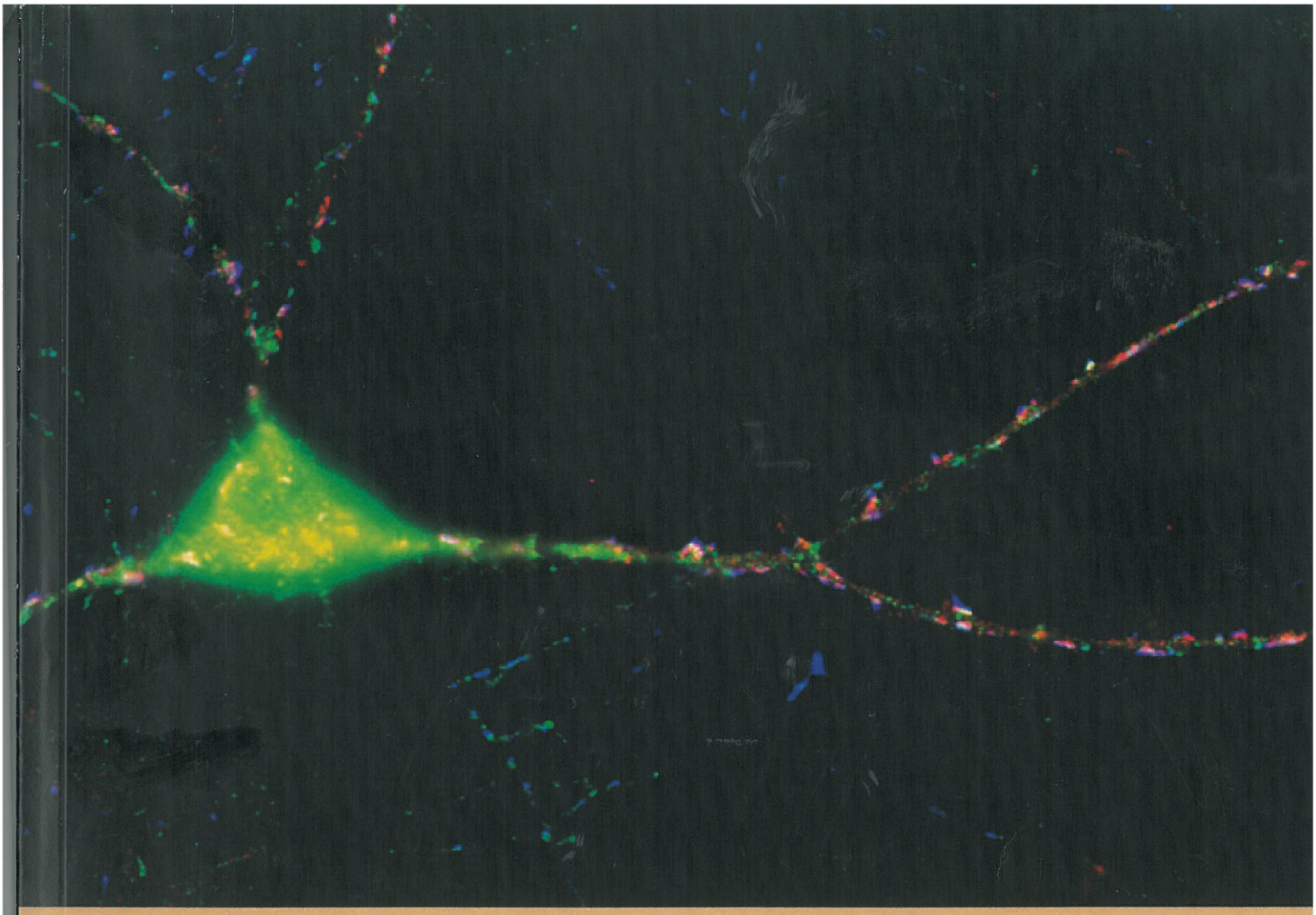

Jimmy George

Purines control Neuron-Glia interaction during Neuroinflammation

Tese de Doutoramento em Biologia Experimental e Biomedicina, ramo de Neurociências e Doença, orientada pelo Prof. Dr. Rodrigo Cunha do Faculdade de Medicina da Universidade de Coimbra e, pelo Dr. Thierry Amédée do Interdisciplinary Institute for Neuroscience da Universidade de Bordeaux, apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra

Junho 2015

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Purines control Neuron-Glia interaction during Neuroinflammation

By

Jimmy George

Coimbra, Junho 2015

Tese apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra (IIIUC), para cumprimento dos requisitos necessários à obtenção do grau de Doutor na especialidade Biologia Experimental e Biomedicina (BEB), realizada sob a orientação científica do Prof. Dr. Rodrigo Cunha (Faculdade de Medicina da Universidade de Coimbra), e do Dr. Thierry Amédée (Interdisciplinary Institute for Neuroscience da Universidade de Bordeaux). Este trabalho foi desenvolvido no Centro de Neurociências e Biologia Celular da Universidade de Coimbra e Interdisciplinary Institute for Neuroscience da Universidade de Bordeaux

Thesis presented to the Institute for Interdisciplinary Research of the University of Coimbra (IIIUC) in partial fulfilment of the requirements for the PhD degree in Biomedicine and Experimental Biology (BEB), under the supervision of Prof. Dr. Rodrigo Cunha (Faculty of Medicine, University of Coimbra) and Dr. Thierry Amédée (Interdisciplinary Institute for Neuroscience, University of Bordeaux). This work was conducted at both Centre for Neuroscience and Cell Biology, University of Coimbra and Interdisciplinary Institute for Neuroscience, University of Bordeaux.

I would like to thank the ENC network for the opportunity and the funding to pursue this doctorate thesis. I also would like to thank the other funding partner FCT (Fundação para a Ciência e Tecnologia) for also financially supporting this work. I would also like to extend my thanks to the Centre for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal and Interdisciplinary Institute for Neuroscience (IINS), University of Bordeaux, France for being gracious hosts for the duration of my work there and, for the opportunity to work and interact with a diverse scientific group present therein.

To my sister Tresa George

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ACKNOWLEDGEMENTS

Foremost I am eternally grateful to my supervisor Dr. Rodrigo Cunha (University of Coimbra) and Dr. Thierry Amédée (University of Bordeaux) for their guidance in this project. I owe them their enthusiasm and their patience in making this project all together an amazing experience. Also, I am thankful for their constant support and their invaluable suggestions that they had to offer for the writing of this thesis. I am extremely honored to have worked under their supervision.

Thanks to Dr. Christophe Mulle (University of Bordeaux) for providing me the chance to carry out majority of my work in his group. Your gracious hospitality, along with your scientific expertise is greatly appreciated.

My sincere thanks to Dr. Catarina Gomes (University of Coimbra), who with patience, took me under her wing and guided me throughout the first year of my work. I shall forever remain grateful for her contributions to my project.

I would also like to thank Francisco Queiroz (University of Coimbra), for been there during all the ATP related experiments, and more, and Vincent Maingret (University of Bordeaux) for taking the time to teach me how to operate the electrophysiological setup. Your generosity and selflessness is appreciated.

My thanks to Silvia Silva for her help in preparing the Portuguese version of the abstract and Joana Ferreira for her time and patience to guide me through the administrative process of submitting this work.

I also would like to thank my team members from Coimbra: Francisco Queiroz, Daniel Rial, Cristina Lemos, Steffania Zapettini, Patirica Simoes, Diana Rodrigues, Paula Agostinho, Paula Canas, Henrique Silva, Angelo Tome, Adalberto, Nuno Machado, Tiago Alfaro, Pedro Garcia, Ricardo Rodrigues, and Dani Pochmann. Your friendly and kind comradery helped me adapt to the new city and the new life.

I also extend my thanks to my lab members in Bordeaux: Bernat Goncalves, Mario Carta, Silvia Silva, Gael Barthet, Adam Gorlewicz Vincent Maingret, Zhang Pei, Sabine Fievre, Meryl Malezieux, Marilena Griguoli, Stefano Zucca, Severine Deforges, Sandrine Pouvreau, Juli Rumi massante, Noelle Grosjean, Virginne Labrousse and Christophe Blanchet for being there to share the excitements and frustrations of performing experiments in the lab.

I am grateful to Virginie Labrousse for her help and expertise with the microscopic images of P2X4, and to Jennifer Peterson (University of Bordeaux) for donating her time in obtaining the cover image.

My thanks for Dr. Teresa Goncalves and her team (CNC, Coimbra), for accommodating me and allowing me to use the teams culture hood. This generosity is appreciated.

I would also like to thank the members of the animal facility in Bordeaux and for me to reap the benefits of the initial hard work you guys did.

My thanks to the two lab technicians of the labs I worked in; Carolina Veloso (Coimbra), and Remi Sterling (Bordeaux). You made working in the lab a little bit easier.

I extend my thanks to the Ms. Silvia Sousa for managing the administrative issues that came along with me, throughout the period of my work.

I was part of the ENC-Network as one of the joint-PhD programme student. Thus I am grateful to the co-ordinator, Maaike Leusden, and the director, Arjen Brussard, as well as to the PIs and colleagues for the discussion and the wonderful time during the meetings and conferences.

My thanks also to Jessie Gutierres (Coimbra) and Steve Carvalho, Ana Quiteria, and Eleonora Lugara (Bordeaux) for being wonderful roommates during the duration of the PhD.

I also would like to thank my sister Tresa George and my friend Ann Erinjeri for always been there to share my happiness and sorrow throughout my doctoral studies.

I am indebted to my parents without whom I wouldn't be writing this thesis at all. It is there everlasting love *and support that has helped me become the person I am today.*

I would like to thank the ENC network and FCT for the funding they provided for me to complete my research work.

Finally I would like to express my gratitude to my family, friends and well-wishers for their constant support and encouragement throughout the duration of my study

Jimmy George

List of Publications:

Published

George J, Gonçalves FQ, Cristóvão G, Rodrigues L, Meyer Fernandes JR, Gonçalves T, Cunha RA, Gomes CA. Different danger signals differently impact on microglial proliferation through alterations of ATP release and extracellular metabolism ***Glia***. 2015; Apr 4. doi: 10.1002/glia.22833 [Epub ahead of print]

Gomes C, Ferreira R, **George J**, Sanches R, Rodrigues DI, Gonçalves N, Cunha RA. Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia ***J Neuroinflammation***. 2013 Jan 30;10:16

In Preparation

Jimmy George, Virginie Labrousse, Christophe Mulle, Rodrigo Cunha, Thiérry Amedee

Role of ATP in P2X4 dependent modulation of frequency facilitation at mossy fiber-CA3 synapse through a microglia based inflammation model. **In Preparation**

Book

Catarina Gomes, **Jimmy George**, Jiang-Fan Chen, Rodrigo A. Cunha. Chapter 5: Role of adenosine A_{2A} receptors in the control of neuroinflammation – relevance for Parkinson's disease. **The Adenosinergic System - A Non-**Dopaminergic Target in Parkinson's Disease. ***Springer***; Still in Press

List of Abbreviations

1. 5-BDBD- 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one
2. [Ca2+] - Free intracellular calcium concentration
3. A1R - A1 receptor
4. A2AR - A2A receptor
5. A2BR - A2B receptor
6. A3R - A3 receptor
7. ACSF - Artificial cerebrospinal fluid
8. ADP - Adenosine-5'-diphosphate
9. AMP - Adenosine-5'-monophosphate
10. AMPA - α-amino-3-hidroxi-5-methyl-4-isoxazolopropionic acid
11. AP - Alkaline phosphatase
12. ARL67156-6-N,N-Diethyl-D-β,γ-dibromomethyleneATP
13. ATP - Adenosine-5'-triphosphate
14. Aβ - β-amyloid
15. BBG - Brilliant blue G
16. BDNF- Brain derived neurotropic factor
17. BrdU –5-Bromo-2'-deoxy-uridine
18. BSA - Bovine serum albumin
19. Bzatp - 2'-3'-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate
20. CaCl2- Calcium chloride
21. CAPS - 3-(Cyclohexylamino)-1-propanesulfonic acid
22. CCPA - 2-chloro-N6-cyclopentyladenosine
23. CNS - Central nervous system
24. CsOH- Caesium hydroxide
25. D-AP5- D-(-)-2-Amino-5-phosphonopentanoic acid
26. DAPI - 4',6-diamidino-2-phenylindole
27. DMSO - Dimethylsulphoxide
28. DPCPX - 1,3-dipropyl-8-cyclopentyladenosine
29. DTT - Dithiothreitol
30. EC50 - Concentration of a ligand eliciting 50% of the maximal response
31. Ecto-5'-NT - Ecto-5'-nucleotidase
32. EDTA - Ethylenediaminetetraacetic acid
33. EGTA - Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
34. E-ntpdases - Ecto-nucleoside triphosphate diphosphohydrolases
35. GABA - γ-aminobutyric acid
36. Gtp- guanosine- 5'-triphosphate
37. GFAP - Glial fibrillary acidic protein
38. HCl- Hydrochloric acid
39. HEPES - N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
40. IL-1β - Interleukin 1-β
41. IP - Intraperitoneal
42. IP3 - 1,4,5-inositol triphosphates
43. JNK - c-Jun amino-terminal kinases
44. LPS- lipopolysaccharide
45. LCCG- 2-(carboxycyclopropyl)glycine
46. LTP - Long-term potentiation
47. MAP-2 - Microtubules-associated protein 2
48. MAPK - Mitogen-activated protein kinase

49. Mepsp - Miniature excitatory postsynaptic potentials
50. MgCl2- Magnessium chloride
51. Mipsc - Miniature inhibitory postsynaptic current
52. Mrna - Messenger ribonucleic acid
53. MTEP- 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine
54. MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
55. N9- murine microglia cell line
56. NaCl- Sodium chloride
57. NBQX- 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
58. NMDA - N-methyl-D-aspartate
59. P1R - P1 receptor
60. P2R - P2 receptor
61. P2XR - P2X receptor
62. P2X4R- P2X4 receptor
63. P2YR - P2Y receptor
64. PBS - Phosphate buffered saline medium
65. PFA- Paraformaldehyde
66. PKC - Protein kinase C
67. PLC - Phospholipase C
68. PMSF - Phenylmethanesulfonylfluoride
69. PNS - Peripheral nervous system
70. PPADS - Pyridoxal phosphate-6-azo(benzene)-2,4-disulfonic acid
71. PSD-95 - Post-synaptic density protein 95
72. RNA - Ribonucleic acid
73. RT - Room temperature
74. SCH58261 - SCH58261 - 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c] pyrimidin-5-amine
75. SDS - Sodium dodecyl sulfate
76. SEM- Standard error mean
77. SNAP-25 - Synaptosomal-associated protein 25
78. TBS - Tris buffered saline medium
79. TBS-T - Tris buffered saline medium with 0.1% Tween-20
80. TNF-α - Tumor necrosis factor-α
81. UDP - Uridine-5'-phosphate
82. UTP - Uridine-5'-triphosphate
83. Vglut1 - Vesicular glutamate transporter 1
84. A,β-meatp - α,β-methyleneatp
85. B,γ-imatp - β,γ-imidoatp

Abstract

Neuroinflammation via glial cells is a feature associated with brain damage. However, whether the involvement of a neuro-inflammatory condition results from dysfunction of glial cells, or by damaged neurons is to be studied. Furthermore, brain damage not only bolsters glial-derived neuroinflammation but also hampers glial-derived neurotrophic support. Thus, the impact on neuronal circuits by neuroinflammation can be due to, either enhanced levels of inflammatory molecules, or low neurotrophic support. The overall aim of this project was to discover the role of purines in controlling neuron-glia interactions, and since most neurodegenerative diseases also involve synaptic dysfunction, this project focused on the role of purines in controlling synapse-microglia interaction.

Purines have shown to be promising candidates to mediate the interaction between synapses and microglia in the early phases of neurodegenerative conditions. ATP acts as a 'danger signal' and an important activator of microglia. The second major role for purines lies in the increased levels of adenosine that occurs from early stages of neurodegeneration. Adenosine A_1 and A_{2A} receptors ($A_{2A}R$) control information salience in neuronal circuits. Furthermore, studies have shown that A2AR antagonists prevent synaptic modifications, preserving brain function in different animal models of disease. $A_{2A}R$ has also shown to control different features of neuroinflammation, namely microglia activation, proliferation, production of pro-inflammatory cytokines and of growth factors such as BDNF. Generally, this suggests that $A_{2A}R$ plays a dual role in controlling both microglia activation and the feed-forward impact of neuroinflammation on neurons

The aim of the project was to: 1) Understand the role of purines (ATP, A_2AR) in the autocrine role of activated microglia and 2) To evaluate the paracrine role of activated microglia in short term synaptic plasticity and the role of purines thereof.

We found that on stimulation with LPS and Glutamate, N9 microglia cells (cell line of microglia) released ATP at different concentrations (decreased ATP release in case of LPS while increase in case of glutamate, with respect to control). The ATP levels were found to be modulated by $A_{2A}R$ expressed on microglia. Moreover, we observed differences in rate of proliferation between microglia stimulated with LPS and Glutamate. Further pharmacological experiments revealed a co-relation (inverse) between microglial ATP release and microglial proliferation, both of which were modulated by A_{2A} receptors.

To study the paracrine effect of this microglial activation on short term synaptic plasticity, we employed a two model system (cell culture and acute slices). LPS treated N9 microglial cells were added onto acute hippocampal slices and changes in short term synaptic plasticity was measured from CA3-mossy fibers. Patch clamp recordings from acute slices with LPS treated N9 microglia showed reduction in both frequency facilitation and paired pulse facilitation, which on further experiments were found to be modulated by P2X4R and A1 receptor respectively. This study also shows for the first time the expression of P2X4 receptors on mossy fibers.

