

The Role Of The Peroxisome Proliferator Activated Gamma Receptor (PPAR- γ) In Reducing Of Neurodegenerative Disorders

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Abstract:

The peroxisome proliferator activated gamma receptor (PPAR- γ) has been shown to be involved in immune differentiation as well as playing a key role in the immune response during the inhibition of the inflammatory response. Neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative changes. This study aims to shed some light on the process of astrocyte proliferation, also explain the specific role of astrocytes in the inflammatory response as well as to attempt to reverse any detrimental changes. The main goal is to follow

the evolution of pathology using organotypic slices culture technique, and then try to improve or reduce the disease using PPAR- γ agonist.

The results on the effect of PPAR- γ agonist and rosiglitazone, after a week of treatment, showed that the PPAR- γ agonist inhibited astrocytes activation in both the cortex and hippocampus of the mutant mice organotypic slice culture. Therefore, PPAR activation has beneficial effects in many pre-clinical models of neurodegenerative diseases and CNS injury.

Key words: PPAR- γ agonist; Astrocytes; Neurodegenerative disorder; Cortex; Hippocampus.

Introduction:

There is growing interest in the importance of peroxisome proliferators in the treatment of brain inflammation (Bernardo and Minghetti 2006; Klegeris, McGeer *et al.* 2007; Saavedra, Sánchez-Lemus *et al.* 2011). More evidence referred that peroxisome proliferator-activated receptors may be useful as a therapeutic target for neurologic disease and CNS injury as their activation affects pathologic mechanisms. (Mandrekar-Colucci, Sauerbeck *et al.* 2013). New evidence has indicated that the presence of PPAR- γ agonists, either via food or drugs are most likely beneficial to human health by acting as anti-inflammatory molecules (Martin 2009). Proliferation of a specific cell type is a response to acute brain injury or infection that involves activation and an increased recruitment of astrocytes and microglia. As a result there is, increased production of cytokines, (chemokines) (Bartholdi and Schwab 2006). The activation of astrocytes has been tracked in order to understand the mechanisms and speed of development of neurodegenerative disorders. For example, Lewy body Dementia is a neurodegenerative disorder affecting

the motor system. Lewy bodies are the abnormal accumulation of protein inside nerve cells resulting in PD (Goedert, Clavaguera *et al.* 2010).

Understanding the causes of neurodegenerative diseases is necessary, given that in many cases, the inflammatory response remains unclear. Researchers have begun to address some of the problems that may cause nervous system degeneration, such as oxidative stress or dysfunction of the proteasome system (Emerit, Edeas *et al.* 2004; Halliwell 2006). A number of studies have investigated the causes of neurodegenerative disorders, some of which have focused on the role of the ubiquitin system (Di Napoli and McLaughlin 2005). Thus, one of the important steps in the emergence of neurodegeneration is the dysfunction of the ubiquitin proteasome system (Hara, Nakamura *et al.* 2006). Currently, inhibiting the proteasome system is one of the methods used in the preparation of neurodegeneration models. Therefore, a 26S proteasome of mouse cortical brain tissue has been inhibited to represent 26S proteasomal dysfunction of neurodegenerative disease (Petrucci and Dawson 2004; Bedford, Hay *et al.* 2008).

This study highlights the importance of PPAR- γ agonists in reducing activated gliosis using rosiglitazone (PPAR- γ agonists) to inhibit astrocyte proliferation in a mouse model representing the neurodegenerative disorder.

To study astrocyte proliferation, organotypic slice culture systems have been utilized *in vitro*. Organotypic cultures have the advantage that they are open to a variety of experimental manipulations. To ensure comparability of the *in vitro* model with animals, *in vivo* paraffin sections were used alongside for identification of astrocyte populations. Since there is no published data on volume-dose ratio in slice culture technique, we

anticipated that 25 μM , 50 μM and 100 μM would be suitable to study organotypic slice cultures. Confocal and light microscopy were used to observe the astrocytes in the organotypic brain culture.

Gliosis:

Gliosis is an accumulation of astrocytes in damaged regions of the CNS. This proliferation of astrocytes occurs as a result of two processes, hypertrophy and hyperplasia. The activation of astrocytes is an indication of damage to the nervous system due to stroke, trauma, growth of a tumor, or neurodegenerative disease, which is one of the most common of sources of damage (Pekny and Nilsson 2005). Gliosis is a reactive cellular process leading to scar formation in the central nervous system, as a result of injury. This action creates a dense fibrous network of neuroglia in areas of damage. Glia scar formation identifies a reactive cellular process including astrogliosis that follows the brain injury, which usually leads to death and disappearance of the neurons, known as gliosis (Djebaili, Guo et al. 2005; Bignami and Dahl 2008).

Mechanism of action of gliosis (proliferation of astrocytes):

Gliosis can be defined as proliferation of astrocytes resulting in an increase of astroglia in the damaged areas of the central nervous system (Logan, Berry *et al.* 2006). It is believed that astrocytes help to retain the structure of cells in addition to the main function of holding neurons together in a 3-D matrix. They also serve many other functions such as maintenance of the extra cellular environment and stabilization of cell-cell communications in the CNS (Bear, Connors *et al.* 2006). Astrocytes become active and start proliferating in response to injury such as trauma,

tumor brain growth or neurodegenerative disorders (Buffo, Rolando *et al.* 2010). Due to brain injuries, astrocytes undergo physiological changes which lead to increased synthesis of glial fibrillary acidic protein (GFAP), which astrocytes express. The expression of GFAP occurs in astrocytes. GFAP is an important intermediate filament (IF) protein that allows the astrocytes to initiate synthesis of more cytoskeletal supportive structures (Franke, Krügel *et al.* 2003) .

Astrocytosis; (astrogliosis) is defined as an increase in the number of astrocytes due to the damage of adjacent neurons (Barbeito, Pehar *et al.* 2004). Due to an injury, astrocytes become activated, which resulted in triggers molecules produced at site drive of further brain injury. This process is called astrogliosis (cell hypertrophy) and is followed by up-regulation of intermediate filaments (IF) and increased cell proliferation. At this stage; the astrocytes migrate towards the injured area to create the glial scar (Figure 1 E), and release factors mediating the tissue inflammatory response (Blackburn, Sargsyan *et al.* 2009). It has been reported that the reactive astrocytes can repopulate stem cell numbers after damage. The cells enter the stage of cell proliferation, while astrocytes can continue to divide and migrate to form the glial scar, and release factors mediating the tissue inflammatory response. The next schematic (Figure 1) illustrates the active mechanisms of astrogliosis (glial scar formation).

