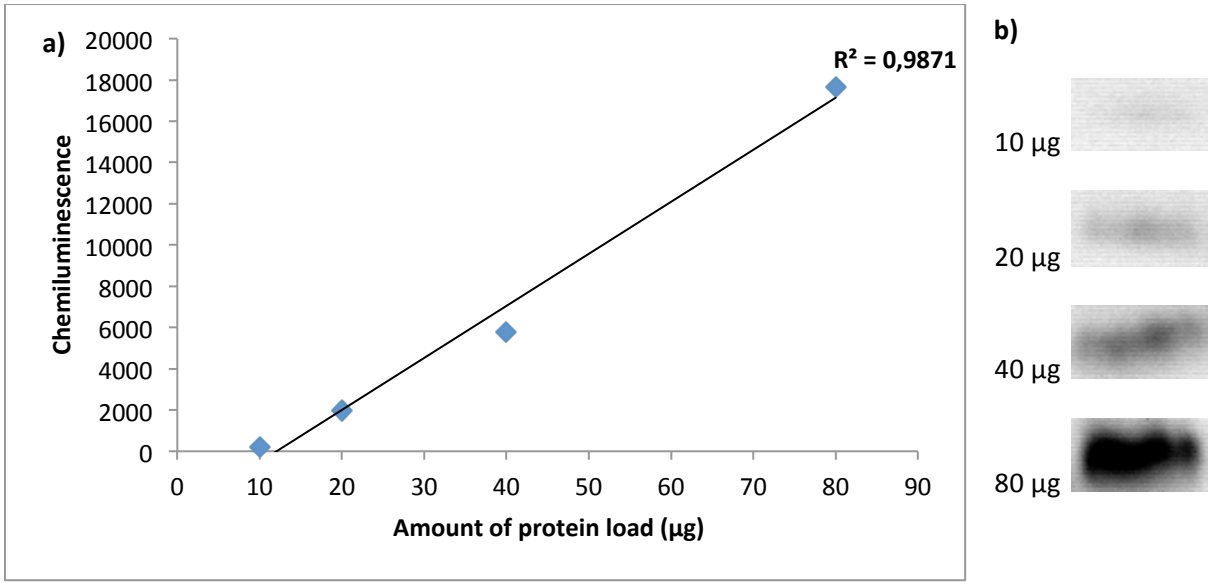

Figure 2. a) Titration from immunoblotting with 2.5 µg/ml of the primary antibody, AT100, on cortex homogenates from 6 month old NMRI mice irradiated on PND 10 with 0.5 Gy. **b)** The different protein loads versus the chemiluminescence.

Immunoblotting with AT100 in samples derived from cerebral cortex of 6 month old NMRI mice, irradiated on PND 10 with 0 respectively 0.5 Gy, resulted in detection of three bands. One of the bands had a strong chemiluminescence around 25kDa (figure 3) and the other two were around 50kDa, but with almost no chemiluminescence at all (figure 3). Those two were excluded (figure 3).

The student's two-tailed t-test of the immunoblotting results from the samples of the neonatally 0.5 Gy irradiated NMRI male mice (n=9) showed a p-value of 0.06, indicating a possible increase of phosphorylation at Ser212 and Thr214 compared to the controls (n=9) (figure 3).

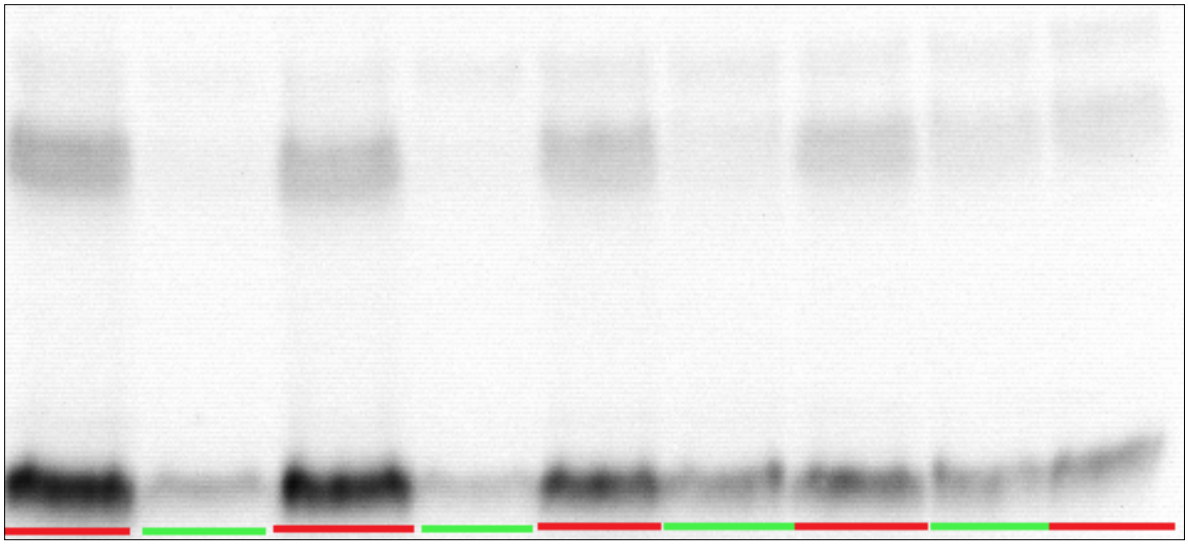

Figure 3. Immunoblotting with AT100 (1µg/ml), from homogenates prepared from cerebral cortex of adult (6-month-old) NMRI male mice neonatally irradiated (at PND 10) to 0 Gy (green mark) and 0.5 Gy (red mark) of gamma radiation. The lower band is around 25kDa. The two upper bands are around 50kDa but have been excluded from the calculations due to low chemiluminescence.

Discussion

Accordingly to Thermo Scientific, the molecular mass of the phosphorylated tau at Ser212 and Thr214 (indicated by AT100) should be approximately at 79kDa, but our results are based on the band around 25 kDa. The bands in the 25 kDa area shows a good correlation, R^2=0.9908 (fig 1a) which can justify its usage. It is known that tau may appear in a wide range of masses (25-80 kDa). Further cleavage of the tau protein may results in truncated fragments of various molecular masses. (Giannetti *et al.,* 2000; Sjögren *et al.,* 2001; Zilka *et al.,* 2012) that might be a possible reason for the observed results.

The results of the immunoblotting showed a tendency ($0.05<p<0.1$) of increased phosphorylation at Ser212 and Thr214 in the adult NMRI male mice, neonatally irradiated with 0.5 Gy, when compared to controls. More samples are needed to strengthen this observation that a low single dose of gamma-radiation increases the phosphorylation of tau at Ser212 and Thr214.

All neurodegenerative diseases, like Alzheimer´s disease, get worse with age. Most of them are classified as proteopathies (diseases were a certain kind of proteins start to misfold and become abnormal), so the time after exposure could also have had an effect on the irradiated NMRI mice's symptoms and therefore also have an effect on the increase in phosphorylated tau levels. Therefore further studies with the same doses should be made, but with sampling at a later time point.

In conclusion, this study has shown a trend in the hyperphosphorylation at Ser212 and Thr214 of the tau protein in adult NMRI male mice, following a neonatal irradiation dose of 0.5 Gy. Further studies are needed to ascertain this observation and also to improve the knowledge about the possible effects of low doses of IR, during critical periods in the brain development, may have for the adults.

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