We have been successful in demonstrating the role of microglial A_{2A} receptors in modulating the ATP release from microglia. Furthermore, we show that the nature of the "danger" signal (Glutamate and LPS) differently modifies purinergic metabolism (in particular ATP conversion into adenosine) and subsequently formats microglia proliferation, by balancing ATP and adenosine signalling (both governed by $A_{2A}R$). This finding points out to the importance of purines (especially A_{2A} receptor and that of ATP-

adenosine metabolism) in governing key neuro-inflammatory events such as microglial ATP release and proliferation.

Moreover our work also defines a role for purines in the interaction between neuron and glia in synaptic transmission, by demonstrating the effect of both microglia derived ATP and its metabolites (adenosine) on neuronal purinergic receptors. LPS treated microglia releases ATP which acts on neuronal P2X4 receptor to reduce frequency facilitation; while the ATP converted adenosine acts on neuronal A_1 receptor to reduce paired pulse facilitation. This dual role of purines (ATP and adenosine) and its receptors in modulating short term synaptic plasticity can prove to be beneficial in further understanding the relation between neuron and microglia in maintaining synaptic regulation. Our work is also the first to demonstrate the expression of P2X4 receptors on mossy fibers.

Thus, our study provides novel insight into the modulatory role of purines in governing the various autocrine functions of microglia (ATP release and proliferation), and the paracrine functions with respect to neuron-glia interactions in events similar to neuroinflammation.

Resumo

A neuroinflamação devida à acção de células gliais é uma característica associada aos processos de dano cerebral. No entanto, não é claro se a neuroinflamação occorre em resultado da disfunção das células gliais ou devido a danos nos neurónios. A neuroinflamação resultante de lesões cerebrais é geralmente associada à acção de células da glia. Isto acontece não apenas devido a uma acção directa das células gliais, mas também devido à falta de apoio neurotrófico que normalmente as células da glia fornecem aos neurónios. Assim, o impacto da neuroinflamação sobre os circuitos neuronais pode ser devido ao aumento dos níveis de moléculas mediadoras de inflamação ou devido à falta de suporte neurotrófico. O objectivo principal deste projecto de doutoramento é investigar qual o papel das purinas no controlo das interacções entre células da glia e neurónios. Em particular, e uma vez que a maioria das doenças neurodegenerativas envolve disfunção sináptica, este trabalho centrou-se sobre a acção das purinas no controlo da interacção entre a microglia e a sinapse.

Estudos recentes têm mostrado as purinas como candidatas a possiveis mediadores da interacção entre sinapses e microglia em fases iniciais do processo neurodegenerativo. O ATP actua como um sinal de 'perigo', activando a microglia. Além disso, as purinas são responsáveis pelo aumento dos níveis de adenosina que ocorre nos estados precoces de neurodegeneração. Os receptores de adenosine A1 e A_{2A} controlam o processamento de informação nos circuitos neuronais. Diversos estudos demonstram que os antagonistas dos receptores A_{2A} têm a capacidade de prevenir alterações sinápticas e preservar a função cerebral em diferentes modelos animais de neurodegeneração. Por outro lado, os receptores A_{2A} têm também a capacidade de controlar diferentes aspectos do processo neurodegenerativo, como a activação e proliferação de microglia, bem como a produção de citoquinas pró-inflamatórias e factores de crescimento como o BDNF. Isto sugere que os receptores A_{2A} desempenham então um duplo papel controlando tanto a activação da microglia como o impacto da neuroinflamação resultante nos neurónios.

Este trabalho procurou 1) entender o papel das purinas (ATP e receptor A_{2A}) na função *autócrina* da microglia e 2) avaliar o papel parácrino da microglia activada (e portanto das purinas) na plasticidade sináptica de curto prazo.

O nosso trabalho permitiu descobrir que a estimulação de células da linha celular N9 (microglia) com LPS ou glutamato induziu a libertação de ATP em diferentes concentrações, com níveis reduzidos de ATP em relação à situação controlo no caso do LPS e aumento dos níveis no caso do glutamato. Nós mostramos também que estes níveis de ATP são modulados pela expressão de receptores A_{2A} na células microgliais. Além disso, o ritmo de proliferação da microglia estimulada com LPS ou glutamato é diferente. Experiencias farmacológicas adicionais revelaram uma co-relação inversa entre a libertação de ATP e a proliferação da microglia, sendo ambos os processos modulados por receptores A_{2A}.

Para estudar o efeito parácrino desta activação microglial na plasticidade sináptica de curto termo, utilizamos um sistema de dois modelos (um de cultura de células e outro de fatias agudas de cérebro de ratinho). As células microgliais N9 tratadas com LPS foram adicionadas às fatias agudas de hipocampo e as alterações na plasticidade de curto termo foram medidas nas sinapses entre as fibras musgosas e as células piramidais de CA3. Os registos electrofisiológicos na configuração *patch-clamp* mostraram uma redução na facilitação neuronal (facilitação por frequência e facilitação de pulsos emparelhados) nas fatias agudas tratadas com N9 activadas por LPS. Em experiencias subsequentes, este trabalho mostra

ainda que estas formas de facilitação neuronal são moduladas pelos receptores P2X4R e A1, respectivamente. Este estudo mostra também, pela primeira vez, a presença de receptores de P2X4 nas fibras musgosas.

Além de tentar compreender o papel dos receptores A_{2A} microgliais na modulação de libertação de ATP pela microglia, este trabalho mostra também que a natureza do sinal de "perigo" (glutamato ou LPS) modifica de forma diferente o metabolismo purinérgico; em especial, a conversão de ATP em adenosina e, subsequentemente, os níveis de proliferação microglial, através do equilíbrio das vias de sinalização do ATP e adenosina, ambos mediados pelos receptores A_{2A}. Esta descoberta chama a atenção para a importância das purinas (especialmente do metabolismo do ATP e adenosina) no mediação dos principais eventos neuroinflamatórios, tais como a proliferação e libertação de ATP microglial.

Este trabalho também define um importante papel para as purinas na interacção entre neurónios e células gliais na transmissão sináptica, demonstrando que o efeito do ATP libertado pela microglia e os seus metabolitos (adenosina) sobre os receptores purinérgicos neuronais. A microglia activada por LPS liberta ATP que, por sua vez, age sobre o receptor P2X4 neuronal para reduzir facilitação de frequência. Por outro lado o ATP é convertido em adenosina que por sua vez actua sobre o receptor A1 neuronal para reduzir a facilitação de pulso emparelhado. Esta dupla função das purinas (ATP e adenosina) e dos seus receptores na modulação da plasticidade sináptica curto termo pode permitir compreender melhor a relação entre neurónios e microglia na manutenção da regulação sináptica.

O nosso estudo proporciona novas pistas sobre o papel modulador das purinas na regulação das várias funções autócrinas da microglia (proliferação e libertação de ATP) e funções parácrinas correspondentes às interações neurónio - glia em estados semelhantes à neuroinflamação.

INTRODUCTION

1.1. Hippocampus

1.1.1. Introduction

The hippocampus is a structure of the limbic system located in the medial temporal lobe. It was named by the neuroanatomist Julius Caesar Aranzi (1564) after its resemblance to a seahorse (from the Greek ιππος = horse, and καμπος = sea monster). Its elongated shape has been compared also to a banana and a ram's horn, the latter leading to the Latin denomination for hippocampal sub-regions as "Cornu Ammonis" (CA)-in association with the ram's horns of the ancient Egyptian god Ammon. Vladimir Bekhterev noted in 1900 the hippocampus involvement in memory after studying the brain of a patient who became amnesic after a bilateral lesion. The hippocampus is involved in formation of new memories, and in the consolidation of previous ones, while playing an important role in pattern separation and completion (Nakashiba *et al*., 2012). Besides its role in learning and memory, the hippocampus is also important for spatial navigation (O'Keefe and Dostrovsky, 1971).

The hippocampus is one of the most studied regions of the brain due to its well-defined cellular architecture, which makes it convenient for physiological research at the synaptic and network level. We know today that this structure plays a pivotal role in humans and other species for the ability to encode and store long-term memory traces (Burgess *et al*., 2002; Kim & Baxter, 2001).

1.1.2. Hippocampal Circuitry

The hippocampus is an elongated tube with its shape being compared to a banana or a sea horse. It's histologically subdivided into four regions: CA1 to CA4 with connections to different pathways and fibres, as shown in the next page.

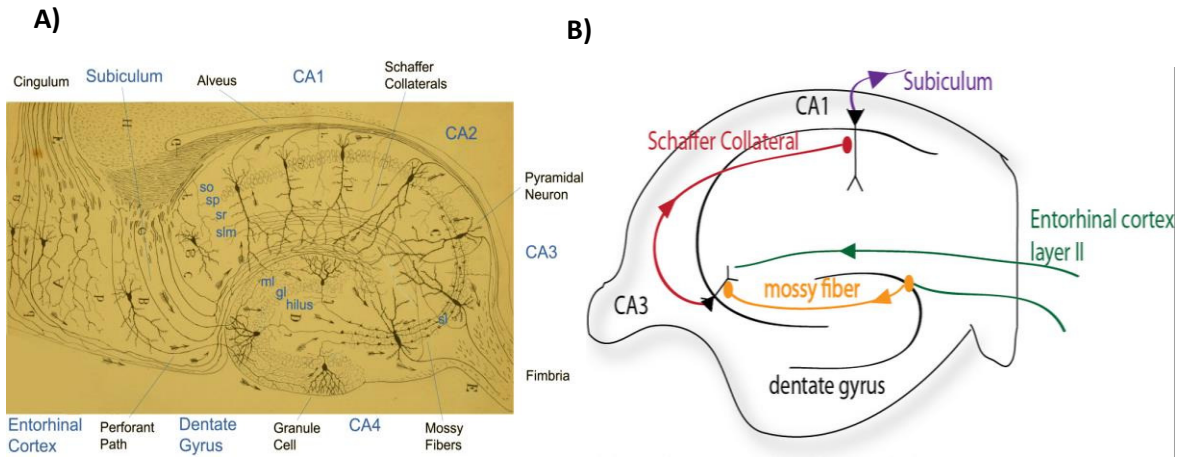


Figure1.1. The hippocampal circuitry A) Diagram of hippocampal structure and connexions (modified from Ramón y Cajal, 1911) **B)** a simplistic cartoon depicting the connections: from the entorhinal cortex of the performant pathway to the dentate gyrus and to CA3 (green). From the dendate gyrus to the CA3 (yellow). From the CA3 to the CA1 through the Schaffer collateral pathway(red) which then leaves the hippocampus towards the subiculm (violet)

Recent discoveries have confirmed and completed Cajal's hippocampal circuit with the following connections (Figure 1.1): EC layer II to GCs, but also to CA3 and CA2 pyramidal cells; EC layer III to CA1 and subiculum; GCs to Mossy cells and vice versa (Scharfman, 2013); GCs to CA4 and CA2 pyramidal cells; GCs in the CA3 area (Szabadics *et al*., 2010); CA3-CA3 recurrent connections 'Associational/Commissural or A/C) (Miles and Wong, 1986; Le Duigou *et al*., 2014); CA3 to GC back projections (Scharfman, 2007); and CA2 to CA1 pyramidal cells (Kohara *et al*., 2013). Besides these excitatory pathways, there are numerous inhibitory interneurons (INs), estimated to be 20% of all cells in the hippocampus (Amaral *et al*., 1990

Lorento de No, way back in 1934 showed that the CA3 region can be subdivided into CA3 a, b and c. CA3a forms the portion of the curve, CA3b forms the region between the curve and the blades of the dentate gyrus and CA3c composes of the blades of the dentate gyrus. The CA3 region also contains an additional stratum called *stratum lucidum*, which contain the mossy fibres. The most noticeable attribute of the CA3 region is that it receives inputs from various pathways. The performant pathway which arises from the entorhinal cortex; the mossy fiber pathway from the granule cells of the dentate gyrus and the third being its own outputs being fed-back as inputs via recurrent collateral pathway (Amaral and Witter 1995).

The cell type of the CA3 region of the hippocampus is the pyramidal cell, which incidentally makes up for majority of the neurons in the pyramidal cell layer. These cells are characterized by having unique and complex dendritic postsynaptic structures called thorny excrescences (Frostscher *et al*., 1991). The cell size (soma) can range from 20 µm to 30 µm in diameter. Although pyramidal neurons comprise the CA3 region, fair amount of interneurons are also present (Witter and Amaral 2004). Both the pyramidal and the interneuron cells receive inputs from the dentate gyrus through the mossy fiber system (Ishizuka *et al*., 1995; Buckmaster *et al*., 1993). However since interneurons are not the focus of this study, much of the following will deal with mossy fibers-CA3 pyramidal cells.

1.1.3. Mossy fibers

The communication between the dentate gyrus to the CA3 region of the hippocampus takes places through granule cells and the axons thereof. This is known as mossy fiber pathway. Ramon y Cajal who coined the pathway, showed that the "fibers" made synaptic contacts onto the pyramidal neurons of the CA3 region (Ramon y Cajal, 1911). This was later confirmed by Blackstad and Kjaerheim in 1961, through the use of electron microscopy.

Mossy fiber (Mf) forms three distinct synaptic contacts with neurons in the hippocampal region. First are the aforementioned expansions that form synapses with pyramidal neurons of the CA3 region through the *stratum lucidum* The synaptic bouton of this area is large and seen at around 150µm distances (Blackstad *et al*, 1970), with a single granule cell connecting with around 15 pyramidal shaped neurons of the CA3 region. While, on the other hand a single pyramidal neuron could have approximately 50 mossy fibers inputs forming a synapse with it (Amaral *et al*, 1990). Filopodia like extensions onto the CA3 interneurons and smaller bouton like terminals of other hippocampal neurons form the second and the third pathway (Amaral 1979; Tashiro *et al*, 2007).

Of the various contacts, this study is solely concentrated on the first synaptic contact mentioned i.e. granule cell to CA3. The basic anatomy of these large mossy fiber terminals is quite unique in various ways. They possess high density vesicles, with very low probability of release. The distance between each release site is said to be as small as 0.45µm (Hamyln 1961). This short distance between release sites indicates a possible crosstalk occurring either presynaptically via Ca^{2+} diffusion, or postsynaptically via glutamate spillover from neighboring release sites. Hamyln (1961) described these synaptic sites being both asymmetrical and symmetrical, indicating their different properties in synaptic transmission.

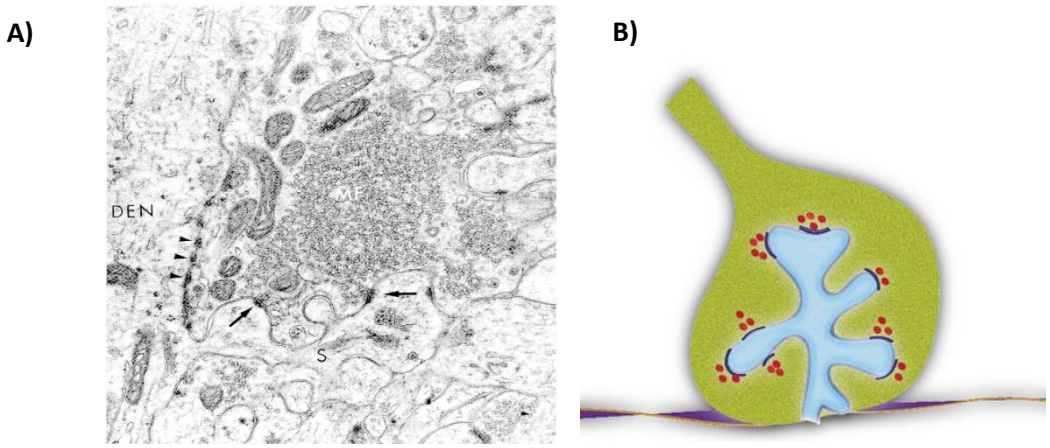


Figure1.2 Ultrastructure of the mossy fiber synaptic complex. (A) Electron micrograph of a MF synaptic complex. The postsynaptic CA3 pyramidal cell dendrite is indicated (DEN) to the left of the presynaptic MF terminal (MF). The presynaptic terminal contains numerous small vesicles and mitochondria. The postsynaptic thorny excrescence is indicated (S) where it penetrates the terminal. Several symmetrical junctions between the dendritic shaft and the terminal are observed (arrowheads) and are representative of puncta adhaerentia. Notice that these symmetrical junctions do not have any vesicles located nearby. In contrast, asymmetrical junctions (arrows) on to the thorn shown a significant clustering of vesicles (From Amaral et al, 1981) **B)** Cartoon depicting the mossy fiber synapse containing multiple release sites (depicted in dark blue) and releasable vesicles (red) (Adapted from Nicoll and Schmitz, 2005)

As shown above (Fig 1.2), Mf contains very large number of vesicles present at their terminals. The number of vesicles at individual release sites is extensive, which might be important to sustain reliable neurotransmission during high-frequency activity (Amaral et al., 1981). Although Mf synapses exhibit a very low release probability (p=0.02-0.2), because of their large number of release sites, the EPSCs can reach unusually high amplitude, such as 1 nA (Kitzing, *et al*., 1994; Bischofberger, *et al*, 2006; Lanore *et al*., 2010).