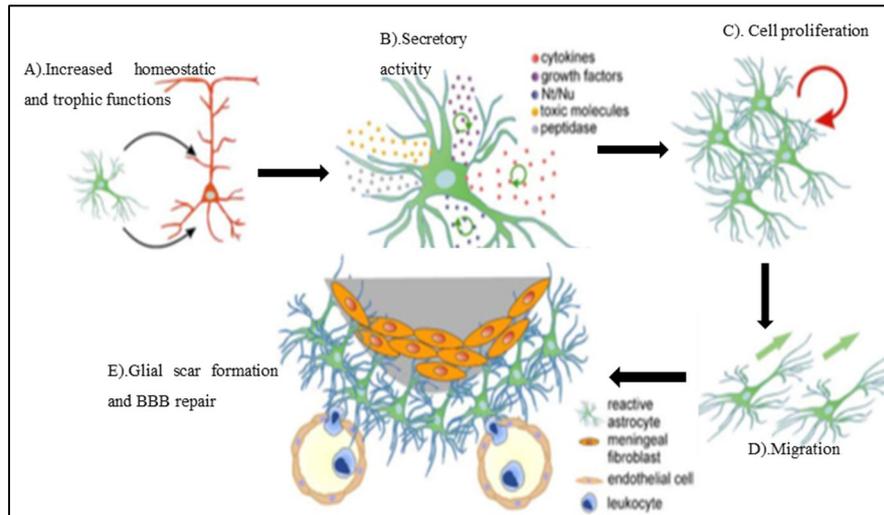


Figure 1; Schematic drawing illustrating astroglial activation (astrocyte proliferation). A: Production of growth factors step (cytokines) and release of nucleotides and toxic compounds. B); Astroglial activation; (cell proliferation). C): Migration towards the lesion. D). Reactive astrocytes contribute in glial scar formation. (Buffo, Rolando *et al.* 2010).

Rosiglitazone; PPAR- γ agonist:

PPAR- γ isoform is a subfamily of the nuclear hormone receptor, named peroxisome proliferator-activated receptor gamma. Thiazolidinediones (TZDs) belong to a group of PPAR- γ agonists, and rosiglitazone is one of the TZDs used in the treatment of type 2 diabetes. The mechanism of the agonist involves binding to the PPAR receptors in fat cells (Malinowski and Bolesta 2000; Culman, Zhao *et al.* 2007).

The current evidence suggests that PPAR- γ may be involved in reducing brain cell inflammation and inhibiting glial cell proliferation (Bernardo and Minghetti 2006; Hirsch, Breidert *et al.* 2006; Schintu, Frau *et al.* 2009; Carta 2013). Moreover, other evidence has emerged, suggesting that PPAR- γ agonists, whether natural or synthetic, may

regulate brain disorder by inhibiting several functions associated with glial cells and microglial activation (Bernardo and Minghetti 2006).

Materials and method:

Animals :

A mouse model of neurodegeneration with 26S proteasome dysfunction was used. It pathologically represents dementia with Lewy bodies disease (*Psmc^{1fl/fl};CaMKII-Cre*) (Bedford, Hay *et al.* 2008). This mouse model depends on a system known as Cre-loxP recombination, which allows the DNA modifications to be targeted to a specific cell type (Kuhn and Torres 2002). This mouse neurodegeneration model (*Psmc^{1fl/fl}; CaMKII α -Cre*) has been used alongside models of wild-type pups (*Psmc^{1fl/fl}; CaMKII α -Wt*). Cre is a 38 kDa recombinase protein derived from bacteriophage P1, which has the ability to cut and recombine DNA between two similarly orientated loxP sites, reference to Figure 2.

It was reported that 26S proteasome function is essential for normal neurological function in mice. In a previous experiment in Bedford's lab, it was shown that these mice are more anxious in open-field analysis and displayed obvious spatial learning deficits in the Morris water maze task at 6 and 8 weeks, respectively. These animals have a short lifespan; they die when aged 3–4 months, as a result of lacking interest in locating food.

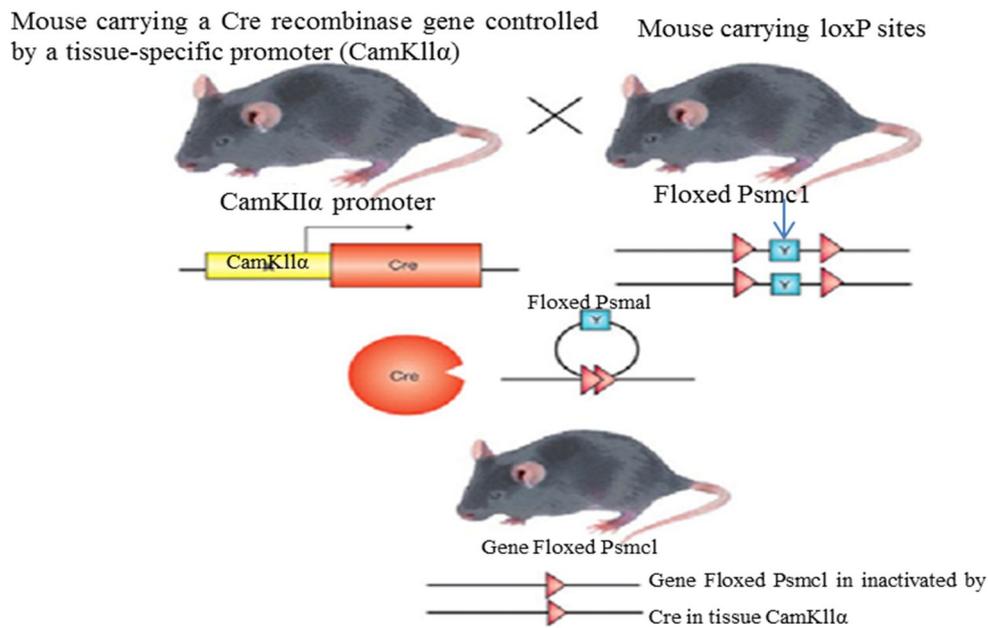


Figure 2; Diagram shows conditional deletion of Psmc1 in mouse, specifically with the CaMKII α promoter (calcium/calmodulin-dependent protein kinaseII α). Expressed cells have 26S proteasome dysfunction, and therefore Ub accumulation pathologically resembling neurodegeneration that occur in Parkinson's' disease and LBD.

Immunohistochemistry materials:

Glial fibrillary acidic protein (GFAP) primary antibodies from thermo scientific were used to identify the astrocytes. GFAP is a common marker used to distinguish astrocytes via intermediate filament proteins (Takamiya, Kohsaka *et al.* 1988; Miguel-Hidalgo, Baucom *et al.* 2000).

Culture mediums:

Hanks' Balanced Salt solution containing sodium bicarbonate, without phenol red, sterile-filtered, PH 7.1-7.5, glucose concentration 0.9 - 1.1 g/l from Sigma was used as a basic salt solution in all sterile

procedures. Culture medium was Dulbecco's Modified Eagle Medium (D-MEM) - 1X, liquid (containing 4500 mg/L; D-glucose), sodium pyruvate, L-glutamine and 25 mM HEPES buffer, Sodium Bicarbonate (PH 7.2) but without sodium pyruvate or phenol red.

