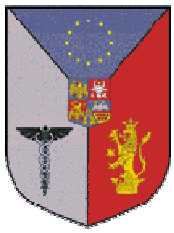
University of Medicine and Pharmacy of Craiova



Ph.D. Thesis

Modulation of microglia motility by cyclooxygenase inhibitor *in vivo*

Summary

Ph.D. SUPERVISOR: Prof. Dr. Maria Iancău

Ph.D. CANDIDATE: Bogdan Cătălin

Craiova

2013

Introduction

Due to a longer life span, in developing and developed countries world population is getting older. As a result new health problems concerning older populations arise.

I Literature review

Chapter 1 Microglia function in CNS

1.1 Introduction

As resident inflammatory cells of the brain, microglia play a key role in normal brain function. Any imbalance in their activity can contribute to neurodegenerative diseases, despite the fact that microglia are only 10% of the total brain cells within the normal brain parenchyma (Lawson et al. 1990).

1.2 Microglal movement in normal brain

Recording microglia activity, in the intact CNS, shown that microglia's ramifications are continuously moving within the surrounding parenchyma (Davalos et al. 2005). This cellular stat describes a microglia with a small body and a large ramification of its processes.

1.3 Microglal movement in injured pharenchyma

No matter the nature of the detected lesion microglia will replay to it. First signs of reaction will be within minutes, when one starts to observe a morphology change is called polarization. It is then rapidly followed by an processes extension towards the lesion site. Body cell migration and in the end phagocytosis will end the microglia inflammatory response (Hanisch and Kettenmann 2007).

Microglia response is based on the equilibrium between ITAM-ITIM signaling and paring of activator/inhibitor signaling receptors that can regulate both magnitude and nature of the response.

Chapter 2 Microglia biology and Alzheimer's disease.

2.1 Introduction

Two main theories regarding microglia cells involvement in Alzheimer's disease exist. First one is the amyloid cascade/neuroinflammation hypothesis. It is centered around the assumption that amyloid activates microglia, that became involved in causing the neurodegeneration and so leading to AD dementia (Streit et al. 2004a).

The second hypothesis is the microglia dysfunction one. It states that microglia have a decreased clearance of beta Amyloid (Aβ), diminished trophic support and increased neurotoxin production (Streit et al. 2004b).

2.2 Dynamics of microglia - Amyloid interactions

Cerebral accumulation of amyloid is one defining feature of Alzheimer's disease (AD). Microglia has long been associated with cerebral Aβ deposits (Stalder et

al. 1999), but still basic questions about it's role in AD pathology have yet to be answered (Morgan et al. 2005).

2.3 Microglia function in Alzeimer's disease

It has been suggested that microglia become dysfunctional and so they are less efficient in removing and degrading Aβ in later Alzheimer's disease (Lee 2010; Hickman et al. 2008), especially because reports shown a progressively down regulation of both receptors and enzymes which are involved in microglial Aβ-uptake and degradation in a transgenic mouse model of Alzheimer's disease (Hickman et al. 2008).

Microglial function can be restored by interventional anti-Aβ approaches, such as Aβ vaccination, even if cell dysfunction develops early in the course of AD in an Aβ-dependent fashion (Krabbe et al. 2013).

2.4. Microglia activation in Alzeimer's disease

Microglia effects on AD seem to have double component. On one hand their activation seems to be neuroprotective at early stages of the disease but at older ages and in severely ill patients the effects could be counterproductive (Solito et al. 2012).

Chapter 3 Non-steroidal anti-inflammatory drugs and Alzheimer's disease.

3.1 Cyclooxygenases - inflammation and cognition

Data suggest that there is an important role for COX-2 derived PGE2 in maintenance of memory function, although higher PGE2 levels can be part of an early inflammatory response (Cunninghan 2012). One longitudinal study reported an early PGE2 increase followed by a declining levels of PGE2 in the same time as memory declined can be detected (Combrinck et al. 2006).