Additionally to these vesicles each mossy fiber bouton contains smooth endoplasmic reticulum and approximately eight mitochondria (Amaral *et al*., 1981; Blackstad *et al*., 1961). The presynaptic MF bouton envelopes a large multi headed postsynaptic spine called a thorny excrescence that protrudes from a proximal dendrites of hilar mossy cells and CA3 pyramidal cells (Cajal, 1911; Chicurel and Harris., 1992). The Mf boutons appear to be anchored around the excrescence to the dendritic shaft of the CA3 pyramidal cells via a series of symmetrical thickenings called puncta adhaerentia (Hamlyn, 1961). The mossy fiber synapse onto CA3 pyramidal cell is believed to be highly efficient, acting as a 'conditional detonator' that is able to evoke action potentails on CA3 pyramidal neurons if activated repeatedly

(Henze *et al.,* 2002). This synapse may also operate as a teacher synapse that triggers spike timing dependent plasticity at associative–commissural synapses between CA3 pyramidal cells (Kobayashi and Poo, 2004). Thus the mossy fiber pathway plays an important role in complex yet vital functions such as storage and recall of information and pattern completion (Nakazaa *et al*., 2002; Bischofberger *et al*., 2006). The mossy fiber syapse is highly plastic in nature. Several forms of short and long term plasticity have been reported. This list includes paired pulse facilitation, frequency facilitation, post tetanic potentiation and long term potentiation (Salin *et al*., 1996; Toth *et al*., 2000). Though some studies describe the LTP at the mossy fiber synapse to be presynaptic in nature (Nicoll and Schmitz 2005), other studies (Kwon and Castillo 2008; Rebola *et al*., 2008) also show a postsynaptic presence.

1.1.4 Synaptic plasticity

One of the most fascinating and vital properties of the mammalian brain is its plasticity, which is its ability to modify neural circuitry and thereby modify future thought, feelings and behavior. Synaptic plasticity can be referred as the activity dependent modification of the strength or efficacy of synaptic transmission at synapses, which has been suggested to play an important role in the capacity of the brain to incorporate transient experiences into persistent memory traces (Citri and Malenka, 2008). It is known to play a key role in the early development of neural circuitry and any impairment could lead to several neuropsychiatric disorders. Because of its importance, huge amount of work has been spent laying out the different forms of synaptic transmission and understanding its mechanism.

Synaptic transmission can be either potentiated or depressed with durations ranging from milliseconds to hours to even days or weeks (Citri and Malenka, 2008). Transient forms of synaptic plasticity have been associated with short term sensory inputs, temporary changes in behavioral states and short term lasting forms of memory. More lasting changes are thought to play important roles in the construction of neural circuits during development and with long term forms of memory in the mature system (Citri and Malenka, 2008). Such diversity in functions can be attributed to the different forms of synaptic plasticity. The following is a brief overview of the various kinds with particular emphasis on the short term forms of plasticity: Frequency facilitation and Paired pulse facilitation.

1.1.4.1. Short Term Plasticity

Many forms of short term synaptic plasticity, lasting for few milliseconds to minutes, have been observed in almost all the organisms ranging from invertebrates to mammals (Zucker and Regehr, 2002). This could be from a short lasting modulation of transmitter release that can take place due to one of two general types of mechanisms. It involves a change in amplitude of the temporary rise in intracellular

calcium concentration that occurs when an action potential reaches a presynaptic terminal (Zucker *et al*, 1989). The second mechanism happens downstream of calcium elevation involving modulation of biochemical properties involved in vesicle exocytosis (Zucker, 1989)

Short term plasticity seen at the Mf projecting onto the CA3 pyramidal cells of the hippocampus, contrast considerably to that seen in other synapses of the CNS. Events such as frequency facilitation and paired pulse facilitation are observed at these synapses as shown by Salin *et al* (1996). Short term plasticity at mossy fibers is activity dependent in manner. Synaptic activity can be increased or decreased depending on the presynaptic stimulation. This lively model of transmission is referred to as "short term plasticity" or STP for short (review Zucker and Regehr, 2002).

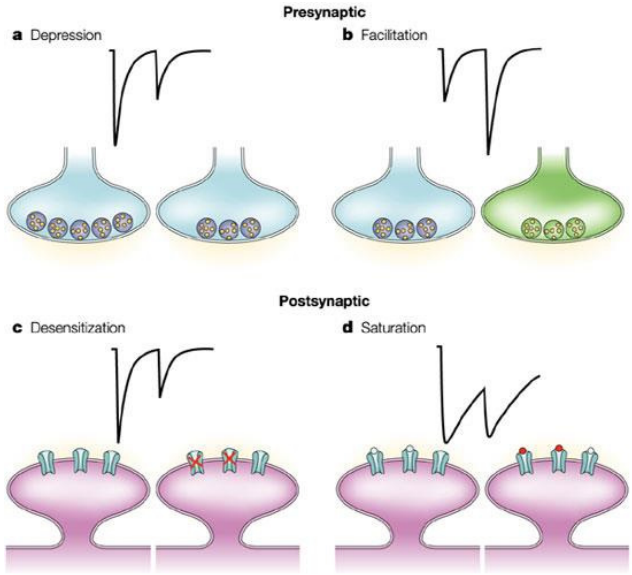


Figure 1.3 Presynaptic and postsynaptic mechanisms of short-term plasticity. Schematized voltage-clamp traces illustrate the influence of two presynaptic mechanisms (a, b) and two postsynaptic mechanisms (c, d) of plasticity on a pair of excitatory postsynaptic currents (EPSCs). Cartoons of presynaptic boutons illustrate possible explanations for presynaptic depression (a) and facilitation (b) and cartoons of postsynaptic spines illustrate desensitization (c) and saturation (d). **a)**| Top, presynaptic depression results in a smaller second EPSC. Bottom, fewer vesicles are available for release on the second (right) stimulus than on the first (left). **b**) Top, facilitation results in a larger second EPSC. Bottom, a residual elevation in intracellular calcium (green shading), combined with the influx of calcium in response to the second stimulus, results in enhanced release. **c)** Top, similar to depression, desensitization results in a smaller second EPSC. Bottom, under prolonged exposure to transmitter, some receptors can enter a non-responsive state (red crosses, right) and be unable to respond to transmitter released during a second stimulus. **d)** Top, channels with slow kinetics (such as NMDA (N-methyl-D-aspartate) channels) that experience saturation can produce a large amount of current following a second stimulus, despite the smaller incremental amplitude of the second EPSC, owing to summation with the previous EPSC. Bottom, for receptors with high affinity for the transmitter, a population of receptors can remain bound with transmitter (red circles) and therefore be unavailable to respond to the transmitter released in response to a second stimulus. (Blitz *et al*., 2004)

Most forms of STPs are triggered by short bursts of activity and they can be mediated by accumulation of Ca^{2+} in the presynaptic terminal (shown in Fig. 1.3). Along with this, post synaptic mediators also affect STP. Figure 1.3, describes a situation where the number of receptors at the postsynaptic membrane limit the size of the EPSCs due to the saturation of postsynaptic receptors (Blitz *et al*, 2004). Also, STP can depend on the gating properties of postsynaptic receptors, if these receptors desensitize (Fioravante and Regehr, 2011). The amount of Ca^{2+} that reaches the multiple low-affinity sites on synaptotagmin and trigger vesicle fusion and neurotransmitter (NT) release depends of several factors [reviewed in (Schneggenburger & Neher, 2005; Südhof & Rothman, 2009)]. One of these factors is the nature of Ca^{2+} binding proteins present at the presynaptic bouton:

Ca^{2+} binding proteins with rapid kinetics are particular effective at intercepting Ca^{2+} before it reaches the release sensors, consequently reducing the probability of release [reviewed in (Amici *et al*., 2009)] . The remaining free Ca^{2+} (also known as residual $Ca^{2+)}$ will be slowly extruded from the terminal being able to affect STPs within few seconds [reviewed in (Fioravante & Regehr, 2011)].

Besides Ca^{2+} dynamics, the nature and properties of the vesicles present at the different synaptic terminals also influence the different forms of STPs displayed by those terminals. Rizzoli and Betz (2005) classify synaptic vesicles at the active zone into 3 categories: the reserve pool (or non-recyclable pool, NRP), the recycling pool (RP) and the ready releasable pool (RRP). The NRP makes up ~80–90% of the total pool of vesicles and is very reluctant to release. The RP is significantly smaller (~10–15%) and can be released with sustained high-frequency stimulation. The readily releasable pool (RRP) consists of few vesicles (~1%) that seem to be docked and primed for release (Rizzoli & Betz, 2005). The number of vesicles released by a single AP will be highly correlated with size of the RRP; if an action potential releases a large fraction of the RRP, a subsequent stimulus delivered before replenishment will lead to the fusion of fewer vesicles.

Facilitation is primarily a presynaptic phenomenon while a post synaptic effect is nearly insignificant (review, Fisher *et al*., 1997; Zucker and Regehr, 2002). The mechanism behind facilitation is largely believed to be due to extracellular Ca^{2+}. Katz and Miledi (1968) reported how extracellular Ca^{2+} played a vital role in synaptic transmission at the neuromuscular junction. It has been theorized that Ca^{2+} ions entering the presynaptic site leads to the release of neurotransmitters, which, depending on the available quanta (vesicle), could also mean increased amount of neurotransmitters released. Katz and Miledi also came up with a residual Ca^{2+} hypothesis, according to which, facilitation at the neuromuscular junction was due to an accumulation of bulk Ca^{2+} ions at the pre synaptic terminal. Furthermore, if Ca^{2+} ions of the previous action potential are not cleared before the next action potential, accumulation of Ca^{2+} ions could take place leading to an increased probability release. Mossy fiber synapses have the ability to undergo

facilitation (***frequency facilitation***) caused by increase in the frequency of stimulation. This means an increase from a stimulation of 0.1 Hz frequency to 1 Hz will lead to a manifold increase in synaptic efficacy. This pattern of stimulation leads to a lasting increase of the EPSP amplitude (Salin *et al*., 1996). However, other synapses such as Schaffer collateral to CA1 pyramidal cells and associational commissural synapses in the adjoining region display relatively low or little facilitation (Dobrunz *et al*, 1999).

Paired pulse facilitation is another form of short term synaptic transmission seen at the Mf-CA3 synapses in which, stimulation is followed with a very short delay (30-40 ms) by second stimulus leading to an increased potentiation at the second stimulation (Salin *et al*., 1996). A simple justification for the occurrence is that residual calcium left over by the previous action potential is carried over and contributes additionally during the second stimulation. Protein kinases of the presynaptic machinery may also have a role in the mechanism, as shown by Rosahl *et al* (1993) where knocked out synapsin (a presynaptic phosphoprotein) resulted in abnormal STP.

In some synapses, repeated activation can lead to synaptic ***short term depression*** that can last for several seconds and or minutes (Betz, 1970). This form of short term depression takes place in synapses with high probability of release occurring due to depletion of readily pool vesicles. This decrease in synaptic strength can also be due to neuromodulator molecules released by the pre or post synaptic terminals or even a signalling cascade that leads to the inhibition of presynaptic release machinery. ***Paired pulse depression*** is usually observed at fewer intervals of 20ms, resulting from an inactivation of voltage gated calcium or sodium channels or as mentioned before due to depletion of readily pooled vesicles at the presynaptic terminal. Since both facilitation and depression depend on the probability of transmitter release (p), synapses which have higher p have a depressed second pulse (Dobrunz and Stevens, 1997) as opposed to synapses with initial lower p have higher second response. Thus manipulating the p can lead to a either depression or facilitation, and or convert a depression to facilitation at the same synapse.

Both frequency facilitation and paired pulse facilitation are key players in understanding pre synaptic activity (review, Urban *et al*., 2001). Since granule cells of the dentate gyrus have low firing (frequency), they have the ability to induce frequency facilitation under physiological conditions (Moser *et al*., 1993; Review Nicoll and Schmitz, 2005; Gundlfinger *et al*., 2007).

1.1.4.2. Long-term plasticity

Longer activity changes are termed as “long-term”, and include potentiation (LTP) and depression (LTD) forms. Classical LTP relies on NMDA receptor activation and subsequent increase in AMPAR responses, but at Mf-CA3, a presynaptic NMDA-independent form of LTP (pre-LTP) also exists (Harris and Cotman, 1986). NMDA/AMPA ratio at Mf’s is lower (Monaghan and Cotman, 1982) and subunit

expression is distinct from other brain areas (Berg *et al*., 2013). Besides reports under experimental conditions, plasticity has also been observed *in vivo* (Hagena and Manahan-Vaughan, 2010), and after more physiologically relevant stimulation by natural spike patterns recorded from GCs during spatial navigation (Dobrunz and Stevens, 1999; Sachidhanandam *et al*., 2009). The following figure is a simplified explanation of LTP and LTD.

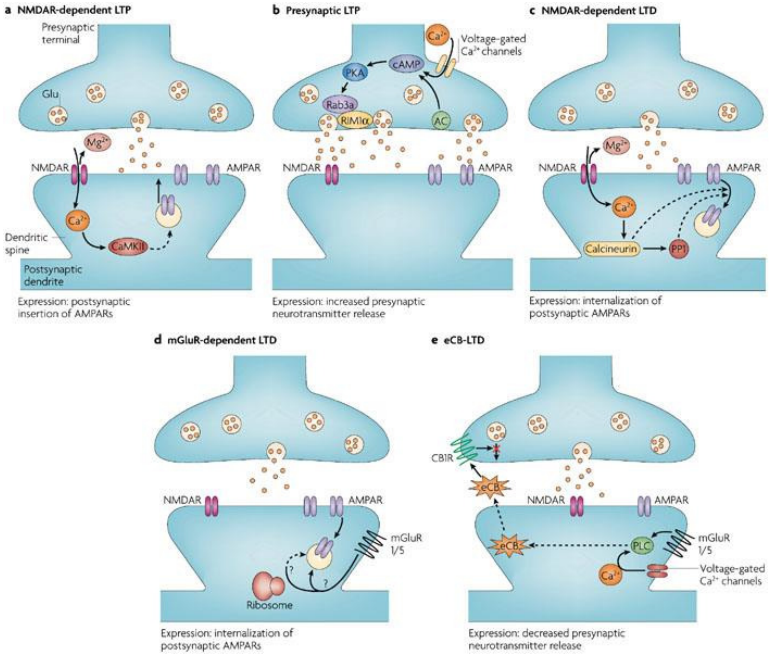


Figure 1.4. Simplified explanation of the induction and expression of LTP and LTD in the rodent. A) NMDAR-dependent LTP has been observed in many different brain regions and is dependent on postsynaptic NMDAR activation and CaMKII for its initiation. The voltage-dependent relief of the magnesium block of the NMDAR channel allows the synapse to detect coincident presynaptic release of glutamate and postsynaptic depolarization. AMPAR insertion into the postsynaptic membrane is a major mechanism underlying LTP expression. **B).** Presynaptic LTP has been best characterized at Mf-CA3 hippocampal synapses. Repetitive synaptic activity leads to the entry of presynaptic Ca^{2+}, which activates a Ca^{2+} -sensitive AC leading to a rise in cAMP and the activation of PKA.This in turn modifies the functions of Rab3a and RIM1α leading to a long-lasting increase in glutamate release. **C).** NMDAR-dependent LTD is triggered by Ca^{2+} entry through postsynaptic NMDAR channels, leading to increases in the activity of the protein phosphatases calcineurin and protein phosphatase 1(PP1). The primary mechanism involves internalization of postsynaptic AMPARs and downregulation of NMDARs by an unknown mechanism **D).** mGluR-dependent LTD has been best characterized at hippocampal synapses. Activation of postsynaptic mGluR1/5 triggers the internalization of postsynaptic AMPARs, a process that under some conditions appears to require protein synthesis. **E).** Endocannabinoid-LTD is the most recently discovered form of LTD, and has been observed in many brain regions. Either mGluR1/5 activation, leading to activation of PLC or an increase of intracellular Ca^{2+} (or both), in the postsynaptic neuron initiates the synthesis of an endocannabinoid (eCB). The eCB is subsequently released from the postsynaptic neuron, travels retrogradely to bind to presynaptic cannabinoid 1 receptors (CB1R) and this prolonged activation of CB1Rs depresses neurotransmitter release via unknown mechanisms. **F).** NMDARs-dependent LTP at Mf is induced and expressed 60postsynaptically. The activation of Ca^{2+} permeable NMDARs leads to the influx of Ca^{2+} at thepostsynaptic site. mGluRs and A_{2A}Rs act synergistically to rise Ca^{2+} via activation of PLC andconsequent activation of PKC. This intracellular cascade will induce insertion of NMDARs at the synapse. Adapted from (Kauer & Malenka, 2007)

Since STP and not LTP is the subject of this study, following will deal more on that.

1.1.4.3. Functions of Short term plasticity.

Various roles have been attributed to short term plasticity in various synaptic systems. Way back in 1965, Kandel and Tauc established how short term plasticity was important in behaviour, from studies of organisms such as aplysia (sea slug). Short term facilitation is shown to inflict high pass filtering characteristics, which is further shown to increase the reliability of transmission of information in periods of high frequency input, as usually seen during bursts of activity (Abbott and Regehr, 2004). While on the other hand, synapses with high probability of release function as low pass filters, since they depress during high frequency with low frequency activity (Abbott and Regehr, 2004). Frequency facilitation is also attributed to presynaptic mechanisms and so any variation in synaptic transmission occurring during this STP is credited as a presynaptic mechanism of action. Other synaptic features such as neuromodulators, presynaptic ionotropic receptors and entry of presynaptic Ca^{2+} affect synaptic transmission and plasticity, making it a highly variable and dynamic process (Abbott and Regehr, 2004). Short term depression, is shown to play a role in regulating behavioural adaptation to repetitive whisker deflection in rodents, by depressive transmission of thalamocortical synapses (Chung *et al*., 2002).