Chemicals and instruments:

Normal goat serum was purchased from Vector, bovine serum albumin (BSA) from sigma. Three types of slide were used; cavity slides (depression slide) for organotypic slices, coated slides (APES) for paraffin embedded sections and chrome gelatinised slides for cryostat sections. The slide sizes were 1.0-1.2 mm; Slides were purchased from Fisher. Paraffin embedded sections were prepared using Microtome-LEICA RM2145. Cryostat sections were prepared on a Cryostat CM1900 and McILWAIN tissue chopper was used to produce the organotypic brain slices. Brains were processed in a Leica-TP 1020 tissue processor prior to paraffin embedded sections being prepared. The organotypic slices were incubated in Galaxy S Co2 Incubator. Axiovert 25 PCR machine was used for amplification of DNA from the brains isolated from the pups.

Inserts and slide types :

TransWell® permeable supports 0.4 µm polyester membrane – 12 mm inserts, 12 well plates was used for organotypic brain slice cultures (Corning Incorporated- Coring, NY 14831 the USA). Micro-plate 96 well purchased from IWAKI / EZ-BirdShut®.

Three types of slides were used in the study: cavity slide, APES (2% 3- aminopropyltriethoxysilane) and gelatin-coated (gelatinised) slides.

Mounting medium:

To cover the slides, DABCO (1-4-Diazabicyclo-2-2-2-octane) in 90% glycerol in phosphate buffered saline (PBS) was used after mounting the fluorescent brain tissue. (100 mg of DABCO in 5 ml of PBS and then add 45 ml of glycerol and mix well, and store at 4⁰C).

Peroxisome proliferator-activated receptor gamma (PPAR- γ) agonist and antagonist:

Peroxisome proliferator activated receptor gamma agonists have been used to investigate the effect of reducing astrocyte activation on neurodegenerative disorders. Rosiglitazone (PPAR- γ agonist) and the antagonist (T0070909) were used in this study, both purchased from sigma.

In order to, Organotypic slice cultures of the transgenic mouse model of dementia with lewy body disease were used to assess the effect of peroxisomal proliferation on pathological injury *in vitro* (Bianco, Ridet *et al.* 2002).

The brains of four day old mice were sliced to a thickness of 350 μ m and cultured on membrane inserts. The hippocampus was separated from the cortex before culturing organotypic slice cultures of the mutant and wild-type mice. Four different control brain slice cultures were used; DMSO vehicle, antagonist (T0070907), antagonist with agonist and untreated medium. The slices were grown for 6 weeks and treatment was started by the end of the fifth week. Three different doses of Rosiglitazone PPAR- γ agonist were used on the slices: (25 μ M, 50 μ M and 100 μ M) every other day for a one week. The culture medium was changed every other.

the end of the sixth week, brain slices were fixed and paraffin sections were prepared in preparation for immunohistochemistry staining.

Microscopes:

The DMRB Fluorescence Microscope Leica^{EM} was used to capture fluorescent images of tissues. These were detected by using DAPI (wavelength 350/50 – 460/50), mCherry filters; (480/40 – 535/50) and Rhodamine RedTM (TRITC, 545/20 – 610/75). The Leica DM4000B light microscope fitted with MicroPublisher 3.3RTV camera was used for pictures. Images were captured on a Confocal Microscope (Leica TCS SP2) and analysed off line on a workstation equipped with Volocity software.

Methodology :

Sample preparation:

For whole brain fixation, mice were injected with Heparin, then anesthetized using isoflurane inhalation, and perfused transcardially. Perfusion was initiated by washing out the blood with 0.9 % saline solution at 37⁰C until the perfusate became clear. This was then followed by perfusion of 4% paraformaldehyde (Bustin, Benes *et al.*) 2009 in 0.1 M PBS, PH 7.4 at 4⁰C. The brains were dissected out and stored in Eppendorf tubes containing 4% PFA overnight for post-fixation.

In vitro, brains from postnatal pups aged 1-5 days were used to prepare organotypic slices at thickness 300 µm and cultured for periods of 2, 3, 4, 5 and 6 weeks. For fixation, slices were washed 4 times in PBS and then fixed for 20 minutes in 4% PFA. The inserts of the organotypic slices were embedded in paraffin prior to being sectioned. Slices were stored in

4⁰C until required for immunohistochemistry with either rhodamine red or DAB stains. The slices were labelled with fluorescent rhodamine red.

Organotypic Slice Culture:

Postnatal slices can be maintained in culture from weeks to months, which is the reason for the use of new-born C57 black 6 modified (*Psmc1^{fl/fl}; CaMKII α -Cre*) and wild-type (*Psmc1^{fl/fl}; CaMKII α -Wt*) mice aged 1-5 days, in this study.(Gähwiler, Capogna *et al.* 1997).

The experiments were performed in accordance with protocols from the Scientific Procedures (Act 1986) and the Home Office recommendations with regards to killing by schedule 1 method, at the designated establishment (BMSU, QMC at the University of Nottingham). Mouse pups were decapitated, the brain removed and cut into 200, 300 and 325 μ m thick tissue slices using a Mcilwain Tissue Chopper. The various thicknesses were checked to select the best for long term survival in culture. Chosen slices were transferred to a sterile petri dish containing Hanks' Balanced Salt solution then dissected slices were detached from each other under the microscope using sterile needles and carefully transferred to the transwell membrane inserts.

Organotypic slice cultures were grown for periods of 2, 3, 4, 5 and 6 weeks in culture harvested from control and mutant pups. 1 or 2 brain slices were placed into each transwell insert with 500 μ l medium (Dulbecco's Modified Eagle Medium) in the base of a 12 multi-well plate. The tissues were strictly controlled in terms of sterilization, temperature and rate of carbon dioxide availability and the media was changed regularly. Therefore, the slices were incubated at 36-37⁰C in 5% CO₂ and the medium changed every second day. The culture medium contained the

following components: 89 ml DMEM, 10 ml FCS and 1 ml glutamine. All procedures were carried out under strict sterile conditions.

Tissue fixation of organotypic slices:

The cultured slices were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes. Organotypic slice cultures were grown for periods of 2, 3, 4, 5 and 6 weeks in culture, once harvested from control and mutant pups. Organotypic slices cultures were re-washed three times in PBS. The slices were immersed in PBS and stored at 4⁰C until required for use. Storage time was kept to a minimum to avoid loss of immunogenicity of the samples. Details of storage time for the slices are mentioned in results section.