3.2. Microglia, PG Synthesis and NSAIDs

Microglia is known to have two important roles at cerebral level: is the preferred target for NSAIDs and is the main source of PGs

Without interacting with COX-1,COX-2, AA or PGES activities, and just by inhibiting the release of COX in LPS-inducting microglial cultures, classical NSAIDs and paracetamol (acetaminophen) reduce PGE2 synthesis (Greco et al. 2003).

3.3. Carprofen as a COX inhibitir molecule

Carprofen (Rimadyl) is a non-steroidal anti-inflammatory drug (NSAID) of the propionic acid class, that includes ibuprofen, naproxen, and ketoprofen.

3.3.2 Clinical pharmacology

The mean terminal half-life of carprofen is approximately 8 hours (range 4.5–9.8 hours), after single oral doses varying from 1–35 mg/kg of body weight. After a 100 mg single intravenous bolus dose, the mean elimination half-life was approximately 11.7 hours.

3.3.3 Dosage and administration:

The recommended dosage for subcutaneous administration is 2 mg/lb (4.4 mg/kg) of body weight daily.

II Personal contribution

4. Aim of this thesis

The main aim of this thesis was to study how cyclooxygenase (COX1, COX2, and COX3) inhibitors affect microglia motility in normal mouse cortex. The second part of the study was to see if microglia motility gets impaired in the presence of beta amyloid, thus confirming the microglia dysfunction hypothesis.

5. Material and methods

5. 1. Animals

Animals were bred and raised to age in a sterile environment, with a constant temperature of 21 °C. The humidity of the rooms was between 40 to 70%.

Studies were done on single and double transgenic mice, 3 to 5 months of age, with an average body weight of 30 grams.

5.1.1. **Green microglia mouse.**

Investigated green microglia mice express a green fluorescent protein in monocytes, macrophages and microglia. This is achieved by the placement of EGFP (enhanced green fluorescent protein) reporter gene into the *Cx3cr1* locus, encoding the chemokine receptor CX3CR (Jung 2000).

5.1.2. β Amyloid and green microglia mouse

Double-transgenic mice that over express amyloid precursor protein (APP) and also incorporate a chimeric human/murine APP construct bearing the Swedish double mutation and the exon-9-deleted PSEN1 mutation (APPSwe þ PSEN1/DE9) provided by Jackson Labs' stock Number: 004462 were acquired. For this study the TG(APPswe,PSEN1dE9)85Dbo/J) mice were crossbred to a homozygous TgH(CX3CR1-EGFP) mice. The obtained mouse had a non fluorescent over expressed amyloid precursor protein and a fluorescent microglia.

In this study a total of 17 animals were used. The majority of the animals were single transgenic mice (15: 8 males and 7 females). Just two double transgenic female mice were use.

Five distinguished groups were investigated:

5. 2. Methods used in the study

5. 2.1. Study Design

This study was designed as an acute one. After a 24 hours accommodation, a subcutaneous injection was done in all mice. Within approximately 12 hours

(between 10 and 14 hours), the animal was anesthetize and a cranial window was implanted above the right somato-sensory cortex of the animal. A single imaging session was done per animal.

After the 5x image taken with a CCD camera and after finding the target with the 20x objective, a baseline of 30 minutes was recorded for each animal. After this 30 minutes recording, a laser induce microlession was made and then an additionaly 60 minutes of recording was done for the same imaging area.

5.2.2. Subcutaneous injection

Before injecting, the mice were anesthetize with volatile anesthetic Isoflurane. The back skin of the mouse covering, the right leg, was pull up and the injection was made.

5.2.3. Anesthesia

Because cranial window implantation needs a lot of maneuvering of the mouse for anesthesia a cocktail of Ketamine (100mg/ml) and Xylasine (20mg/ml) was used.

5.2.4. Surgery

First, the skin covering the skull needs to be removed, subcutaneous fat, remaining on the bones needs to be removed. Once the bones are clean, a craniotomy is done on the right parietal bone.

After the craniotomy is completed and the durra has been clean Kwik-Sil was applied. A small, round cover slip is dropped on the Kwik-Sil, cyanoacrylate (superglue) is applied to it's edges

When the superglue has hardened dental cement can be applied all over the exposed bones and over the edges of the glass. A period of minimum 2 hours should pass until first imaging session.