Short term plasticity also plays a central role in information processing and working memory. It has been described particularly to help in information processing in the auditory pathway (von Gersdorff and Borst, 2002; Macleod, 2011). The interplay between facilitation and depression also play important roles in the auditory information processing at the Calyx of Held synapses (Hermann *et al*., 2009). The short term increase in presynaptic calcium levels are used as a memory buffer by storing memory of an item, which is then extracted by a timed external signal. This process is involved in working memory; a system which temporarily stores and manages information required for complex cognitive tasks such as learning, reasoning and comprehension (Baddeley *et al*., 1974). Short term increase in residual presynaptic calcium levels is used as a memory buffer that temporarily stores the memory of an item , which is then extracted from synaptic to spiking form by appropriately timed external signal (Deng and Klyachko, 2011). The duration of such working memory can be regulated by modulating the levels of spontaneous network activity (Barak *et al*., 2010). At least from a theoretical standpoint, this opens up the possibility that the molecular factors that control presynaptic calcium dynamics might be involved in the modulation of working memory.

Decision making, another cognitive process, is also been proposed to be influenced by STP. This process involves comparing two sets of stimuli that occur sequentially at different times (Wang 2008). A study by

Deco *et al* (2010), showed that neurons encode the stimuli by the pattern of action potential firing and then use synaptic facilitation not only to temporarily store the first set of stimuli during the delay period (which would represent working memory) but also during presentation of second stimuli. In other words during decision making process facilitation based working memory in partial differential neurons is involved in storing information about the first set of stimuli , thus allowing comparison with the second set of stimuli, which is then read out by applying an external non-specific stimulation to all the neurons in the network. However, this theoretical model of decision making will require experimental verification. A brief figure summarizing the above is shown below

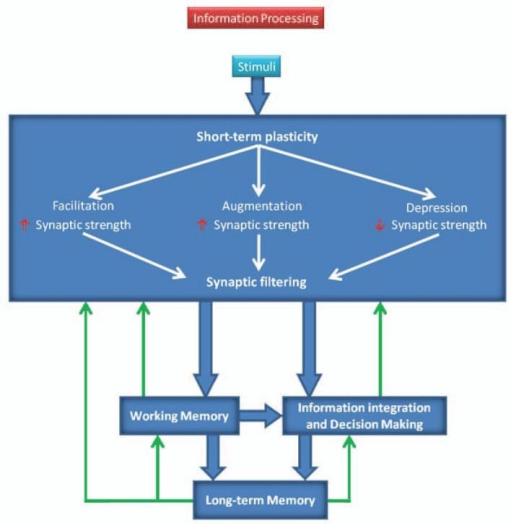


Figure 1.5. Model of STP functions in synaptic computations and information processing. Wide blue arrows and white arrows indicate information flow directions; green arrows show the factors that may influence information processing; and red arrows represent increase or decrease in synaptic strength. As shown, depending on the kind of stimuli the plasticity is either facilitative, augmentative or depressive in nature. Each kind either enhances of represses synaptic strength, which is then utilized for working memory and decision making (Deng and Klyachko, 2011)

Furthermore, synaptic plasticity (both short and long) and synapses in general, have shown to be involved in glial functioning and vice versa, forming an integral part of the central nervous system complex

1.2. Synapse and Glia

1.2.1. Astrocytes and synapse: The 'tripartite synapse'

The term tripartite synapse refers to a model of bidirectional communication between astrocytes and neurons. It represents a concept where in addition to the information flow between pre and the post synaptic part of the neuron, astrocytes too exchange information with the synaptic elements, responding to synaptic activity and regulating synaptic transmission (Araque *et al.*, 1999).

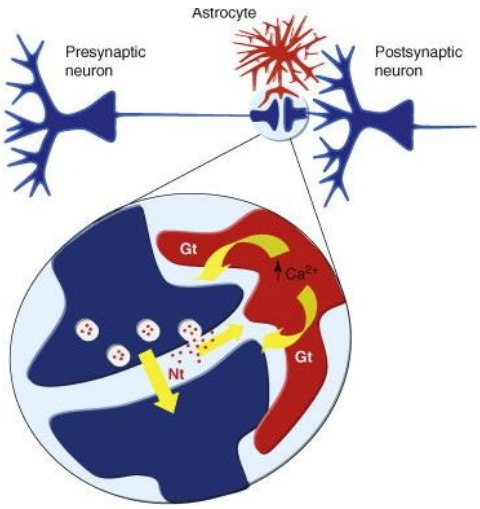


Figure1.6. Scheme of the tripartite synapse. Cartoon representing the transfer of information between neuronal elements and astrocyte forming the tripartite synapse. Astrocytes respond with Ca^{2+} elevations to neurotransmitters released during synaptic activity and, in turn, control neuronal excitability and synaptic transmission through the Ca^{2+}-dependent release of glia-transmitters (Gt) (From Perea *et al.*, 2009)

Experimental evidence strengthens the idea that astrocytes integrate and process synaptic information elaborating a complex non-linear response coming from the adjacent synapse (Perea *et al.*, 2009). It has been previously established that astrocytes respond to synaptic activity with elevations in their Ca^{2+} levels (Fig. 1.6) (Perea *et al.*, 2009). The astrocyte Ca^{2+} signal does not result from a nonspecific spillover of neurotransmitters, but through activity of specific selective synaptic terminals (Schipke *et al.*, 2008). With respect to synaptic transmission, astrocytes play an important role too. Several mechanisms underlying the astrocyte effects on long term potentiation (LTP) have been described. Studies indicate a passive or tonic mode of action in which astrocytes tonically suppress or potentiate synaptic transmission (Beattie *et al.*, 2002; Pascual *et al.*, 2005; Panatier *et al.*, 2006). Astrocytes through the release of ATP control the strength of the basal hippocampal synaptic activity by tonic suppression of neurotransmission which eventually leads in an increase of LTP (Pascual *et al.*, 2005). Changes in the astrocytic coverage of

synapses influences NMDA-receptor –mediated synaptic response via changes in the released ambient D-serine levels, in the hypothalamic supraoptic nucleus (Beattie *et al*., 2002). There are of course many other ways astrocytes can influence synaptic physiology, however since our study deals with microglia-synapse dynamics, much of the explanation will be focused on that.

1.2.2. Microglia and Synapse- The Quadripartite synapse

Before we introduce the concept of the 'Quadripartite synapse', it seems only right to point out some of the basic features of microglia: its origin, morphology and physiological functions, its role in diseases and finally, followed by microglia-synapse interactions.

1.2.2.1. Microglia: An Introduction

Since the discovery by Pio del Rio-Hortega in 1920, several studies have shed light on the importance of microglia in the nervous system. Microglia is the resident tissue macrophage of the immune system. Unlike other glial cells, microglia originates from the primitive hematopoiesis within the yolk-sac (YS) and enters the neuronal tube during embryogenesis (Schulz *et al*, 2012). Microglia has a special place in the central nervous system as they are derived from the mesoderm as opposed to the neuroectoderm from which other cells are formed (Ransohoff and Cardona, 2010). During the process of development, microglial cells of the brain and spinal cord get distributed throughout the brain matter and thus form a close link with neurons and other glial cells. Kreutzberg (1996) using microscopic images of fixed tissues showed that microglia would transform or "activate" in response to basically all pathological conditions of the CNS. Thus the concept of "active" and "inactive" or "resting" microglia was developed. However various studies on microglia in the non-pathological brain have questioned the theory of an "inactive" or "resting" microglia.

Resting/Surveying microglia

Since microglia is never resting and is constantly in guard of its surrounding, the term "resting microglia" has been rebranded to "surveying microglia". The microglial processes in this state are in constant motion as they survey their environment. The processes do not have a fixed movement path but move in a radical way, from the cell body to the surrounding parenchyma (review Catalin *et al*, 2013). Surveying microglia has two principal movements in their processes; 1) extending and 2) retracting. The speed of these distinct movements can reach to around 1.5µm/min (Nimmerjahn *et al*, 2005). Some of these processes contain filopodia like protrusions which are highly mobile in nature, forming bulbous endings and having a life period of around four minutes. Aside from their surveying role, microglia conducts housekeeping functions such as getting rid of all cellular debris in the surrounding (Davalos *et al*, 2005).

Active microglia

On encountering a stimuli or an unhealthy cell or debris thereof microglia changes its morphology to an amoeboid state. This is followed by migration towards the site in question and eventual phagocytosis (Linnartz and Neumann, 2013).

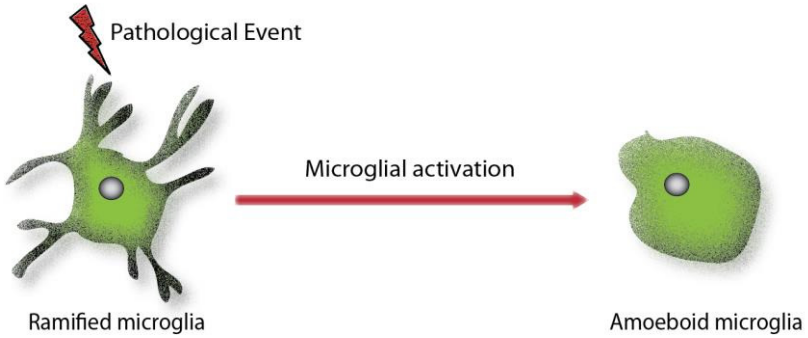


Figure 1.6 Activation of microglia Cartoon representing the change observed in microglia. As shown on encountering a stimulus or a pathological event, a 'resting microglia' is transformed into an amoeboid state, termed as 'active microglia'. This process is known as microglial activation.

Tissue damage by high pulse laser can cause microglia to react within three to five minutes in terms of processes movement, followed by cell migration. Known chemo-attractants such as ATP and NO play an important role in microglial motility and within 24 hours, microglia will surround the damaged tissue (Dibaj *et al*, 2010). Microglia will surround the lesion site by forming a shielding ring around the site of injury (Hines *et al*, 2009); shown to last from few days to weeks (Fenrich *et al*, 2012).

1.2.2.2. Microglia: Yin and Yang

Several factors influence whether microglia is beneficial or harmful to its surrounding cells. Factors such as type of stress, kind of danger signal, time period of the said danger signal impact, association with other cell types and surprisingly even the age of the organisms (Walter and Neumann, 2009) are some of them. Both the beneficial and harmful characteristics of microglia though interconnected are independent of each other. Thus they can be compared to the Chinese philosophy of yin-yang where both the yin and yang balance each other out. Just like any imbalance between the two can cause disorder, similar can be said about the two states of microglia. The microglial activation pathway can be divided into two types: 1) classically activated and 2) alternatively activated.

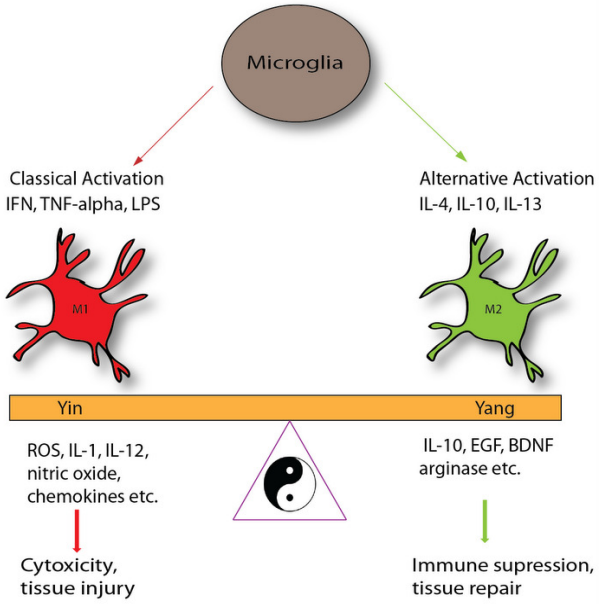


Figure 1.7 Yin -Yang of microglial activation Cartoon representing the two forms of microglial activation. As shown above, microglia can be classically or alternatively activated. The classical form of activation is due to compounds like Interferon (IFN), TNF-α or LPS which can result in release of ROS, Interleukin 1, 12, nitric oxide among others leading to cytotoxic injury. On the other hand presence of IL-4,10 or 13 etc. can cause it 'alternatively' activate causing release for IL-10, BDNF, arginase etc..., used for immune suppression and tissue repair.

Classically activated M1 macrophage/microglia is due to the presence of compounds such as LPS and pro-inflammatory cytokine IFN-γ. This type of microglia express CD16/32 and CD86 and produce increased amounts of nitric oxide and other oxidative metabolites, such as superoxides, along with proteases and further more pro-inflammatory cytokines. This pathway plays a central role in host defense mechanism against pathogens and tumor like cells, even sometimes affecting other healthy cells such as glia and neurons in the process. On the other, in complete distinction to the classical pathway, **alternatively activated** pathway M2 macrophage or microglia are activated by IL-4, IL-3 and express CD206 and arginase 1, which is known to down-regulate inflammation and enhance tissue repair.

This dual activation of pathway is also dependent on various transcription factors (Mantovani *et al*, 2004; Kigerl *et al*, 2009). Sawada *et al*, (2008), show that, in a LPS stimulated young mouse model of chronic inflammation, microglia protected dopamine producing neurons against toxicity, while promoting cell death of the same cells in adult conditions. Phagocytosis can be both detrimental and beneficial to the nervous system. Toll-like receptor (TLR4), a receptor expressed in microglia is shown to play an important role in bacterial infections, in both neuroprotective and harmful ways (Walter and Neumann, 2009). TLR4 can cause neurodegeneration by releasing pro-inflammatory molecules; which can also be protective by recruiting oligodendrocyte progenitor cells by enhancing remyelination (Glezer *et al*, 2006).

Other receptors such as prostaglandin receptor of type 2 (PGE_2) can cause microglia induced neurotoxicity through its activation (Cimino *et al*, 2008). Microglia, through the release of cytokines such as IL-10, tumor growth factor (TGF-β), and TNF-α can be neuroprotective, and simultaneously be detrimental through Il-1 and IFN-γ, among others (Walter and Neumann, 2009). TNF-α can be neuroprotective at low levels, and toxic at high (Bruce *et al*, 1996). Isoforms of receptors such as TNFR can be harmful (TNFR-1) and defensive (TNFR-2) (Fontaine *et al*, 2002). MMP-3 and MMP9 also have a similar characteristic (del Zoppo *et al*, 2007). This neuroprotective and neurotoxicity conditions mentioned depends on the activation of these individual receptor by microglia. Some of these receptor activation can also lead to neuro-inflammatory diseases

1.2.2.3. Microglia in disease

Microglial role in disease has been a complex, yet widely studied topic over the years. Several studies, such as Taharaoui *et al*, 2001 and Dommergues *et al*, 2003, have shown the ability of activated microglia to induce neuronal death in white matter. Though precise mechanisms behind microglia's harmful characteristics are poorly known, glial-neuronal, and glial-glial (astrocyte-microglia) crosstalk seem to be a key component in their understanding (Walter and Neumann, 2009). Upon activation, microglia can release a hoard of neurotoxic and cytotoxic substances such as IL-1β and Reactive Oxygen Species (ROS) (Lee *et al*, 1993), TNF-α (Sawada *et al*, 1989), MMP and glutamate (Walter and Neumann, 2009). Superoxides and nitric oxide, which are forms of ROS, can induce neuronal loss and reactive gliosis. The glutamate release by microglia can lead to excitotoxicity, especially when released from chronically activated microglia after ischemia (Yenari *et al*, 2010).

Alzheimer's disease (AD) is one of the most widely present forms of neurodegenerative disorders. Loss of memory, impaired cognitive functions and various behavioral and psychiatric impairments are some of the symptoms of this disease (Saijo and Glass, 2011). AD is characterized by accumulation of amyloid- β plaques, comprising of cleaved products of APP and presence of neurofibrillary tangles comprising of hyper-phosphorylated tau protein (Saijo and Glass, 2011). The pathological forms of Aβ are shown to activate microglia, which in turn could release pro-inflammatory factors or mediators leading to neurotoxicity (Halle *et al*, 2008; Yan *et al*, 2009). Using two photon imaging, Fuhrmann *et al* (2010) showed microglia involvement in neuronal loss, in brains of living mice with a transgenic expression of APP, presenillin 1 and tau. Microglial effect in the said neuronal loss was further confirmed with the use of CXCR1 deleted mice, which showed decreased neuronal loss.

In Parkinson's disease, characterized by loss of dopaminergic neurons, signs of inflammation such as microglial activation and increased levels of pro-inflammatory factors are seen in the cerebral spinal fluid (CSF) of diseased patients (Hirsch *et al*, 2003). It was also observed using animal models, that

intraperitoneal LPS injection was enough to induce massive microglial inflammatory response in the *substantia nigra*, followed by dopaminergic neuronal death (Dutta *et al*, 2008). Similar result was also seen using in-vitro experiments, where Saijo *et al* (2009) used TLR-4 activated microglia conditioned medium inducing loss of dopaminergic neurons.

Preventing and or inhibiting these activation pathways appear to be an attractive therapeutic option for preventing neurodegeneration. An antibiotic currently used to inhibit microglial activation is a tetracycline antibiotic called minocycline. Its ability to cross the blood brain barrier and inhibit microglia activation is noteworthy. It inhibits the production of pro-inflammatory cytokines and NO and decreases its migration (Machado *et al*, 2006), another important function of microglia. Furthermore, studies have also pinpointed at minocycline's ability to be neuroprotective in adult models of neurodegenerative disorders (Yrjänheikki *et al*, 1998; Du *et al*, 2001; Popovic *et al*, 2002; Carty *et al*, 2008). Though, all the above studies project minocycline as a useful tool against microglial activation, some studies have reported its adverse effects too. Tsuji *et al*, (2004) stated that the use of minocycline worsened hypoxic-ischemic brain injury in a neonatal mouse model. Though the reason behind such inconsistency is still unclear, it can be said that inhibiting microglia activation can be beneficial at times.