Effect of PPAR- γ agonist rosiglitazone on the reduction of astrocyte proliferation:

Organotypic slices from the cortex and hippocampal areas were cultured separately. The concentrations of T0070907 PPAR- γ antagonist used were 25, 50 and 100 μ M. T0070907 PPAR- γ antagonist was used separately, and with an agonist as a negative control. In this assay 4 controls were used, which are DMSO vehicle, T0070907 alone, free drug medium, and agonist followed with antagonist. Organotypic slices were grown up to the fifth week. On the first day of the sixth week, experiments were conducted as outlined in the following diagram. The medium was changed every 2 days. Figure 3 shows time course of treating organotypic slice culture with PPAR- γ agonist & antagonist and vehicles.

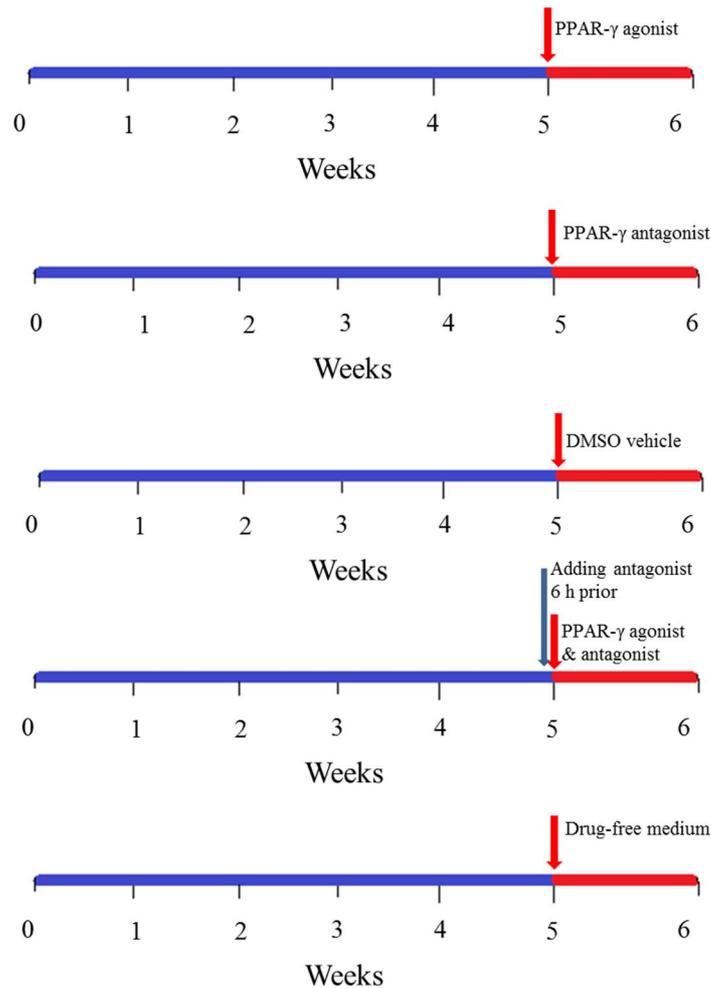


Figure 3; Schematic diagram indicates the time courses of treating the organotypic slice culture with PPAR- γ agonist & antagonist, DMSO vehicle and free drug medium. The slices which were treated with agonist alongside antagonist were pre-treated with the antagonist alone, for 6 hours. The organotypic slices were allowed to grow for 5 weeks, and treatment started at the end of the fifth week for a week. The medium was changed every second day. Organotypic slices were prepared from the cortex and hippocampus of both the mutant and wild-type pups.

Statistics :

One way and two-ways ANOVA, followed by a post hoc student t-test were performed for statistical analysis, where P values <0.05 were considered significant.

Results:

Validation of GFAP marker to stain astrocytes of paraffin sections:

The aim of this study was to detect astrocytes *in vitro*, using an organotypic slice culture. To achieve this, it was important to ensure that astrocytes are detected reliably *in vivo* and then *in vitro* using a Glial Fibrillary Acidic Protein (GFAP) marker. Paraffin-embedded brain tissues of mice and rats were prepared for this experiment. The rat brain sections have been employed as a positive control. A technical challenge was to improve the captured image, and to improve the access of the primary antibody into the cell, by varying the temperature to 25°C Celsius during this process. The underlying mechanism can be described as antigen retrieval, which facilitates the break of protein cross-links that have been formed by formalin during tissue fixation; thereby uncovering hidden antigenic sites. Result shows that GFAP is detectable with good signal-to-noise ratio in both mice and rats sections, Figure 4.