5.2.5. Two photon laser scanning microscopy

High resolution *in vivo* imaging was performed using our custom-made two-photon laser-scanning microscope (2P-LSM), equipped with a fs-pulsed titanium-sapphire laser having a peak power higher than 3.3 W and a tuning range between 680nm to 1080nm, able to simultaneously record up to four channels.

5.2.6. Imaging protocol.

Parallel, uniformly spaced planes were recorded, digitized and processed to obtain z-stacks of images. The total acquisition time for a stack of 50 to 60 images was approximately 2 min. Recordings of, at most, 60 µm stack depth were obtained 30 minutes before a lesion and 60 minutes after.

5.2.7. Sholl analysis

Morphological analysis of neuritis (dendrites and axon) has been performed of surveying microglia.

5.2.8. Statistical processing of the obtained data was performed.

6. Results

We investigated microglia behavior both in the intact adult brains during the surveying state and immediately after a local injury done, using the laser used for two-photon microscope.

6.1 Control group

The speed, analysis of surveying microglia process and activated microglia process shows an approximately 50% increase in the activated microglia process speed compared to the surveying state ($p<0.0001$). No statistical difference was found when analyzing the variance ($p=0.2$) of the two microglia forms.

The linear Sholl analysis done on surveying microglia showed a maximum microglial surveying territory of about 45 µm. The mean process microglia length was about 25 µm.

6.2 Group treated below therapeutic dose

Surveying microglia process and activated microglia process shows an increase in the activated microglia process speed, compared to the surveying state ($p<0.0052$).

The linear Sholl analysis done on surveying microglia showed a maximum microglial surveying territory of about 40 µm. The mean process microglia length was about 35 µm

6.3 Group treated with therapeutic dose

Time-lapse recordings of the same brain region show a decrees in the activated microglia process, compared to the surveying state ($p=0.007$).

The linear Sholl analysis done on surveying microglia showed a maximum microglial surveying territory of about 38 µm. The mean process microglia length was about 35 µm

6.4 Group treated above therapeutic dose

The only notable difference between surveying and activated microglial process speed was that the individual cell variance was increased once activation started ($p<0.0001$). The linear Sholl analysis done on surveying microglia showed a maximum microglial surveying territory of about 38 µm.

6.5 Alzheimer model group

Using *in vivo* imaging of TG(APPswe,PSEN1dE9)85Dbo/J)xTgH(CX3CR1-EGFP) mouse cortex we found microglia processes where extending or retracting processes in the brain intact parenchyma.

No significant increase in the activated microglia process speed, compared to the surveying state ($p=0.15$).

6.6 Control compared to treated animals

No physiological difference was observed microglia behavior towards the necrotic induce lesion done in all mice groups.

6.7 Control compared to app mouse group

Extending or retracting microglia process speeds were not significantly different in between the control group and the Alzheimer's disease-like pathology mouse ($p<0.05$)

After a laser induce lesion, we compared the speed of processes send by microglia towards the necrotic lesion site. We found that there is a significant difference in between the two groups ($p=0.15$).

The linear Sholl analysis done on surveying microglia showed no statistical difference in between the branching of the two groups ($p=0.54$).

6.8 All treated groups compared

The aim of this study was to see how non-steroidal anti-inflammatory drugs influence microglia motility, thus shading some light on how the use of NSAID is beneficial in the treatment of Alzheimer's disease.

The results show no significant difference in extensions and retractions speeds of microglia processes ($p>0.05$)

After the lesion all groups had the same microglia response. ANOVA testing showed a significant statistical difference with a $p<0.0001$, when comparing speeds of activated microglia.

The most interesting part of the analyzing data was when we compared variance in the processes speeds in the intact brain. A dose dependent effect was observed. As the dose increased the variance of the processes got smaller.

7. Discussion

Most scientists believe that microglia cells are involved in AD pathology, although there is still a debate on how microglia actually affects brain function the case of AD.