Microglia has established itself as a key player in its maintenance and well-being. Possibly the most commonly known function of the microglia is its ability to phagocytose dead cells and debris (review Napoli and Neumann 2009). This function is visible in both disease and normal pathology (Penfield 1952; Ferrer *et al*, 1990). This process of phagocytosis is of paramount importance as the dead or dying cells could release cytotoxic substances into the surrounding which could affect the nearby healthy cells. Quick and efficient removal of the cells by phagocytosis is thus necessary for proper nervous system development and maintenance. Not just in humans, microglial phagocytosis is present in other species of animals, most importantly in mouse where Sierra *et al* (2010) observed microglia phagocytose newly apoptotic neurons in the adult mouse hippocampus. Engulfment of a phagocytic cell took around 60 to 90 minutes, while another study demonstrated that microglia could engulf newly dying cells on an average of around 33 minutes (Petersen and Dailey 2004). Irrespective of the different time variations, these studies do prove that microglia are efficient agents at clearing and or removing both naturally occurring apoptotic cells and or foreign entities. Microglia also promotes neurogenesis and restoration of myelin sheath (Ziv *et al*, 2006: Franklin *et al*, 2008).

Not just in normal conditions, but microglia has shown to play a vital, protective role in neurodegenerative brain conditions. In AD brain, microglia promotes protection via the secretion of proteolytic enzymes that can degrade amyloid-β and its clearance by phagocytosis (Mandrekar *et al*, 2009). Furthermore, bone derived microglia were shown to be able to phagocytose/eliminate amyloid-β deposits (Simard *et al*, 2006). Microglial released cytokines have also neuroprotective properties. Il-6 can

act on astrocytes to start up brain tissue repair (Streit *et al*, 2000), while IL-10 can inhibit microglial apoptosis (Strle *et al*, 2002). TGF-β, another cytokine released by microglia, is reported to reduce plaque loads in mice model of Alzheimer's disease (Wyss-Coray *et al*, 2001).

Microglia, through its many functions, has cemented its place in many aspects of the nervous system. Its ability to modulate neuronal function is also one of them. Studies implicating microglia's ability to govern neuronal activity showcases its potential in the modulation of synaptic activity (mentioned in the following paragraphs) and, a potential role in understanding the changes in neuronal activity taking place thereof.

1.2.2.4. Microglia-Synapse Interactions:

The term quadripartite synapse has been loosely used to denote a model in synaptic physiology where microglia and astrocyte surrounding a neuron, are involved in information transfer between the synapse causing changes in synaptic activity, transmission and physiology.

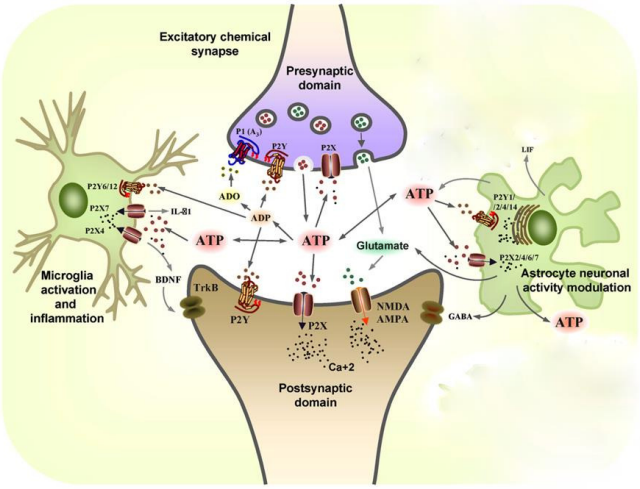


Figure 1.7 The quadripartite synapse: As shown above a model describes the mechanism of microglia and astrocyte in maintaining synapse regulation. Activated microglia and astrocyte can release compounds (BDNF, IL-1β, ATP) which can activate receptors on the pre and postsynaptic neuron. While ATP and glutamate released by the pre synaptic side can not only activate the receptors on the post synaptic side but also the purinergic receptors present on microglia and astrocyte leading to various functions from them. This bidirectional flow of information from both glia (microglia and astrocyte) to neurons and vice versa is termed as 'the quadripartite synapse' (Modified from del Puerto *et al*., 2013).

As shown in the Fig. 1.7 through the release of compounds such as BDNF, IL-1β and ATP both microglia and astrocyte can modulate synaptic function. However ATP and glutamate released form the synapse can also affect glial (astrocyte and microglia) functioning. We shall however dwell on the microglial aspect of this quadripartite synapse.

In vitro experiments (Biber *et al*., 2007) show that microglia have the capacity to express receptors or respond to neurotransmitters such as glutamate, acetylcholine, GABA, ATP, etc. Such studies have reported that these neurotransmitters can affect microglia *in vitro* in numerous ways, including change in membrane potential, intracellular calcium, cytokine release, and cell mobility (Kettenmann *et al*., 2011). Imaging studies have greatly helped understand the relationship between microglia and synapse. Through use of two-photon live imaging, it was reported that microglial processes briefly (5 min) contacted synaptic elements in layers II-III of the somatosensory and visual cortices at the rate of ~1 structure per hour and that microglial processes seem to enlarge once presynaptic terminals were contacted (Feng *et al*., 2000; Hirasawa *et al*., 2005; Wake *et al*., 2009). Additionally Wake *et al* (2009) induced ischemic injury by photochemical occlusion of the middle cerebral artery (MCA) and demonstrated that under these conditions microglia contact with presynaptic structures was prolonged and was often followed by their disappearance. Tremblay *et al* (2010), in a landmark study, revealed that spines often changed size upon microglia contact, hinting a role of microglia as key regulators of structural spine plasticity. They also showed that spines which changed sizes upon microglial contact tended to be smaller and were often eliminated. These data lead to a possible function of microglial phagocytosis as a mechanism to mediate response to sensory experience. Consistent with these data glia has been shown to engulf destabilized synaptic bouton and presynaptic debris, in developing drosophila (Fuentes-Medel *et al*., 2009), which corroborated the early work by Berbel and Innocenti (1988).

Regulation of microglia due to neuronal activity is also possible. Since microglia is equipped with various receptors, it is only wise to say that some extent of neuronal activity is required for their activation (review Mahe *et al*, 2005 and Selmeczy *et al*., 2008). Although typical levels of synaptic activity inhibit microglial activation, increase or decrease of the said activity can lead to an activated form of microglia (Biber *et al*., 2007). Pilocarpin and or kainic acid, known to cause epileptic seizures in mice when injected, resulted in microglial activation and subsequent proliferation (Lu *et al*., 2008; Shapiro *et al*., 2008). This was later supported by studies which used chemical seizure models (Aronica *et al*., 2001; Bonde *et al*., 2006). Addition of glutamate receptor antagonist like NBQX and GABA receptor inhibitor like bicuculline has shown to decrease microglial motility (Lee *et al*., 2011). Though microglia themselves possess glutamate receptors, such reduction in motility has not yet been observed by a direct application of either of the inhibitors onto a pure microglial cell line, suggesting an indirect effect on microglia when slices or tissues are treated by the drugs.

1.2.2.4.2 Microglia and synapse: Maturation

Recent work in developing cortex and hippocampus has suggested a vital role of microglia in remodeling and/or maturation of synaptic circuits (Paolicelli *et al*., 2011; Roumier *et al*., 2004, 2008). Paolicelli *et al* (2011), using fractalkine receptor (CXC3R1) (exclusively expressed by microglia) demonstrated the importance of microglia in hippocampal synapse development. In disease conditions, CX3CR1 (chemokine receptor) have the ability to modulate microglial quantity, activation and recruitment to site of injury by binding to its ligand CX3CL1 (expressed by injured neurons) (Cardon *et al*., 2006; Jung *et al*., 2000). Paolicelli *et al* (2011) also showed increase spine density and increased PSD-95 immuno-reactivity. Enhanced hippocampal LTD, and decrease of duration and latency to pentylenetetrazol (PTZ)-induced seizure response, are associated with less mature synapse and abnormal pruning, in CX3CR1 KO mice. In accordance with above, earlier *in vitro* studies established that maturation of hippocampal synapses were altered in mice containing mutation in KARAP/DAP12 (DAP12 ^{KI}), a transmembrane receptor expressed by microglia from birth (Tomasello *et al*., 2000; Hammerman *et al*., 2005; Roumier *et al*., 2008). Roumier *et al* (2004) using acute hippocampal slices from P22 DAP12 KI mice, showed an increase of NR_{2B}- containing NMDA receptors as assessed by ifenprodil sensitivity, along with an increased AMPA receptor calcium permeability which are the signature of younger synapse. In a later study by Roumier *et al* (2008), slices prepared from P18-DAP12 Ki mice had increased AMPA/NMDA ratios and increase in perforated synapses at the *stratum radiatum* of CA1. Cristavao *et al* (2014) have found that, when they co-cultured LPS treated microglia with hippocampal neurons, there was an increased density of axonal proteins in the pre-synaptic nerve terminals, as opposed to neurons cultured with non-activated microglia, implying microglial role in synapse formation.

These new insights offer a glimpse of the relationship between microglia and synapse with respect to synapse maturation in the developing brain.

1.2.2.4.3 Microglia and Synaptic Plasticity

Previous studies describe microglial role in synaptic pruning and/or maturation. However, in addition to them, several studies also describe a potential role of microglia in regulating different forms of synaptic plasticity.

The microglial receptor (CX3CR1) for the neuronal fractalkine ligand (CX3CL1) highlights this point. As mentioned above, it has a complementary expression on both neurons and microglia as a ligand and receptor respectively. Loss of this receptor in microglia is shown to impair hippocampal long term potentiation (LTP) in mice (Rogers *et al*, 2011). This impairment is accompanied by cognitive deficits in

the CX3CR1 KO mice. Paolicelli *et al* (2011) showed that mice lacking the same receptor showed diminished frequency of its excitatory post synaptic currents (EPSCs) implying a weakened synaptic connectivity, a fact concurred by Hoshiko *et al*., (2012). Another example is Connexins (Cx) (proteins found in gap junctions), which connect adjacent cells. Each Cx isoform forms oligomers, forming a complex known as hemichannel (Willecke *et al*., 2002). Blocking this hemichannel results in an inhibition of microglial release of glutamate, which further leads to an over activation of neurons; also known as excitotoxicity (Maezawa and Jin 2010).

CD200 receptor (CD200 R) is another instance of how microglia governs neuronal activity. Costello *et al* (2011) showed that CD200 R deficient mice resulted in a decreased LTP. Since CD200 R is solely present on microglia, with its ligand part being expressed on neurons and other neuronal cells (Barclay, *et al*, 2002; Costello *et al* 2011), the decreased LTP strongly implies the impact of microglia in long term synaptic potentiation. DAP12 (receptor expressed only in microglia, Roumier *et al*., 2004) mutant mice are an established model of Nasu-Hakola disease; a disease associated with progressive dementia (Hakola *et al*., 1972) which is shown to cause microglial dysfunction (Ohgidani *et al*., 2014), impact mouse behavior (Kaifu *et al*., 2003), and higher brain functions in humans (Paloneva *et al*., 2000). Expression of synaptic molecules for adhesion such as protocadherin and SynCAM1 were decreased in neurons incubated with microglia as opposed to neurons without (Ji *et al*., 2013), implicating microglial role in synaptic adhesion.

More than a few studies have been conducted by specifically stimulating microglia and analyzing its effects in synaptic transmission. Meucci *et al* (1998) used fractalkine to study microglia-neuron interaction. On addition of fractalkine onto neuronal cultures a rapid modulation of calcium currents was also observed. Although it was first misleadingly credited to be a role of neurons, it was later found to be that of microglia. This result was later corroborated by the use of hippocampal slices where the stimulation of microglia by fractalkine resulted in a significant reduction in the amplitude of EPSCs in the CA1 region (Ragozzino *et al*, 2006).

In addition to the above *in vitro* examples, studies using *in vivo* techniques have also been conducted to analyze the effect of microglia on neuronal activity. Using zebrafish larvae, it was demonstrated that microglia surveys and monitors spontaneous and/or visually evoked neuronal activity and helps in sending bulbous processes toward the most active neurons, depending on the amount of ATP surrounding the neuron (Li *et al*., 2012). It was also observed that contacts between microglia and neuron result in a swift reduction in both the frequency and amplitude of synaptic calcium events (Li *et al*., 2012). The role of ATP and other neurotransmitters like dopamine have been implicated in microglial motility due to

regulation of inward and outward K+ currents (Farber *et al*., 2005). Though this aspect is not well understood, its role in governing neuronal physiology cannot be denied. All together, these studies strongly suggest a role for microglia in modulating and/or impairing synaptic transmission in physiological and pathological conditions.

Studies have also implied a reverse dynamic between microglia and neurons. For example recent researches have been very suggestive in the control of microglial motility by neuronal activity (Schafer *et al*., 2012). Fontainhas *et al* in 2011 with the use of live cell time lapse microscopy showed that in *ex vivo* retinal implants, microglial motility can be regulated by ionotropic glutamatergic transmission and decreased by ionotropic GABAergic neuronal transmission.

1.2.2.4.4. Microglial proteins modulate synaptic transmission.

Microglia releases a vast range of neurotransmitters and cytokines which can regulate synaptic activity (Elkabes *et al*., 1996). Thus a causative relation between microglia and synaptic regulation through these molecules can be predicted.

BDNF, a neurotrophic factor is one of the main neurotransmitter proteins released by microglia (Gomes *et al*, 2013). Thus any change induced by BDNF in synaptic activity may be attributed, at least in part, to microglia. For instance it was observed that upon neuropathic injury, microglia present in the dorsal horn released BDNF which induced a shift in the chloride gradient in nociceptive neurons (Coull *et al*., 2005). These changes were earlier found to increase the excitability of neurons through $GABA_A$ receptor depolarization (Coull *et al*., 2003).

TNFα is also a key player in regulating synaptic activity. As shown by Santelo *et al*. (2011), TNFα regulates and controls basal synaptic functions, as well as synaptic plasticity (Stellwagen and Malenka, 2006; Costello *et al*, 2011).

IL-1β has been implicated in consolidation of memory (Goshen *et al*., 2007). Nistico *et al* (2013) showed that just as seen in mice with Experimental Autoimmune Encephalomyelitis (EAE), IL-1β inhibited GABAergic spontaneous inhibition of post synaptic currents (sIPSC). To prove that IL-1β was released from microglia, they added activated microglia on acute hippocampal slices which exhibited changes in GABAergic transmission similar to those observed in EAE brains.

Toll-like receptors (TLRs) recognize conserved pathogen-associated molecular patterns (PAMPs) of bacteria, viruses, yeast, fungi, and parasites (Janeway Jr, 1992). Microglial TLRs are crucial as a first line of defense against bacterial or viral infection. While TLR expression is hardly detectable in resting

microglia in a healthy CNS, multiple TLRs rapidly appear upon activation of the cells. Primary microglia *in vitro* constitutively express a wide array of TLRs (TLRs 1–9) at varying levels (Bsibsi *et al*., 2002). It was observed that both TLR-4 and TLR-2, subclasses of TLRs, show opposite effects in synaptic stability (Freria *et al*., 2012). TLR-4 helped in the preservation of synaptic terminals whilst TLR- 2 led to synaptic loss and increased astrogliosis. Since TLR-4 is only expressed in microglia (Habich *et al*., 2005; Gondokaryono *et al*., 2007; Midwood *et al*., 2009), this is consistent with the involvement of microglia in the synaptic preservation. Furthermore, LPS, a ligand of TLR-4 has recently established itself as a regulator of neuronal activity (Pascual *et al*., 2012). It was observed that addition of LPS-activated microglia in acute slices resulted in a rapid and transient increase in the frequency of spontaneous synaptic AMPAergic currents in the post synaptic region of the CA1 neurons. This effect was not present in Pu.1 deficient mice, which lack microglia, thus implicating the role of activated microglia in the observed change.

Nitric Oxide (NO) is a free radical, widely known as a messenger molecule, released by microglia, along with other cell types. Earlier studies have already depicted the role of NO in regulation of synaptic activity. Use of NOS inhibitors such as N-methyl –L- arginine and N-nitro –S- arginine inhibited hippocampal LTP (Schumann and Madison, 1991). Production of NO by activated microglia was shown to block transport of precursors of synaptic vesicles which could cause synaptic and axonal dysfunction (Stagi *et al*., 2005). Other proteins such as interleukin 1-6, prostaglandins, C1q and C3 (complement cascade proteins) have also been shown to play a role in memory and learning processes (review by Blank and Prinz 2013). Since the action of these proteins is principally modulated by microglia (review Yirmiya and Goshen 2011), an indirect influence of microglia on synapse through these proteins cannot be ignored.

1.2.2.5. Microglia and Behaviour:

Proper microglia function has shown to be a prerequisite for proper brain function. Two studies (Derecki *et al*., 2007; Chen *et al*., 2010) have highlighted this point. Brain dysfunction associated with microglia is the pathological grooming behavior seen in Hoxb8-deficient mice. These mice are used as a mouse model for the human disorder known as trichotillomania (compulsive hair pulling disorder). Using this model, Chen *et al* (2010) demonstrated rescue of the behavioral phenotype by transplantation of wild type bone marrow cells into mutant mice. They argue that the bone marrow derived monocytes populate the brain and transform into cells with microglial properties. The rescue experiment also suggested that the dysfunction was not a developmental defect but due to microglial dysfunction. The other study by Derecki *et al*, (2012) used a mouse model of Rett syndrome, a form of autistic disorder characterized by

impaired synaptogenesis, resulting in severe disturbances of motor, language and cognitive functions (Kettenmann *et al*., 2013). The disease is known to be linked with mutations in the gene for methyl CpG binding protein 2 (MECP2), a transcriptional receptor present in all cell types of the brain (Kettenmann *et al*., 2013). Mice with MECP2 deletion show impaired locomotor function along with reduced life span. Derecki *et al* (2012) demonstrate that microglia deficient in MECP2 show a strong reduction in phagocytic activity. However, the phenotype of MECP2 knockout mice could be partially rescued by selective expression of MECP2 in cells of monocytic lineage, including microglia. Thus it was shown that microglial phagocytic ability was essential for the development and /or maintenance of neuronal circuit.