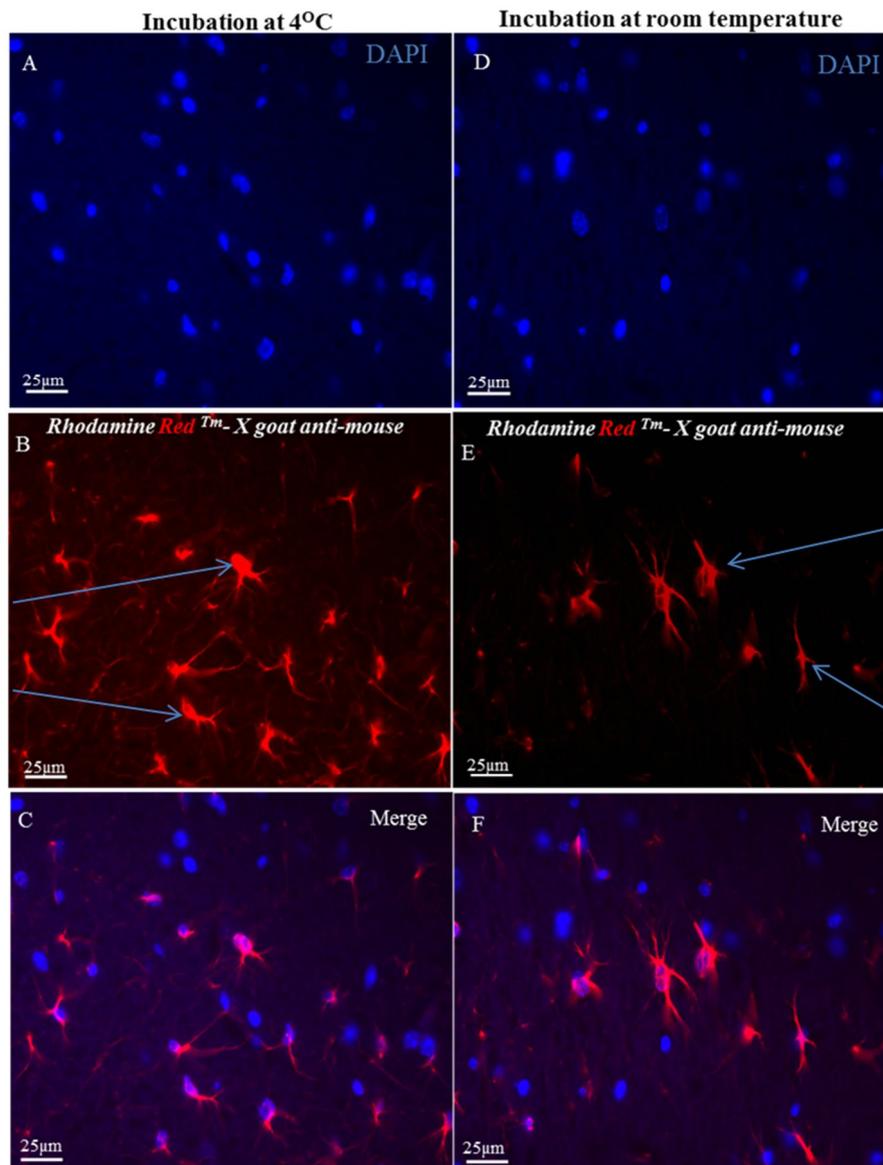


Figure 4; Rat brain paraffin section stained with GFAP (Thermo scientific). A, B and C illustrate slices (mouse brain sections) that were treated with a GFAP antibody and incubated over night at 4⁰C.; D and E: (rat brain sections), Slices were treated with a GFAP antibody and incubated over night at room temperature. The arrows refer to GFAP⁺ cells Magnification X63, scale bar 25 µm

Validation of the use organotypic slice cultures to observe astrocytes, and detect proliferation:

To assess pathological injury in the brains of mice, and ultimately investigate whether the development of gliosis was comparable *in vitro* and *in vivo*, organotypic brain slices were cultured for 2, 3, 4, 5 and 6 weeks. The slices were harvested from brains of mutant and wild-type postnatal mice aged 3-4 days. Brain tissues were sliced at 250 µm thickness and cultured then fixed with 4% PFA for 20 minutes according to target ages. Immunohistochemistry was performed using anti-GFAP antibody and Rhodamine Red™. The results showed that at all ages, gliosis was detectable under both ordinary fluorescent or confocal microscopy. Interestingly, it was observed that nuclei from the astrocytes in the mutant brains were different from those in the control animals. The astrocyte nuclei in the controls looked smaller in weeks 2, 3, and 4, but became identical during the fifth and sixth week (Data not shown at all ages). Additionally, they adopted an oval form in the mutants while remaining spherical in the wild-type slices (it can be seen in Figures 5).

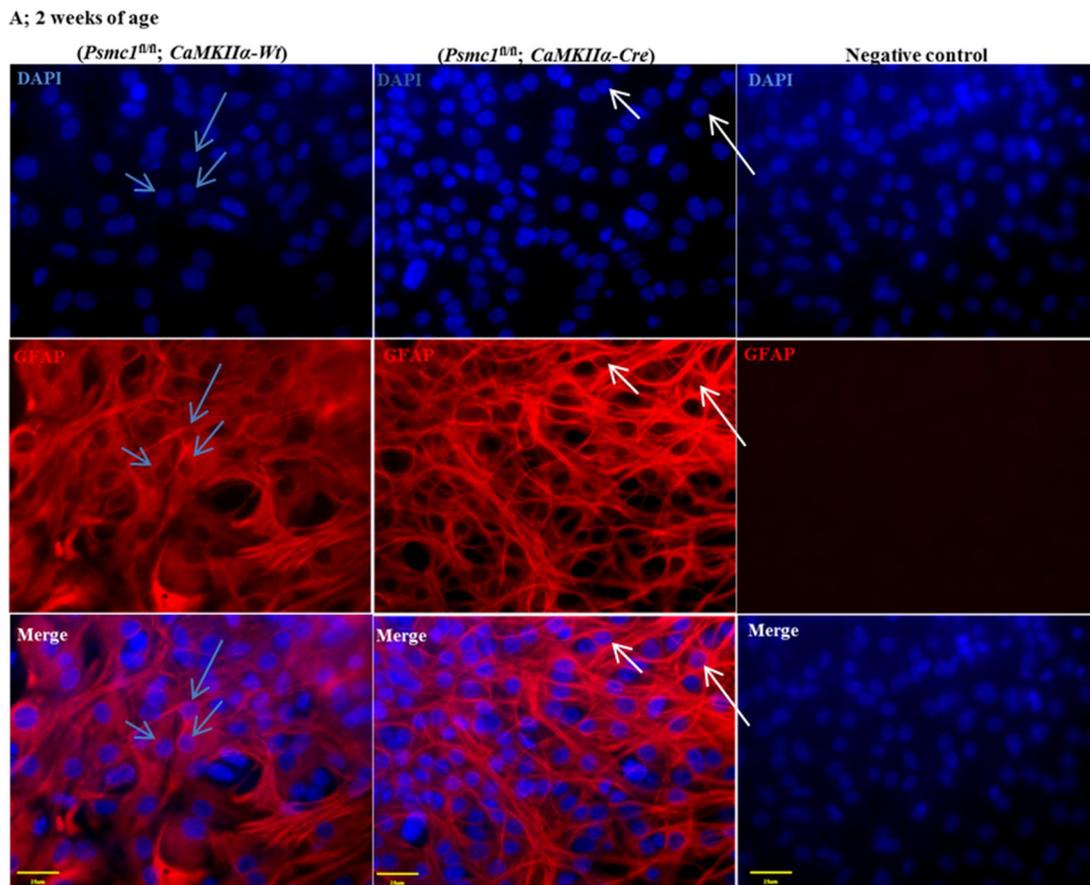


Figure 5; Organotypic slice cultures of mutant and wild-type mice at 2 weeks of age. Brain slices from mice at two weeks of age were grown in an organotypic slice culture system. Slices were fixed and left for 24 hours in PBS at 4°C, and immunohistochemistry performed using anti- **GFAP** antibody. The blue arrows indicate examples of single body's of astrocytes and its nucleus in control animals, while white arrows represent the same cells in mutant slices (magnification 63X). **GFAP** in red and **DAPI** in blue at magnification X40

Progression of neurological disease *in vivo*:

The results obtained showed that the number of astrocytes started to increase in the brains of mutant animals at the third week in both the cortex and hippocampus. The population of astrocytes was greater in the fourth week. Therefore, the disease was more pronounced when the mutant

animal entered the fourth week of age. It was noted that the difference in the number of astrocytes was greater in the area of the cortex than in the hippocampus, especially at weeks 4, 5 and 6.

Reactive gliosis was observed by the third week of age in mutant animals, and gradually increased to the sixth week of age, especially in the cortex. Subsequent statistical analysis showed the results of each week separately represented graphically.