7.1 Principal findings

7.1.1 Normal microglia motility

The speed analysis of surveying microglia process and activated microglia process shows an approximately 50% increase in the activated microglia process speed compared to the surveying state ($p<0.0001$), thus showing that the model chosen is perfect to evaluate microglia motility both in normal and altered brain parenchyma.

7.1.2 Microglia motility under cyclooxygenase inhibitors treatment

For all animals groups treated with cyclooxygenase (COX) inhibitors, we found no statistical difference in between microglia surveying speeds and the control one. This can mean that beta amyloid is cleared via microglia normal housekeeping function even under NSAIDs treatment, confirming the neuroinflammatory theory. When analyzing process speeds send towards a necrotic lesion we found, to our surprise, that all treated groups did not increase their speeds, although the normal reaction was kept.

7.1.3 Microglia motility and beta amyloid

In our experiments we saw little to no activity in clustered microglia in the normal brain parenchyma, but when we did a necrotic lesion near this clusters we could see microglia processes send by clustering cells towards the necrotic lesion, clearly showing that microglia that are clustered are capable of reacting.

In contrast to our findings other reports done on baseline dynamics of plaque-associated microglia showed that microglia motility gets impaired in the presence of Aβ (Koenigsknecht-Talboo et al. 2008, Krabbe et al. 2013).

7.2 Clinical relevance

In vivo transcranial measurements of microglia dynamics under NSAIDs reveals that in the normal brain parenchyma the surveying state of microglia is not affected making COX inhibitors an ideal treatment in early stages of AD.

Conclusions

8. Conclusions

1. Analyzing surveying and activated microglia process speeds, recorded for the control group, showed an approximately 50% increase of activated microglia speeds compared with surveying state ($p<0.0001$) and no overall significant difference between our findings and previous results described by other scientists ($p>0.05$), thus ***validating our experimental model.***
2. Trying to record the anti-inflammatory modulation of microglia via cyclooxygenase inhibitors, we treated a **group of animals with a below therapeutic dose of Carprofen (1mg/kg)** and found ***no statistical difference for the surveying microglia*** speed compare with the control group ($p>0.05$), however when ***activated microglia speeds were significant lower ($p=0.0012$) compared with surveying state.***
3. Microglia behavior, in the **therapeutic dose group (5mg/kg)** is similar to control and 1mg/kg treated animals, with ***no statistical difference recorded for the surveying state ($p>0.05$), but with significant statistical difference in the activated state ($p=0.007$).*** While one hour later, after a necrotic lesion was made, microglia, in both control and 1 mg/kg groups, were able to form a shielding ring around the lesion, in the ***5 mg/kg treated mice microglia was unable to completely surround the lesion***.
4. Microglia surveying process speeds, for the **over dose treated mice (10mg/kg)** were similar with control surveying speeds and all other treated mice group speeds ($p>0.05$). Microglia of the over dose mice also ***failed to completely surround the lesion***.
5. Our results revealed, **for the first time**, that microglia in mice treated groups fail to increase their process speeds when they get activated by a neighboring new lesion, suggesting that ***cyclooxygenase inhibitor treatments affect the motility of activated microglia and not surveying ones***, so clinical trials

using NSAIDs as therapeutic drugs against Alzheimer's disease should consider smaller doses of cyclooxygenase inhibitors.

6. Microglia are capable to respond in the same manner in both normal and APP mouse brain with **no morphological or behavior changes, except clustering, detected in the Alzheimer's disease-like mouse model, compared with normal animals.** Surveying process speeds analysis show ***no significant difference in between control and APP mouse (p>0.05), showing no signs of microglia dysfunction in the intact brain***. However, activated microglia process speed failed to increase as seen in the control group, ***no significant difference was recorded between activated and surveying process speeds (p=0.15),*** showing that ***directed process motility and phagocytic activities. are two functions that seem to be impaired in mice with pathology, compared to age-matched non-transgenic animals.***
7. By using Sholl analysis on all animal groups, we were able to show that ***anti-inflammatory treatment using a COX inhibitor seems to have a direct effect on microglia branching, decreasing the maximum length of microglia for all treated groups.***
8. Our results suggest that ***NSAIDs treatment is beneficial only in very early stages of AD and can be detrimental in late stages of AD*** and ***should be initiated and maintained in a low doses***, because it seems that higher doses have a more negative effect on surveying microglia speeds that low doses.