The receptor CX3CR1 (described above) has also shown to play a role in regulating behaviour by at least three different studies. According to the first study (Rogers *et al*., 2011), CX3CR1 deficient adult mice displayed motor learning difficulties, as well as impaired cognitive function. This was achieved by using contextual fear conditioning and Morris water maze. The behavioural phenotypes were accompanied by increased levels of Il-1β levels which were reversed by the addition of IL-1 receptor antagonist. Thus, this study suggested a possible signaling mechanism between microglia and neurons through CX3CR1. In another study (Zhan *et al*., 2014) using CX3CR1-deficient mice, synapse maturation defects and impairments in functional connectivity between the prefrontal cortex and hippocampus were observed along with decreased social interactions and increased repetitive behavior, usually associated with autism. Investigators (Parkhurst *et al*., 2013) specifically manipulated gene expression in microglial using a tamoxifen-inducibe Cre-ER protein, driven by CX3CR1. A week after tamoxifen injection, depletion of either microglia or the neurotropin BDNF was observed, inducing in turn significant defects in motor learning and fear conditioning, along with changes in spine morphology (decreased spine formation in motor cortex).

Lenz *et al* (2013) through an interesting study presented evidence to suggest sex-based differences in microglia in the preoptic nucleus of the hypothalamus (region regulating expression of sexual behaviours) in healthy rats. They found sex-based differences in the number of activated microglia. Male rats were shown to have more activated microglia, while the female had less. Increase in microglia activation in females led to 'masculine behaviours' and, conversely decreasing activation in males led to more 'feminine behaviours' (Lenze *et al*., 2013). Together, these studies establish microglial role in the regulation of CNS functions such as learning and behavior (social, cognitive and sexual).

The above studies demonstrate the potential role of microglia in synaptic formation, maturation and transmission, behaviour and its regulation thereof. Thus it is very important to consider microglia-neuron

interaction as a dynamic system, in which receptors, mechanisms of release, and morphology could play a vital role in the understanding of microglia associated disease progression. Understanding microglia relationship with various receptors (neuronal or otherwise) is a key factor for this; and purinergic receptors are one of them.

1.3. Purines and Neuromodulation

Purines and their receptors form an integral complex, which play a vital role in the functioning of the nervous system. One of the key players of this complex is ATP.

1.3.1. ATP-the ubiquitous neurotransmitter

Adenosine 5'-triphosphate (ATP) was first discovered by Karl Lohmann in 1929, as a purine nucleotide composed of an adenine attached to a ribose sugar molecule (in this case adenosine) with links to a chain of three phosphate groups (Lipmann 1941). ATP is the main source of chemical energy used for the metabolism of all living cells (Lipmann 1941; Yu *et al*., 2008). The concept of ATP as a neurotransmitter was first introduced by Burnstock *et al*., (1970). They found that ATP was the signaling molecule released during non-adrenergic and non-cholinergic transmission in the smooth muscle. Further research into its role in the CNS was propelled by the work of Phillis *et al*., (1975), where they discovered ATP to temporarily facilitate cortical excitability, an effect not seen in other purines. Years later the depolarizing effects of ATP in the CNS were demonstrated in a subpopulation of dorsal horn neurons (Jahr and Jessel, 1983) and in the caudal trigeminal nucleus (Salt and Hill, 1983). It is a well understood fact that ATP is released from all cell types and is not only a fast neurotransmitter but also a modulator of neurons, glial and neuron-glia interaction (Burnstock, 2007a). It also had potent roles in proliferation, differentiation and cell death and regeneration as well as disease (Abbracchio and Burnstock, 1998). ATP was found to be co-released and co-stored with other classical neurotransmitters both in the CNS and the PNS (Burnstock, 2004). In the brain terminals, ATP is mainly stored and released from a distinct pool of vesicles (Pankratov *et al*., 2006).

1.3.1.1. ATP: Storage, release and hydrolysis

Under physiological condition, intracellular ATP can be found in the concentration range of 2-5 mM (cytoplasm of cells) and sometimes even higher concentration of up to 100 mM (synaptic vesicles) (Burnstock *et al*., 2007a), whereas it is found in the nanomolar range in the extracellular space (Agtersch *et al*., 1999). The accumulation of ATP into vesicles can be modulated by a Cl^--dependent vesicular nucleotide transporter (VNUT), belonging to the SLC17 anion transporter family, which also includes

vesicular glutamate transporters (VGLUT) (Sawada *et al*., 2008). VNUT is highly expressed in the brain and seen distributed to chromaffin granules and sub-population of astrocytes (Abbracchio *et al*., 2008). ATP is probably present in every synaptic and secretory vesicle, albeit at different concentration, which can be co-released with other neurotransmitters such as gamma-amino-butyric acid (GABA), noradrenaline or glutamate (Abbracchio *et al*., 2008).

It is unclear whether ATP and other nucleotide-receptor agonists (ADP, UTP, and UDP) are released by a common mechanism. However there is strong evidence for exocytotic neuronal vesicular release of ATP (Pankratov *et al*., 2007). Furthermore, studies also demonstrate a vesicular release of ATP from astrocytes (Bowser *et al*., 2007, Zhang *et al*., 2007) and microglia (Imura *et al*., 2013, George *et al*., 2015). Additionally, evidence of other forms of release has been provided. ATP was also reported to be released in a frequency and Ca^{2+} dependent manner from hippocampal slices (Wierasako *et al*., 1989; Cunha *et al*., 1996c). Mechanisms of nucleotide release, including ATP-binding cassette transporter, connexins or pannexin hemichannels, plasmalemmal voltage-dependent anion channels, in addition to P2X7 receptors (Dubyak, 2006; Pankratov *et al*., 2006) have also been discovered. Hemichannels have been reported to be involved in ATP mediated intracellular communication in taste buds (Huang *et al*., 2007) and in ischemia-induced neuronal ATP release (Thompson *et al*., 2006).

Once released into the extracellular space, ATP and other nucleotides undergo rapid enzymatic degradation by ectonucleotides. This is a key mechanism because ATP can act as an important physiological ligand to various purinergic receptors (Abbracchio *et al*., 2008). The ATP released is then hydrolysed into its metabolites by a class/group of enzymes known as ecto-nucleotidases. As shown in the fig. 1.8, they include the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphates and/or phosphodiesterase's (E-NPPs), alkaline phosphatases and ecto-5'-nucleotidase (Fields and Burnstock, 2006). E-NTPDases and E-NPPS hydrolyze ATP and ADP to AMP, which is further hydrolyzed to adenosine by ector-5'-nucleosid while, alkaline phosphatases equally hydrolyze nucleoside tri, di and monophosphates (Fields and Burnstock, 2006). Furthermore to the catabolic pathways, nucleotide interconverting enzymes exist for nucleotide rephosphorylation and extra cellular synthesis of ATP. Ectonucleoside diphosphate kinase and adenylate kinase are examples of this. Although, adenosine is produced by enzymatic breakdown of ATP, some neurons and astrocytes have shown to directly release them (Wall and Dale 2007).

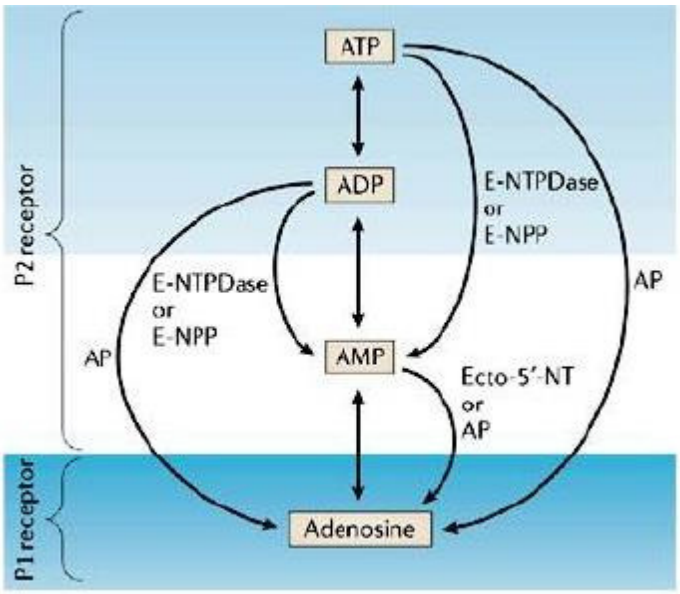


Figure 1.8 Extracellular catabolism of ATP into its metabolites by ecto-nucleotidases. Once ATP is released into the extracellular spaces, it is rapidly degraded into its metabolites by a group of enzymes known as ecto-nucleotidases. Four major types of theses enzymes are present in the catabolism of ATP: the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases) and ectonucleotide pyrophosphatse and/or phosphodiesterases (E-NPPs) convert ATP into ADP and AMP; ecto-5'-nucleotidase (Ecto-5'-NT/CD73) family that hydroyses AMP to adenosine and lastly alkaline phosphatases (AP) which cataylse ATP to adenosine (Figure:Fields and Burstock, 2006)

1.3.1.2. ATP as a danger signal

Growing evidence suggests role of increased levels of extracellular ATP in several brain conditions. Presence of increased levels of extracellular ATP is seen in conditions of trauma (Wang *et al*., 2004; Franke *et al*., 2006), epilepsy associated seizures (Wierasko *et al*., 1989) and also in hypoxia or ischemia (Lutz and Kabler, 1997) Melani *et al*., 2005). This enhancement of extracellular ATP and adenosine can be attributed to systematic mechanisms rather than ATP leakage (Rodrigues *et al*., 2015). However, this mechanism or source of ATP release is still unclear (Rodrigues *et al*., 2015).

The idea of ATP as a danger signal implies a role of released ATP and its receptors in the involvement of brain disorders.

1.3.2. Purinergic receptors

Separate receptors for adenosine (P1 receptors) and ATP (P2 receptors) were recognized and cloned (Lustig *et al*., 1993; Webb *et al*., 1993; Brake *et al*., 1994, Valera *et al*., 1994) to characterize and understand their mechanism.

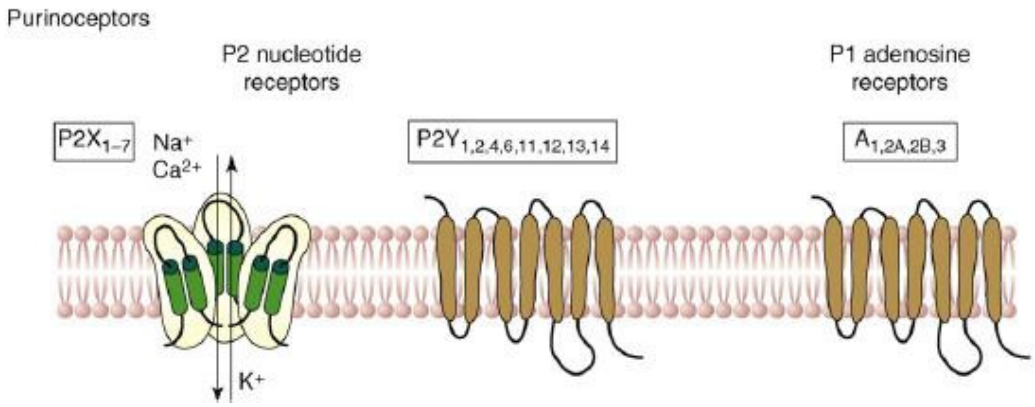


Figure1.9 Classification of purinergic receptors. Purinergic receptors are classified into two types P2 nucleotide receptors and P1 adenosine receptors. The P2 class of receptors is further divided into P2X (P2X1-7) and metabotropic P2Y (1,2,4,6,11,12,13,14). (Abbracchio *et al*., 2009)

Purinergic receptors may be the most abundant of receptors as they can be found in all types of cells, including neurons (Burnstock and Knight, 2004). Since ATP is the primary source of adenosine we shall begin by discussing about its receptors.

1.3.2.1. ATP/P2 receptor

Burnstock and Kennedy (1985) first proposed the existence of two kinds of P2 receptors, which was later confirmed by cloning, with differences in protein structure, physiology and signaling mechanisms: **P2X receptors** or ion channel receptors and **P2Y receptors** or G-protein coupled receptors (Lustig *et al*., 1993; Webb *et al*., 1993; Valera *et al*., 1994). The way to distinguish between both the receptors is their affinity to ATP, α, β- methylene ATP and 2-methyltioATP (Abbracchio and Burnstock 1994).

1.3.2.1.1. Ion channel gated P2X receptors

P2X receptors are cationic ligand gated channels that on binding with ATP (minimolar range) open the pore permeable to Na^+, K^+ and Ca^{2+} (Abbrachio *et al*., 2008). These receptors can from trimers from individual subunits encoded by seven distinct genes, designated as P2X1 to P2X7 (Brake *et al*., 1994)

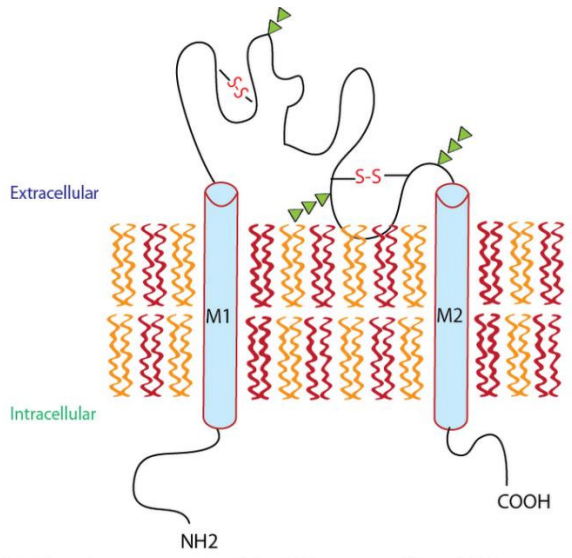


Figure1.10 Primary structure of P2X receptor: The structure of PX receptor contains two transmembrane domains M1 and M2. S-S denotes the disulphide bonds while the 3N linked glycosyl chains present are shown in green. Both N and C terminal are in the intracellular part of the membrane (Brake et al., 1994)

As shown in the Figure 1.10, each of the subunit comprises of two transmembrane domains with an extracellular binding loop. Both the -NH_2 and -COOH ends are located inside the cytoplasm (Brake *et al*., 1994). The -COOH terminal deviates significantly in sequence between subunits and binds the motifs for protein kinases responsible for the receptor kinetics and desensitization. The -NH2 terminal is glycosylated and is required for trafficking of receptors to the plasma membrane. The disulphide bridges is the site where ATP, antagonists and other modulators have their binding site (Khakh 2001) P2X1 to P2X5 subunits can form homomers or heteromers while the P2X6 receptor seems to only form as a heteromer (Torres *et al*, 1999; Egan *et al*., 2006). Each combination of homomer or heteromer assemble can thus provide with various distinct biophysical properties. Though it was widely assumed to assemble as a homomer, studies (Guo *et al*., 2007) showed a presence of a P2X4/7 heteromer in macrophages derived from bone marrows. These different receptor subunit assemblies can sometimes have different sensitivities to pharmacological agonists and antagonists.

Each of the receptor subunit has different sensitivity to ATP or their analogues. P2X1 and P2X3 receptors have the highest sensitivity among all the subtypes to ATP and their analogue (αβmeATP) (EC_{50}~1 μM) (Khakh, 2001), while P2X2, P2X4 and P2X5 receptors have a lower sensitivity to ATP (EC_{50}~10μM) (Abbracchio *et al*., 2008). The P2X7 receptor subunit is unique to others as it can only be activated by high concentrations of ATP (Duan and Nearu, 2006). Various P2X receptor subunits can be co expressed within the same neuron and can lead to heterogeneous kinetics when ATP mediated currents are recorded from them.

P2X receptors have higher calcium permeability than AMPA and kainate receptors, while similar to those of NMDA and nicotinic Ach receptors. Endogenous ATP induced currents in several neurons (medial habenula and somatosensory cortex) have high Ca^{2+} permeability, which is comparable to the Ca^{2+} permeability of NMDA receptors (Abbracchio *et al.*, 2008). Thus, P2X receptors may represent the most important route for Ca^{2+} influx at the postsynaptic density at resting membrane potential, because of the unavailability of NMDA receptors due to Mg^{2+} block (Guo *et al.*, 2007). Studies have shown that stimulation of P2X receptors trigger cytosolic Ca^{2+} signals (Pankratov *et al.*, 2008) along with presynaptic Ca^{2+} signals which can regulate neurotransmitter release (Sperlagh *et al.*, 2007). Postsynaptic P2X receptors interact with several ionotropic receptors including nicotinic acetylcholine receptors, $GABA_A$ receptors and NMDA receptors. These phenomenon maybe be modulated by intracellular Ca^{2+} activating the kinases, which in turn phosphorylate the receptors (Pankratov *et al.*, 2008). Since P2X4 receptors became a highlight of this study a short discussion on them is warranted.