The histograms and figures of each age group will now be analysed. Figure 6-1 and 6-2 are comparison GFAP⁺ cells in the cortex, CA1 and dentate gyrus

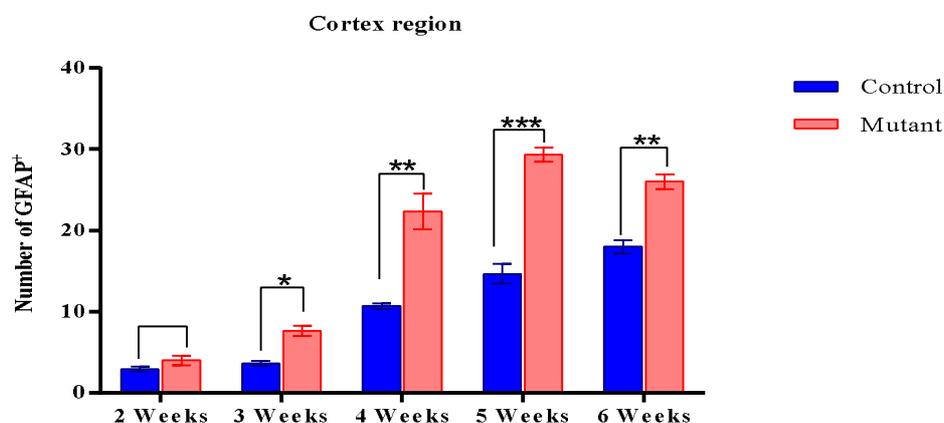


Figure 6-1 Number of astrocytes in wild-type and mutant mice between 2 and 6 weeks of ages in the dentate gyrus. Bar graph showing GFAP⁺ cells in the cortex of control and mutant mice. The mean (\pm SD) number of the GFAP⁺ cells per control (n=3) or mutant (n=3) was determined from three coronal sections per mouse. Asterisks refer to the results of 2-way ANOVA using original cell counts. There was an increase in GFAP⁺ cells in the cortex of mutant mice. Analysis shows a statistically significant difference using unpaired Student's t-tests between control and mutant at 3, 4, 5 and 6 weeks of age. P* $<$ 0.05, P** $<$ 0.001 and P*** $<$ 0.0001

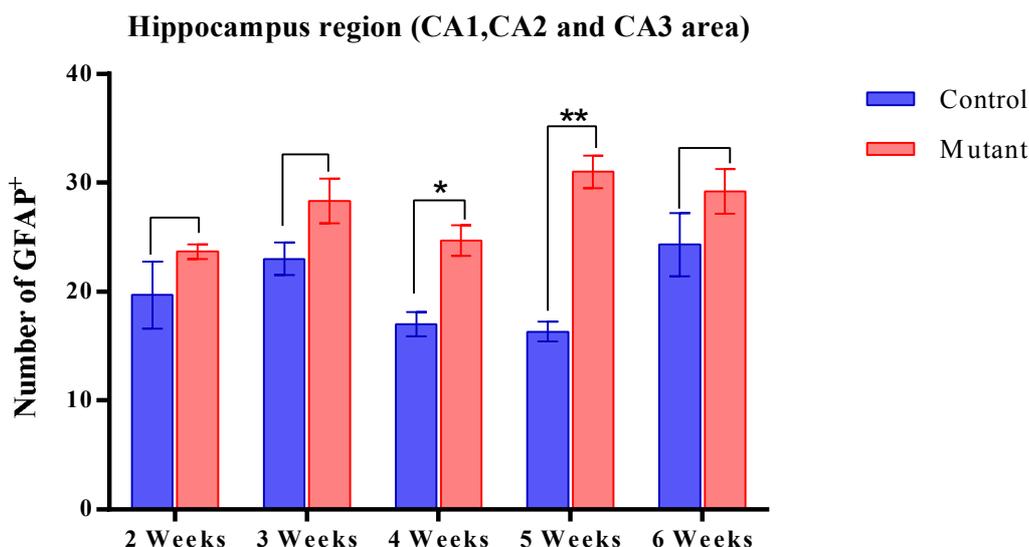


Figure 6-2 Number of astrocytes in wild-type and mutant mice between 2 weeks and 6 weeks of age in the CA1 area of the hippocampus. Bar graph showing GFAP⁺ cells in the hippocampal region of control and mutant mice. The mean (\pm SD) number of the GFAP⁺ cells in the control (n=3) or mutant (n=3) animals was determined from three coronal sections per mouse. Asterisks refer to the results of 2-way ANOVA using original cell counts. There was a significant increase in GFAP⁺ cells in the hippocampal region of mutant mice, compared to the controls, at 4 and 5 weeks of age Where $P^* < 0.05$ and $P^{**} < 0.001$, respectively in an unpaired Student's test

Effects of PPAR- γ agonist on astrocyte population during astrocyte reactivation :

The doses 50 μ M and 100 μ M showed a positive result in the cultures, while the 25 μ M dose did not. We investigated the effect of PPAR- γ agonist, rosiglitazone, in the cortex and hippocampal areas after a week of treatment. The results showed that PPAR- γ agonist inhibited astrocyte activation in both the cortex and hippocampus of the mutant mice

(Figure; 9). The number of GFAP positive astrocytes was significantly decreased in mutant mice slices treated with 100 μ M rosiglitazone in both areas. 50 μ M rosiglitazone caused decreases in the number of astrocytes in both the hippocampus and the cortex, yet was only significant compared to controls, in the cortex. (Figures 7 and 8).

Investigating the effect of two different concentrations of the PPAR- γ agonist rosiglitazone on astrocyte numbers in cortical organotypic slice cultures of wild-type mice.

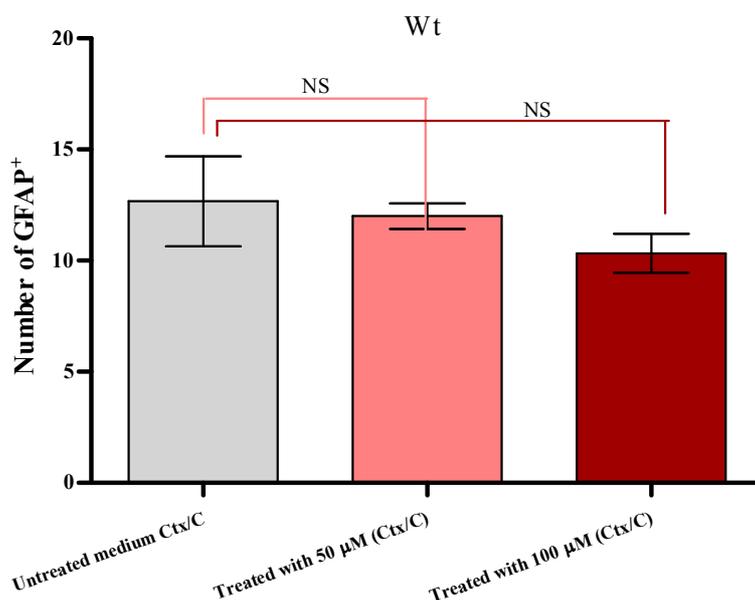


Figure 7; Investigation of the effect of the PPAR- γ agonist; rosiglitazone on astrocyte numbers in the cortex region, using wild-type organotypic slice cultures. The bar chart represents the varying effects of two different concentrations of rosiglitazone on astrocyte numbers in organotypic slice cultures of wild-type mouse cortex. Data are expressed as mean \pm SD. Slices were treated with either 50 μ M or 100 μ M rosiglitazone (PPAR- γ agonist). There is no significant difference between the concentrations and controls when analysed using one way ANOVA with Post-hoc; Dunnett's multiple comparison test, (n=3) P< 0.05.