9. Selected references

1. Combrinck, M, Williams, J, De Berardinis, MA, Warden, D, Puopolo, M, Smith, AD and Minghetti, L 2006, 'Levels of CSF prostaglandin E2, cognitive decline, and survival in Alzheimer's disease', *J Neurol Neurosurg Psychiatry,* vol. 77, no. 1, pp. 85-8.
2. Cunningham, C and Skelly, DT 2012, 'Non-Steroidal Anti-Inflammatory Drugs and Cognitive Function: Are Prostaglandins at the Heart of Cognitive Impairment in Dementia and Delirium?', *Journal of Neuroimmune Pharmacology* vol. 7, no. 1 , pp. 60-73
3. Davalos, D, Grutzendler, J, Yang, G, Kim, JV, Zuo, Y, Jung, S, Littman, DR, Dustin, ML and Gan, WB 2005, 'ATP mediates rapid microglial response to local brain injury in vivo', *Nat Neurosci,* vol. 8, no. 6, pp. 752-8.
4. Greco, A, Ajmone-Cat, MA, Nicolini, A, Sciulli, MG and Minghetti, L 2003, 'Paracetamol effectively reduces prostaglandin E2 synthesis in brain macrophages by inhibiting enzymatic activity of cyclooxygenase but not phospholipase and prostaglandin E synthase', *J Neurosci Res,* vol. 71, no. 6, pp. 844-52.
5. Hanisch, UK and Kettenmann, H 2007, 'Microglia: active sensor and versatile effector cells in the normal and pathologic brain', *Nat Neurosci,* vol. 10, pp. 1387–1394. doi: 10.1038/nn1997.

6. Hickman, SE, Allison, EK and El Khoury, J 2008, ‘Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer’s disease mice’, *J Neurosci*, vol. 28, pp. 8354–8360, doi: 10.1523/JNEUROSCI.0616-08.2008.

7. Jung, S, Aliberti, J, Graemmel, P, Sunshine, MJ, Kreutzberg, GW, Sher, A and Littman, DR 2000, 'Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion', *Mol Cell Biol,* vol. 20, no. 11, pp. 4106-14.

8. Koenigsknecht-Talboo, J, Meyer-Luehmann, M, Parsadanian, M, Garcia-Alloza, M and Finn, MB et al. 2008, ‘Rapid microglial response around amyloid pathology after systemic anti-Abeta antibody administration in PDAPP mice’, *J Neurosci,*vol. 28, pp. 14156–14164.

9. Krabbe, G, Halle, A, Matyash, V, Rinnenthal., JL and Eom, GD et al. 2013, ‘Functional Impairment of Microglia Coincides with Beta-Amyloid Deposition in Mice with Alzheimer-Like Pathology’, *PLoS ONE*, vol.8, no.4: e60921. doi: 10.1371/journal.pone.0060921.

10. Lawson, LJ, Perry, VH, Dri, P and Gordon, S 1990, 'Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain', *Neuroscience,* vol. 39, no. 1, pp. 151-70.

11. Lee, CY and Landreth, GE 2010, ‘The role of microglia in amyloid clearance from the AD brain’, *J Neural Transm*, vol.117, pp. 949–960, doi: 10.1007/s00702-010-0433-4.

12. Solito, E, McArthur, S, Christian H, Gavins F, Buckingham JC and Gillies, GE 2008, ‘Annexin A1 in the brain – undiscovered roles?’, *Trends Pharmacol. Sci.*, vol. 29, pp. 135–142, doi: 10.1016/j.tips.2007.12.003.

13. Streit, WJ 2004a, 'Microglia and Alzheimer's disease pathogenesis', *J Neurosci Res,* vol. 77, no. 1, pp. 1-8.

14. Streit, WJ, Mrak, RE and Griffin, WS 2004b, 'Microglia and neuroinflammation: a pathological perspective', *J Neuroinflammation,* vol. 1, no. 1, pp. 1-4.