1.3.2.1.1.1. P2X4 receptors

P2X4 class of receptors of the P2X receptor family are widely expressed in the brain and spinal cord (Rubio and Soto 2001; Burnstock and Knight, 2004). Activation of P2X4 receptor leads to induction of electrical activity, along with stimulation of voltage gated Ca^{2+} influx (He *et al*, 2003). Calcium currents were measured at around 8% of baseline levels for recombinant (human) P2X4 receptors (Garcia-Guzman *et al*, 1997). The presence of P2X4 receptor is also observed in microglia and other macrophages (Bowler *et al*, 2003). Activation of spinal cord microglia through the up-regulation of P2X4 receptor is shown to contribute to neuropathic pain (Tsuda *et al*, 2003; Ulmann *et al*, 2008). P2X4 receptor knockout mice display decreased amplitude of long term potentiation (Sim *et al*, 2006). Neuronal P2X4 receptors are expressed in superoxide dismutase SOD1 mutation mouse model of ALS leading to the recruitment and their eventual engulfment by activated microglia (Casanovas *et al*, 2008). Also contrasting to apoptotic cells, P2X4 positive neurons show no sign of chromatin condensation or caspase-3 activation, but do exhibit loss of neuronal NeuN marker and eventual recruitment by microglial cells (Casanovas *et al.*, 2008).

Microglia P2X4 receptor is also shown to be associated with increased neurophagic activity (Cavalier *et al*, 2003). Removal of P2X4 receptor resulted in weaker microglial activation and loss of PGE2 associated inflammatory pathway (Ulmann *et al*, 2010). Robinson and Murell-Lagnado (2013) show how P2X4 receptors form a large conductance pore on the cell surface which affects the ionic balance and consequently the release of pro-inflammatory substances. P2X4 receptors are resistant to lysosomal degradation through being trafficked into late endosomes. This prevents microglial action on healthy neurons and its own activation under normal conditions. Guo *et al*, (2005) observed increased expression

of P2X4 receptor in spinal cord microglia, following formalin injection. Similar increased expression was also observed followed by LPS injection (Guo *et al*, 2006) and previously via a mechanical lesion to the spinal cord (Schwab *et al*, 2005). Rats suffering from experimental autoimmune neuritis also showed increased expression of P2X4 receptor (Zhang *et al*, 2008). Varma *et al* (2009) observed that toxic effect of Aβ was increased in cultures over expressing P2X4 receptors, whereas it was decreased in cultures with decreased expression of P2X4 receptor, suggesting its role in Aβ induced neuronal cell death. Reduced expression of P2X4 receptor was also seen in the medial frontal gyrus in patients of end stage AD pathology, but also increased just before the advent of neuronal death, as observed in an in vitro model of Aβ induced cell death (Varma *et al*, 2009).

P2X4 receptors have the highest permeability to calcium among the P2X receptor family (Soto *et al*, 1996; North, 2002), suggesting that release of ATP near the synapses could lead to changes in synaptic transmission and synaptic strength, induced by continuous activity.

1.3.2.1.2. Distribution of P2X receptors

P2X receptors are present in the central nervous system, both in neuronal and glial cells. P2X2 receptors are extensively present in brain regions such as cerebral cortex, substantia nigra, hippocampus and hypothalamus (Norenberg and Illes, 2000; Illes and Riberio, 2004). The P2X3 receptors are present a high density subpopulation of the neurons of the dorsal ganglia and in structures involving pain transmission (Norenberg and Illes, 2002). It was also observed in the presynaptic sides of the hippocampus (Rodrigues *et al*., 2005b) and the midbrain (Diaz-Hernandez *et al*., 2001). P2X4 receptors, on the other hand, are expressed in the rat cerebellum and spinal cord, cerebral cortex, hippocampus , the thalamus and the brain stem (Norenberg and Illes, 2000; Illes and Riberio, 2004). P2X2, P2X4 and P2X6 receptors are expressed in the CA1 region of the rat hippocampus, at the post synaptic membrane opposed of terminals of Schaffer collateral, precisely where the density of glutamate ionotropic receptors decrease (Rubio and Soto , 2001). The P2X7 receptor is mostly found in activated glial and immunocompetent cells (Collo *et al*., 1997, Butt, 2001), and also in inhibitory hippocampal neurons (Atkinson *et al*., 2002). It is also reported to be expressed in the synaptosomes of the cerebral and midbrain granule cells (Miras-Portugal *et al*., 2003).

1.3.2.2. P2Y Receptors

P2Y receptors consist of seven G protein coupled transmembrane domains with and extracellular $–NH_2$ domain and an intracellular –COOH domain (Abbracchio *et al*., 2006).

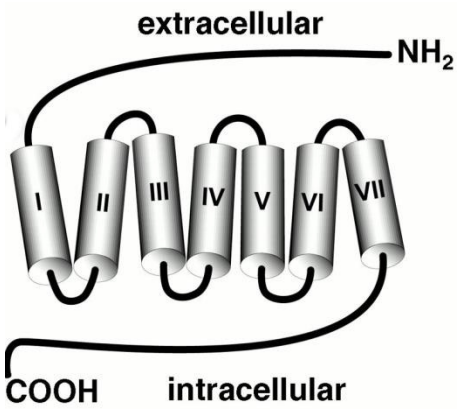


Figure1.10. Primary structure of P2Y receptor: The structure of P2Y receptor contains seven transmembrane domains. N terminal part of the structure is in the extracellular space while the C terminal is in the intracellular part of the membrane (Lazarowski and Boucher , 2001)

Depending on the presence of amino acids required for ligand binding, and selectivity of G-protein coupling, two distinct P2Y receptor subtype can be identified: the P2Y1, P2Y2, P2Y4, P2Y6 and P2Y11 form the first one and P2Y12, P2Y14, and P2Y16 form the other (Abbracchio *et al*., 2006). receptors of the first subtype use Gq/G11 to activate the phospholipase C/inositol triphosphate (InsP3) endoplasmic reticulum Ca^{2+}- release pathway (Abbracchio *et al*., 2009), while the second subtype couple to the Gi/o, which inhibits adenylyl cyclase and modulate ion channels (Abbracchio *et al*., 2006). P2Y receptors can be also subdivided based on their ligand they respond to. These four groups are: P1Y1, P2Y12 and P2Y13, which respond to ADP and ATP; human P2Y4 and P2Y6, which mainly respond to UTP or UDP; rodent P2Y2 and rodent P2Y14, which have mixed selectivity; and finally P2Y14 which respond to sugar nucleotides like UDP-glucose and UDP-galactose (Abbrachio *et al*., 2006). Coupling of the same P2Y receptor to different G proteins can be done, indicating agonist specific signalling involving distinct active receptor conformations. For example, activation of P2Y11 receptors by ATP increases cAMP, InsP3 and cytosolic Ca^{2+}, while activation by UTP produces only Ca^{2+} mobilization and not InsP3 or cAMP increase (White *et al*., 2003). Sometimes P2Y receptors can interact directly with other proteins without any G protein modulation (Erb *et al*., 2001)

P2Y receptors can form homomers or heteromers with other P2Y receptors or even other transmitter receptors (Ecke *et al*., 2008). These P2Y receptors are included in various pathways and mechanisms.

Some of them are: nucleotide-induced transactivation of tyrosine kinase receptors (which include receptors for epidermal growth factor, platelet-derived growth factor and vascular endothelial growth factor), activation of soluble tyrosine kinases (Camden *et al*., 2005) and interaction with integrins or the nerve growth factor (NGF), among other signalling pathways (Milenkovic *et al*., 2003, Abbracchio *et al*., 2009). Another type of ion channels modulated by P2Y receptors are glutamate NMDA receptor. Adenine and uridine nucleotides enhance NMDA currents in the rat prefrontal cortex, in the pyramidal neurons of the V layer, through activation of P2Y2R (Wirkner *et al*., 2002) and inhibit these currents through P2Y1 receptor (Luthardt *et al*., 2003). However it's been shown that ATP can directly inhibit these currents through binding with glutamate at the NR_{2B} subunit of NMDA receptors (Ortinau *et al*., 2003). An important mechanistic feature of P2Y receptors is their signalling capacity to regulate nuclear gene transcription and protein synthesis. For example, Gs protein coupled to P2YR can induce and increase of cAMP levels and subsequently cause the activation of cAMP response element binding transcription factor (CREB) , which in turn activates gene transcription (Wilkin *et al*., 2001; Marteau *et al*., 2004).

1.3.2.2.1. Distribution of P2Y receptors

The P2Y1 Receptor and P2Y11 receptor are known to be particularly abundant in number in the brain as opposed to other tissues (Moore *et al*., 2001). The human P2Y1 receptor mRNA was reported at neuronal structures in the basal ganglia, hippocampus, cerebellum, and many regions of the cerebral cortex (Moore *et al*., 2000a), with similar distribution of P2Y1 receptor in the rat brain (Moran-Jimenez and Matute, 2000). Lastly, both P2Y12 and P2Y13 receptors are present in high density in the neurons of the cerebellum, cerebral cortex, hippocampus, substantia nigra and the caudate nucleus (Laities *et al*., 2001)

Both the P2X and P2Y receptors through its activation by ATP are shown to regulate synaptic plasticity

1.3.3. ATP and Synaptic Plasticity

Upon stimulation of nerve terminals, ATP is released (White, 1997). Synaptic transmission can be modulated by either controlling the release of neurotransmitters or by modulating the excitability of post-synaptic membranes (Hussl and Boehm, 2006). However, the sole role of ATP is difficult to decipher due to its rapid degradation by enzymes (Cunha and Ribeiro, 2000). The effect of ATP can be confused with the activation of A_1 receptors by adenosine (Ribeiro 1995; Cunha *et al*., 1998) or that of adenosine on A_{2A} receptors (Cunha and Ribeiro 2000). Nevertheless, the hippocampus is one of the brain areas with the highest density of P2 receptors and evidence to back the claim of ATP's role in synaptic transmission (Inoue, 1998).

It was observed that P2X1, P2X2/3 and P2X4 receptors mediated a facilitatory effect in the release of glutamate, while P2Y1, P2Y2 or P2Y4 had inhibitory effect in this release (Mendoza-Fernandez *et al.*, 2003, Rodrigues *et al.*, 2005b). A pre-synaptic P2X2 receptor mediated facilitation of transmission was seen in interneurons of the *stratum radiatum* in the hippocampal CA1 region (Khakh *et al.*, 2003). ATP was also found to activate inward currents at the post-synaptic CA1 and CA3 hippocampal sub regions through P2X2 like receptors (Pankratov *et al.*, 1998; Mori *et al.*, 2001). Along with contributing at the excitatory post synaptic currents of the CA1 region (Pankratov *et al.*, 1998; Rubio and Soto, 2001), P2X receptor also mediate the inactivation of NMDA receptors, through a Ca^{2+} dependent mechanism (Pankratov *et al.*, 2002). P2X receptor sensitization also helps to increase the NMDA mediated current, causing an increase in LTP by phosphorylation of the extracellular domain of NMDA receptors (Wierasko *et al.*, 1989); Chen *et al.*, 1996). Nevertheless, P2X receptors are not always excitatory, as shown by Sperlagh *et al* (2002), where activation of P2X1 receptor induced a release of GABA. Another study reported a long lasting inhibition of glutamatergic transmission from CA3 mossy fibers, on addition of a potent P2X7 receptor agonist (Armstrong *et al.*, 2002). Pougnet *et al* (2014) demonstrated P2X mediated depression of field potentials at the CA1 neurons, along with reduction in the expression of surface AMPA receptors at dendrites and synapses.

P2Y receptors can also either potentiate the currents of NMDA receptor, through P2Y2 receptor (Wirkner *et al.*, 2002) or inhibit those currents, through P2Y1 receptor activation (Khakh *et al.*, 2000; Liu *et al.*, 2000).

The end product of ATP catabolism; adenosine, is another important player in the neuromodulation by purines.

1.3.4. Adenosine and Neuromodulation

Since adenosine is not stored in vesicles, or released in response to action potential, it predominantly does not act as a classical neurotransmitter. Adenosine is generated by the conversion of ATP into its metabolites serving as a critical neuromodulator to control neuronal excitability, release of neurotransmitters including glutamate, GABA, acetylcholine, and dopamine along with modulation of synaptic plasticity (Sebastiao and Ribeiro, 1996). Extracellular adenosine has physiological and pathological effects on its four receptors, known as P1 or adenosine receptors. They are subdivided into four subtypes: A1, A_{2A}, A_{2B} and A_3 (Cirera and Fredholm, 2001; Fredholm *et al.*, 2001). Each of this subtype has a unique tissue distribution, pharmacological properties, different G-protein coupling and distinct signaling pathways (Fredholm, Ijzeman *et al.*, 2001, Fredholm *et al.*, 2011, Chen *et al.*, 2014). Adenosine has different potency levels (EC50) for each of the receptors: A_1- 73 nM, A_{2A}- 150 nM, A_{2B} –

5100 nM and A_3- 6500 nM (Daly and Padgett, 1992; Peakmann and Hill, 1994). In physiological condition, the concentration of extracellular adenosine is reported to be around 20-200 nM in the brain, which is enough to activate its receptors (Fredholm *et al* 2001). The effect of adenosine depends on its levels and the expression level of its receptors.

1.3.4.1. Adenosine - Source and metabolism

Unlike in some cases of ATP, adenosine is not stored and released form vesicles but rather created by highly regulated intracellular pathways (including ATP production via AMP by adenosine kinase (ADK), nucleotide/DNA synthesis and S-Adenosylhomocysteine pathway (Chen *et al*., 2014). Most cells possess nucleotide transporters, ENT1 and ENT2 which are efficient in bidirectional transportation of adenosine across the cell membrane (King *et al*., 2006). Through many studies over the past many years (Delaney and Ginger 1998; Fredholm *et al*., 2007), it is well known that extracellular ATP undergoes rapid conversion (1ms) through a two-step process: first conversion of ATP/ADP to AMP by the ectonucleoside triphosphate diphosphophohydrolase-1(CD39), which is then followed by AMP hydrolysis to adenosine by ecto-5'-nucelotidase (CD73). Because of this enzymatic conversion and the nucleotide transporters mentioned above, the concentration of extracellular adenosine can never be zero and has been estimated to be around 25-250 nM under basal conditions (Ballarin *et al*., 1991; Dunwiddie and Masino, 2001; Pedata *et al*., 2001). However, under conditions of metabolic imbalance this can increase significantly to 1-10 µM (Cunha, 2001). Thus, the maintenance of these concentrations depend on the balance between the release and removal of the nucleoside by the membrane transporters or/and its formation by the ecto-enzymes in the extracellular space (Dunwiddie *et al*., 1997). The nucleotide transporters can also function as adenosine scavengers outside the cell promoting *de novo* synthesis of nucleotides in the cytosol (Baldwin *et al*., 2004). Both ENT1 and ENT2 are highly expressed in the rat brain (especially the hippocampus, cerebellum, cortex and striatum) and variety of cell types such as neurons and astrocytes among others (Parkinson *et al*., 2011).

In hippocampal slices, the application of a high frequency stimulation burst leads to increase in the extracellular levels of adenosine, derived from the catabolism of ATP (Cunha *et al*., 1996). Antagonist studies have shown that adenosine exerts a tonic inhibitory effect on synaptic transmission, implying that under basal conditions, adenosine levels are sufficient to tonically activate relevant adenosine receptors (Cunha, 2001).

1.3.4.2. Adenosine receptors

Adenosine receptors are mainly classified into four subtypes A_1 (A_1R), A_{2A} ($A_{2A}R$), A_{2B} ($A_{2B}R$), A_3 (A_3R), with different ligand affinity of 70 nM, 150nM, 5.1 µM and 6.5 µM respectively (Dunwiddie and Masino, 2001). They were initially classified based on the pharmacological profile to inhibit (A_1 and A_3) or stimulate (A_2 subtype) adenylate cyclase (AC) by adenosine analogues and antagonism by methylxanthines (Londos *et al*., 1980; van Calker *et al*., 1979). The A_2 receptor subtype was subsequently further categorized according to the presence of high affinity (A_{2A}) or low affinity (A_{2B}) binding sites to the brain (Daly *et al*., 1983). To date the four different subtypes have been identified, purified and cloned from mouse, rat, human and other mammalian and non-mammalian species (Fredholm *et al*., 2000; Fredholm, Ijzerman *et al*., 2001). Molecular cloning has not only definitely verified the presence of these receptors but has also helped in the creation of the genetic knockout (KO) to further elucidate the function of each receptor subtype (Chen *et al*., 2014). Each of the four receptors is metabotropic in nature; G protein coupled receptors with seven transmembrane domains (Furlong *et al*., 1992).

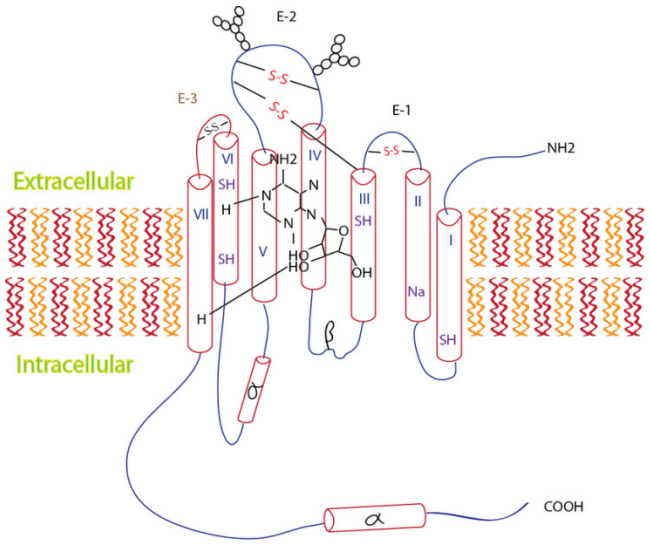


Figure 1.11 Primary structure of P1 receptor: The structure of P1 receptor contains seven transmembrane domains (I-VII) arranged in a clockwise manner Histidyl residues (H) in the sixth and seventh helices are proposed to hydrogen bond to adenosine with ribose 2',3' positions . the locations of cysteinyl residues (SH) and hypothetical disulphide bridges (S-S) are indicated. Glycosylation occurs in the second extracellular loop (E-II). Cytoplasmic segments show a hypothetical secondary structure (α,β) predicted using computational algorithm (Adapted from Fredholm et al., 1994).