Investigating the effect of two different concentrations of the PPAR- γ agonist; rosiglitazone on astrocyte numbers in cortical organotypic slice cultures from mutant mice.

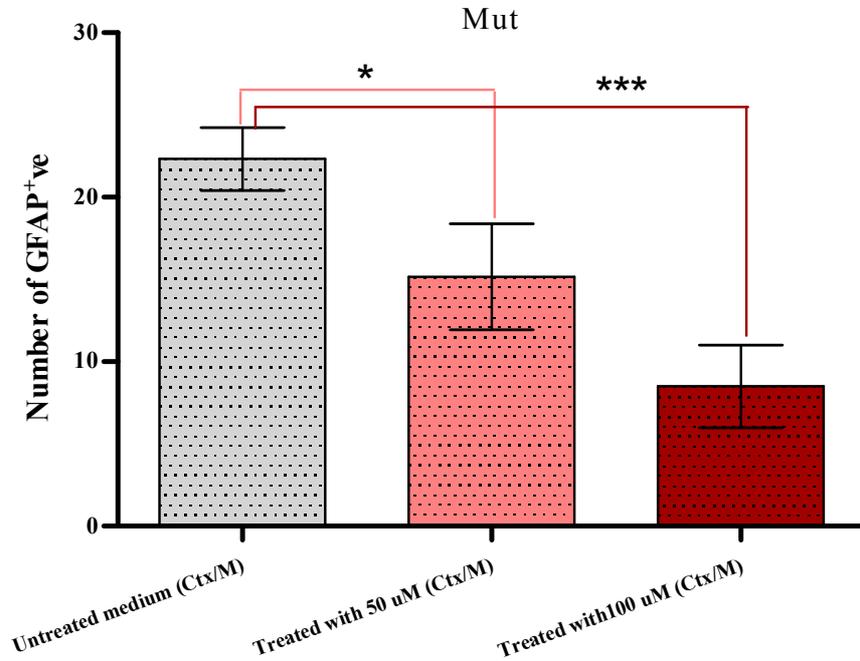


Figure 8; Investigation of the effect of the PPAR- γ agonist; rosiglitazone on astrocyte numbers in the cortex region of cultures from mutant mice. The bar chart represents the varying effects of two different concentrations (50uM and 100uM) of rosiglitazone on astrocyte number in organotypic slice cultures of mutant mouse cortex. Data are expressed as mean \pm SD. A statistically significant difference was seen between control treated cultures and rosiglitazone treated cultures when analysed using ANOVA with post-hoc Dunnett;s multiple comparison test (n=3) *P< 0.01 and ***P< 0.001.

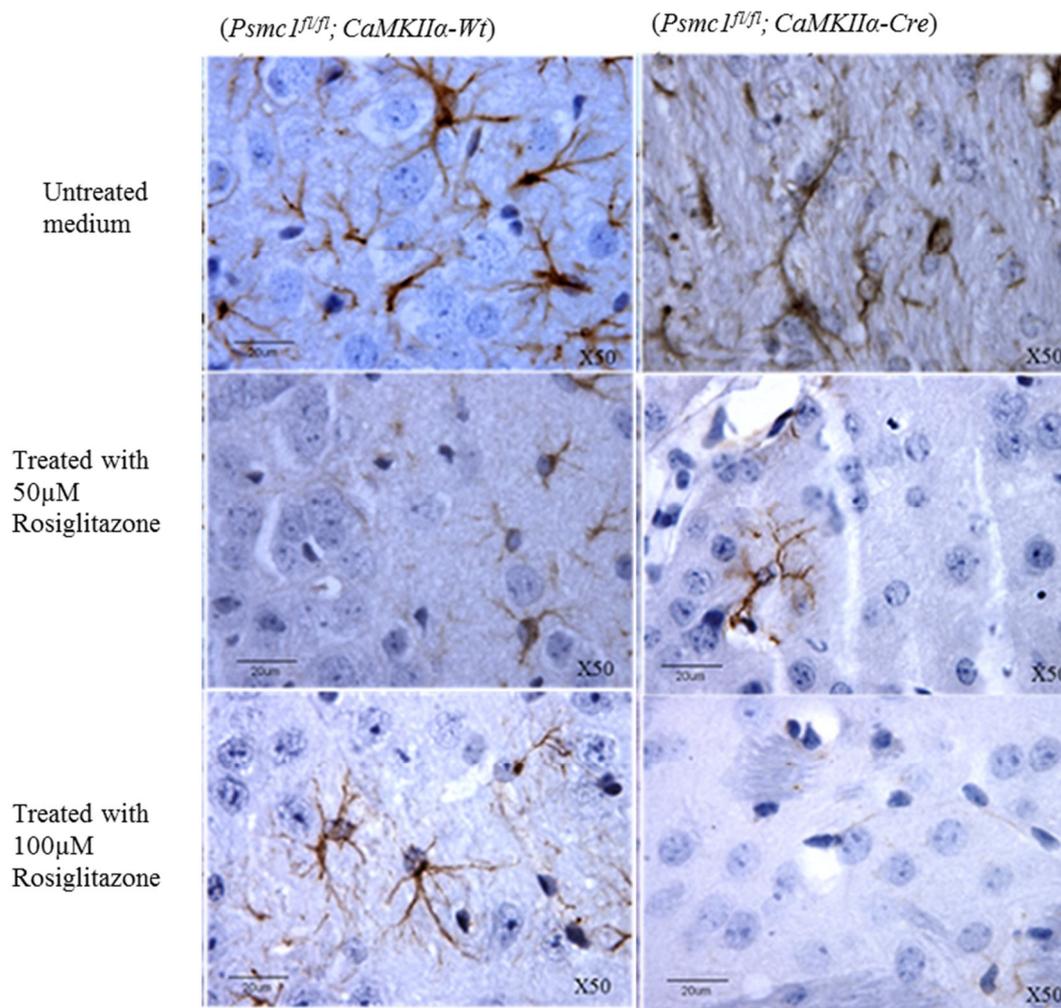


Figure 9; Immunohistochemical staining of GFAP through the cortical region of a brain slice from a wild-type and mutant mice, treated with 50 and 100 µM rosiglitazone. Micrographs illustrate the differences in the number of labelled astrocytes when comparing the control condition with the two concentrations of PPAR-γ agonist, rosiglitazone. Micrographs were captured at magnifications and X50

Discussion:

The development of neurodegenerative diseases *in vivo*:

A transgenic mouse model of neurodegeneration with 26S proteasome dysfunction was used. The model depends on a system known as Cre-loxP recombination which allows the DNA modifications to be targeted to a specific cell type. This model represents Lewy Body Dementia (LBD) in humans.

Paraffin sections were prepared from the brains of *Psmc1^{fl/fl}; CaMKII α -Cre* (mutant) (Ardley and Robinson) and *Psmc1^{fl/fl}; CaMKII α -Wt* (control) mice. Immunohistochemistry was performed for GFAP, to determine at which age astrocyte activation is triggered, following 26S proteasome dysfunction. This is the hallmark of the onset of neurodegeneration. Identification of the age of disease onset was vital so that it could be correlated with start of drug administration. There was a gradual increase in number of astrocytes in the mutant mice, compared to the control mice at three weeks of age which became significant at four and six weeks. This correlates well with the reports of Bedford, Hay *et al.* (2008) who used the same mouse model to study 26S proteasomes in neurodegeneration (Bedford, Hay *et al.* 2008).

Evaluating the use of organotypic slice cultures study neurodegenerative diseases:

Organotypic slice cultures proved to be a useful tool for understanding aspects of neurodegenerative disorders. The major advantage is that the glia cell grows in a 3-D configuration, helping to maintain both microcircuitry connections and relationships between neurons, glia and the extra cellular matrix (ECM). It closely resembles the

in vivo state and is a relatively simple to prepare. However, the slices need to be treated carefully to avoid damage to the slice when transferring to the trans-well inserts.

Rosiglitazone (Peroxisome proliferator activated receptor- γ agonist) may be effective in the treatment of human diseases:

Evidence indicates that astrocyte activation is implicated in the pathogenesis of some neurodegenerative disorders. Astrocytes undergo changes during the process of neurodegeneration, including altered morphology, gene expression and cellular function. These can be collectively encompassed by the term ‘reactive astrogliosis’. However, it is still not fully understood when and how proliferation arises in the developmental course of a neurodegenerative disorder (Sun, Xu *et al.* 2004).

Dose-dependent effects of rosiglitazone, a PPAR- γ agonist:

It was necessary to focus on the role of PPAR- γ agonists in reducing astrocyte proliferation, as this may contribute to knowledge for the future therapy of neurological degeneration. Rosiglitazone is an agonist of PPAR- γ , also known as thiazolidinedione (TZD). It is classified as a non-steroidal anti-inflammatory compound and is widely used in recent neurodegenerative experiments. Rosiglitazone acts as an agonist for PPAR- γ and inhibits secretion of pro-inflammatory molecules (Sastre, Klockgether *et al.* 2006). Few studies have addressed the development of therapeutic strategy, with regards to inhibiting astrocyte activation in an attempt to reduce inflammation and neurotoxic effects in the CNS.

More recently, PPAR- γ agonists have been shown to affect production of pro-inflammatory molecules by primary microglia and

astrocytes (Lovett-Racke, Hussain *et al.* 2004; Storer, Xu *et al.* 2005). Likewise, PPAR- γ agonists have also been shown to reduce disease pathology in animal models that previously presented neurodegenerative disorders (Racke and Drew 2008).

The mouse model chosen for this study has a proteasome deletion that causes neurodegeneration. In this model, astrocyte number increases in response to the neurodegenerative disorder. In this study, the finding that astrocyte activation was high at week 4, that interpreted as proteasome deletion in neurons, causing neuronal death. This is consistent with the findings of Bedford, (2008). At week 4, the neurons may send a signal to astrocytes, causing them to enter proliferation (Bedford, Hay *et al.* 2008). Another possibility is that as a result of neuronal death, the astrocytes increase in number to fill the gaps left by the neurons. Three different concentrations of rosiglitazone were administered. The high concentrations (50 μ M and 100 μ M) have shown significant reduction in astrocyte activation in mutant animals. It is known from the literature that, PPAR- γ regulates gene transcription heterodimers with the retinoic acid receptor (RXR), by binding to a precise site of DNA sequences, termed peroxisome proliferator response elements (PPREs). The mechanism of PPAR- γ agonists rely on the increase in the binding of PPAR- γ to PPRE's, resulting in enhanced translational activity of PPAR- γ (Yi, Park *et al.* 2007).

It was expected that rosiglitazone would reduce proliferating cell number in brain slices, as shown by GFAP⁺ cells staining. Rosario Luna *et al.* (2005) reported that PPAR- γ receptor played a vital role in the regulation of cellular proliferation and inflammation. The majority of the results from the present study agreed with this, as it showed that rosiglitazone did alter proliferation in the hippocampus. This may be consistent with another finding, that a 50 μ M dose did not cause a

reduction in proliferation in the hippocampus, while increasing the dose to 100 μM , caused a significant effect. The number of astrocytes was decreased and statistically different in mutant brains, compared to wild-type brains in both the cortex and hippocampus. However, lower doses of 25 μM had no effect on the astrocyte number in mutant mice in either regions (Luna-Medina, Cortes-Canteli *et al.* 2005).

The current study produced similar findings to studies that have also utilized this PPAR- γ agonist. For example, rosiglitazone has also been shown to prevent cognitive impairment by inhibiting astrocyte activation and oxidative stress in a rat epilepsy model of neurodegenerative disease (Chuang, Lin *et al.* 2012). Similar to the current study it was concluded that the PPAR- γ agonist caused decreased numbers of immune cells, astrocytes, oligodendrocytes, neurons and microglia and may therefore be effective as a therapeutic drug used to inhibit neurodegeneration (Chuang, Lin *et al.* 2012).

Conclusion:

Peroxisome proliferator-activated receptor gamma is protective against neurodegeneration, by its action in preventing astrocyte proliferation. This effect could be reversed by administration of the antagonist T0070907. In the current study, it was hypothesized that treatment with the PPAR- γ agonist rosiglitazone could be used to inhibit astrocyte proliferation, and therefore enhance neuro-protection against neurodegenerative disorders. Organotypic slice cultures were treated with 25 μM , 50 μM and 100 μM doses of Rosiglitazone after five weeks of culture for 7 days. This experiment showed a therapeutic effect of rosiglitazone, after it was bound to PPAR- γ . Rosiglitazone is capable of reducing astrocyte proliferation, which could reduce the effects of neurodegeneration in this particular model.

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