As shown in the Fig.1.11, the N terminal of the receptor is present outside the membrane while the C terminal is intracellular. The seven transmembrane helices (I to VII) are arranged in counter clockwise direction. In actuality the domain I and VII are in proximity to each other forming a barrel shape, which

surrounds the ligand binding site. Histidyl residues (H) in the sixth and seventh helices are proposed to hydrogen bond to adenosine with ribose 2', 3' positions. The locations of cysteinyl residues (SH) and hypothetical disulphide bridges (S-S) are indicated. Glycosylation occurs in the second extracellular loop (E-II). Cytoplasmic segments show a hypothetical secondary structure (α, β) predicted using computational algorithm (Fredholm *et al*., 1994).

1.3.4.3. Adenosine receptors expression in the brain

A_1 receptor is expressed throughout the body, highest being in the brain, notably in the neurons of the cortex, hippocampus and cerebellum as well as dorsal horn of the spinal cord (Dixon *et al*., 1996; Fredholm *et al*., 2000). In neurons, A_1 receptor is found at both pre and post synaptic sites (Rebola *et al*., 2003). It is also expressed in various cell types, such as astrocytes, microglia and oligodendrocytes (Gebicke-Harter *et al*., 1996, Biber *et al*., 1997, Othman *et al*., 2003, Luongo *et al*., 2014).

A_{2A} receptor expression is highly concentrated in dorsal and ventral striatum as well as olfactory tubercule (Rosin *et al*., 1998, Schiffmann *et al*., 1991; Fink *et al*., 1992). A_{2A} receptors are also present in the hippocampus and cerebral cortex (Cunha *et al*., 1995, Dixon *et al*., 1996; Svenningsson, Hall *et al*., 1997). In the hippocampus they are mainly expressed at presynaptic sites (Rebola *et al*., 2005). Furthermore, A_{2A} receptors were also found to be localized at the excitatory striatal synapse where neurons express VGLUT (Hettinger *et al*., 2001). Sometimes A_{2A} receptors at presynaptic sites are co-localized with A_1 receptors to fine tune the release of glutamate at the glutamatergic striatal terminals (Ciruela *et al*., 2006). A_{2A} receptors are also present in other cell types such as astrocytes (Nishizaki, 2004) and microglia (Saura *et al*., 2005).

A_{2B} receptor is expressed in the eye, in the median eminence, along with neurons and glial cells of the pituitary gland, although at low levels (Daly 1977; Fredholm *et al*., 2005). Recently a work published by Goncalves *et al* (2015), demonstrated for the first time the expression of A_{2B} receptors at the glutamatergic synapses of the hippocampus.

The expression of A_3 receptors is seen at low levels in cortex, striatum, olfactory bulb, nucleus acumbens, hippocampus, amygdala, hypothalamus and cerebellum (Fredholm *et al*., 2005).

1.3.4.4. Adenosine and synaptic plasticity

Adenosine, an abundant neuromodulator have been shown to modulate synaptic plasticity at various synapses. Adenosine can be obtained from catabolism of ATP. The source of adenosine may determine the receptor subtype activated. For example, adenosine originating form adenine nucleotides seem to

activate preferentially excitatory A_{2A} receptors (Cunha *et al*., 1996). However, adenosine do play a vital role in the various kinds of synaptic transmission.

1.3.4.4.1 Modulation of glutamatergic transmission:

Presynaptic inhibition of glutamatergic transmission via adenosine has been known for nearly three decades (Dunwiddie and Fredholm *et al*., 1984). Though the possible involvement of cyclic AMP in this process had been debated at first (Dolphin and Prestich, 1985; Ribeiro and Sebastiao, 1986), there is now an agreement that presynaptic inhibition by adenosine involves inhibition of voltage-gated calcium channels (Ribeiro *et al*. 1979), possibly by G_0 coupling (Dolphin *et al*., 1986) or even via direct interaction of beta-gamma subunits with the channels (Haas and Selbach, 2000). Due to pharmacological selectivity of antagonists it could be said that the receptor that inhibits excitatory synaptic transmission at the hippocampus is an A_1 receptor (Sebastiao *et al*., 1990). A_1 receptor has also shown to inhibit NMDA receptor-mediated synaptic currents (de Mendonca *et al*., 1995). Hippocampal AMPA receptor mediated currents following A_1 receptor activation has also been reported (Dias *et al*., 2012). LTP in the mossy fibre pathway was shown to be impaired in A_1 receptor KO mice, an effect mimicked by the pre-treatment of A_1 receptor antagonist (Moore *et al*., 2003). Addition of the A1 antagonist (DPCPX) showed increase in levels of basal transmission of CA3-Mf, while conversely reduction though the use of its agonist (Rebola *et al*., 2008). In Schaffer collateral-CA1 pathway of the hippocampus the deletion of A_1 receptor did not produce alteration in LTP or LTD, but did alter paired pulse facilitation (Gimenez-Llort *et al*., 2005).

A_{2A} receptor, in spite of their limited distribution in the hippocampus (Schiffmann *et al*., 1991; Cunha *et al*., 1994) may modulate glutamate mediated excitability. Activation of A_{2A} has been shown to increase population spike amplitude (Sebastiao and Riberio, 1992), along with glutamate release (Lopes *et al*., 2002) and even weaken A1 receptor related inhibition of excitatory synaptic transmission (Cunha *et al*., 1994). A_{2A} receptors can also influence glutamate levels at synapses, since their activation inhibits glutamate transport into astrocytes (Matos *et al*., 2012). At the post synaptic side, A_{2A} receptors facilitate AMPA-mediated currents and increase the amplitude of miniature excitatory postsynaptic currents (EPSCs) in the CA1 pyramidal neurons (Dias *et al*., 2012). This was found to be due to a mechanism involving PKA activation and increase in the membrane levels of GluR1 subunits phosphorylated at the PKS site (Ser845). A_{2A} receptors promoted synaptic recruitment of calcium permeable AMPA receptors, contributing to a lasting form of synaptic plasticity that occurs as a result of ischemic events (Dias *et al*., 2013b). A_{2A} receptors also modulate facilitation of metabotropic glutamate receptors (mGluR5) at hippocampal NMDARs (Tebano *et al*., 2005), while also shown to be required for LTP mediated by

postsynaptic NMDA receptors at the mossy fiber synapses of the hippocampus (Rebola *et al*., 2008). Conversely, A_{2A} receptors can inhibit NMDA receptor mediated synaptic currents in rat neo-striatal neurons via the PKA pathway (Norenber *et al*., 1998; Wirkner *et al*., 2000). Thus antagonism of A_{2A} receptor potentiates the excitotoxic effect of direct NMDA receptor stimulation, while reducing neurotransmitter release (Robledo *et al*., 1999; Popoli *et al*., 2002) . A_{2A} receptors present at the cortico-striatal projection terminals (Rosin *et al*., 2003; Rebola, Rodrigues *et al*., 2005) modulate glutamate release and control synaptic plasticity (Rebola, Rodrigues *et al*., 2005).

A recent study by Goncalves *et al* (2015) demonstrated for the first time the expression of A_{2B} receptors at hippocampal glutamatergic synapses. They also reported that the activation of A_{2B}R partially occluded A_1 receptor-mediated inhibition of synaptic transmission, suggesting a primary fine tuning role for A_{2B} receptors.

1.3.4.4.2. Role in GABAergic transmission

Immuno-histochemical experiments have revealed the expression of A_1 receptor, not only at glutamatergic synapses of pyramidal cell dendrites, but also in the somatic cell membrane of CA1 pyramidal neurons and some GABAergic interneurons (Rivkees *et al*., 1995; Ochiishi *et al*., 1999). Adenosine A_1 receptors selectively modulate tonic inhibition mediated by extra-synaptic $GABA_A$ receptors (Rombo *et al*., 2014a). This function is seen not only in CA1 pyramidal cells but also in CA1 GABAergic interneurons. A_1 receptors were also reported to attenuate the depolarizing $GABA_A$ receptor responses during seizures through the activation of potassium channels and subsequently, increasing the cell membrane conductance (Ilie e*t al*., 2012). This resulted in a shunting effect on $GABA_A$ R currents in post synaptic pyramidal neurons (Sebastiao and Ribeiro, 2014)

A_{2A} receptors have been shown to enhance GABA release from isolated hippocampal nerve terminals (Cunha and Ribeiro, 2000). Rombo *et al* (2014b) recently showed (using optogenetic afferent stimulation and whole cell recording from postsynaptic neurons) that A_{2A} receptors located at the nerve terminals of a subset of interneurons, enhance GABAergic inhibitory synaptic transmission between interneurons of the CA1 area, causing disinhibition of pyramidal cells. Cristovao-Ferreira *et al*., (2009) reported that A_{2A} receptors enhanced GABA transport into isolated nerve endings. Interaction between A_1 and A_{2A} receptor to control GABAergic activity is shown by the study of Cunha-Reis *et al*., (2008) where enhancement of extracellular GABA levels caused by VIP in the hippocampal nerve terminals was dependent on the tonic activity of both the receptors. Selective blockade of either of the receptor would prevent the action of VIP while conversely selective activation would partially restore it. A_{2A} receptor might also interact with A_3 to receptor to regulate GABAergic transmission, as blockade of either of the receptor subtype decreased the

desensitization of human $GABA_A$ receptors expressed into Xenopus oocytes (Roset *et al*., 2009, Sebastiao and Ribeiro, 2014). Additionally A_{2A} receptors are also required for the facilitatory action of BDNF upon GABA transport into astrocytes (Vaz *et al*., 2011).

1.3.4.4.3. Modulation of cholinergic transmission

Cholinergic system regulates another form of neurotransmission seen in the hippocampus (Ji *et al*., 2001). Remarkably, it was the cholinergic system that prompted a possible role of adenosine in the inhibition of synaptic activity (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975; Gustafsson *et al*., 1978). Years later, studies reporting that role of A_1 receptors inhibiting acetylcholine from the hippocampus appeared (Jackish *et al*., 1984; Pedata *et al*., 1986). Furthermore, it was the cholinergic pathway that demonstrated the excitatory action of A_{2A} receptor upon neurotransmitter release, first at the striatum (Brown *et al*., 1990), followed by neuromuscular junction (Correia-de-Sa *et al*., 1991) and at the hippocampus (Cunha *et al*., 1994). Within the hippocampus, the facilitatory action of A_{2A} receptor is more prominent at the CA3 area, as compared to the dentate gyrus or the CA1 (Cunha *et al*., 1994). It is also significant that A_{2A} receptor activation by endogenous adenosine is required for the fast negative modulatory action of BDNF upon nicotinic α7 acetylcholine receptor responses in interneurons at the *stratum radiatum* region of the CA hippocampus (Fernandes *et al*., 2008).

Many studies have concentrated on a direct effect of adenosine upon synaptic plasticity (Sebastiao and Ribeiro, 2014). The inhibitory impact of A_1 receptor activation on LTP at the CA1 synapse as first reported nearly 25 years ago by two different and independent groups, that is, Arai *et al*., 1990; de Mendonca and Ribeiro, 1990 is one of them. The inhibitory effect is not only observed on the application of adenosine (or stable adenosine analog) before LTP induction (de Mendonca and Ribeiro, 1990), but also when applied within 1 min of LTP induction (Arai *et al*., 1990). This hints at a possible role of A_1 receptor in interfering with the biochemical processes resulting in LTP expression. The inhibition of LTP by adenosine analog was previously demonstrated *in vivo* (Dolphin, 1983). Moreover, it was shown by de Mendonca and Ribeiro (1994) that the LTP can be facilitated or depressed by drugs that inhibit A_1 receptor activation or by enhancing extracellular adenosinergic tonus, respectively. The same study also reported the first evidence of A_{2A} receptor mediated facilitation of high frequency induced LTP, via the use of a selective receptor agonist.

It was revealed that a slow onset NMDA-independent form of LTP could be induced at CA3-CA1 hippocampal synapse in the presence of an A2 receptor agonist DPMA (Kessey and Mogul, 1997). Incidentally, the NMDA-dependent LTP observed at the mossy fiber synapses was shown to be due to postsynaptic A_{2A} receptor (Rebola *et al*., 2008). Caffeine, a non-selective antagonist of adenosine

receptors can attenuate frequency-induced LTP at the CA1 pyramidal cells in a manner similar to the selective blockade of A_{2A} receptors (Costenla *et al*., 2010). This effect of caffeine is shown to be present only at low micromolar concentrations and in aged animals (Costenla *et al*., 2010). Furthermore an *in vivo* study (Batalha *et al*., 2013) showed the beneficial effect of A_{2A} receptors blockade in reverting stress induced changes in synaptic plasticity, dendritic atrophy and learning. This was only observed in aged animals as A_{2A} receptors blockade was likely impairing learning in young animals (Sebastiao and Ribeiro, 2014).

When it comes to LTD and depotentiation (reversal of LTP), very little information is available. An early form of depotentiation can be induced shortly after LTP induction (<3 min), which largely results from tonic A_1 receptor activation by endogenous adenosine (Larson *et al*., 1993; Huang *et al*., 1999; Liang *et al*., 2008). On the other hand, A_2 receptors have an opposite effect on this form of depotentiation (Huang *et al*., 1999). Depotentiation induced by prolonged and moderate (1Hz) stimulation long after the LTP is fully expressed is another kind of depotentiation. This form of depotentiation is NMDA receptor dependent and requires activation of protein phosphates and attenuated by endogenous adenosine through A_1 receptors (de Mendonca *et al*., 1997; Huang *et al*., 2001). Endogenous activation of A_2 receptors also impacts this form of depotentiation (Fujii *et al*, 1999, 2002).

In contrast to depotentiation, LTD (long lasting inhibition of synaptic transmission) in neonatal rats is inhibited by endogenous A_1 receptors (de Mendonca *et al*., 1997). Remarkably, LTD can only be induced, in adult (Kemp and Bashir, 1997) and even aged animals (Costenla *et al*., 1999) by blocking the activation of A_1 receptor. This suggests a major role for adenosine to hamper long lasting inhibition (Sebastiao and Ribeiro, 2014). However, recent data from freely behaving rabbits showed LTD evoked in the somatosensory cortex to be prevented by the blockade of A_1 receptors (Marquez-Ruis *et al*., 2012).

When it comes to A_3 receptors, their activation has been shown to enhance LTP and attenuate LTD (Costenla *et al*., 2001). A_3 receptors may also help in the impairment of the mechanisms leading upto LTP (Maggi *et al*., 2009).

Thus purines, (in this context ATP and adenosine) just like microglia play a crucial role in modulating synaptic plasticity. Additionally, purines have also shown to regulate glial functioning (in this case microglia).

1.4. Microglia and Purines

1.4.1. Expression of Purinergic receptors in microglia

Microglia expresses multiples functional purinergic receptors. With respect to P2 receptors, they express both the ionotropic (P2X) and metabotropic (P2Y) receptors. . These include P2X4, P2X7, P2Y4, P2Y6, P2Y12 and P2Y16 (James and Butt, 2002; Boucsein *et al*., 2003; Tsuda *et al*., 2003; Kobayashi *et al*., 2006; Koizumi *et al*., 2007). On the other hand, with respect to P1 receptors, microglia has shown to express all the four subtypes of adenosine receptors: A_1, A_{2A}, A_{2B}, A_3 (Fredholm *et al*., 2001; Luongo *et al*., 2014). Although the expression of these receptors is found on microglia, their density might depend on the environment and thus depends on the microglial phenotype (Koizumi *et al*., 2013). Interaction between P1 and P2 or within the same class of receptors can also be observed in some cases (Koizumi *et al*., 2013). Thus it can be said, that microglia alters the purinergic receptor system according to need and uses it to transform itself accordingly.

1.4.2. Role of Purines in Microglial function

In a normal healthy brain, microglia is present in the so called “resting” form with highly branched processes known as ramified microglia. As mentioned above, microglia even in the “resting” form does perform functions from scanning the environment to housekeeping (Davalos *et al*., 2005; Nimmerjahn *et al*., 2005; Wake *et al*., 2009). Through this scanning and surveying state it is immediately transformed into an active one depending on the environmental changes, and purinergic receptors have a pivotal role in this process (Koizumi *et al*., 2013). Microglia in the pathological brain shows several distinct phenotypes such as process extension, retraction, migration, proliferation and phagocytosis (Kreutzberg, 1996; Hanisch and Kettenmann, 2007). For such physiological functions, the extracellular release/leakage of nucleotides or nucleosides and their action on receptors is necessary (Koizumi *et al*., 2013). The expression levels of these receptors shown complex spatiotemporal patterns according to the changes in the environment and microglia uses these signals co-operatively or distinctively, and control complex and dynamic changes of microglial phenotype (Koizumi *et al*., 2013).

1.4.2.1. Purines in surveying and process-extension in microglia

As previously mentioned, microglia even in their "resting" state are constantly scanning the environment, including synapses (Wake *et al*., 2009). Microglia makes direct contact with synapses at the frequency of about once per hour, depending on neuronal excitability (Wake *et al*., 2009). This suggest of an intimate communication between neurons and microglia at synapses, by which surveying behaviours are

controlled. When acute brain injuries occur, microglia significantly increases its motility and is transformed into a process-extending phenotype (Koizumi *et al*, 2013). These processes are then immediately extruded to the injury site, by which they prevent the spread of lesion in the brain (Hines *et al*., 2009). Haynes *et al* (2006) showed that this process extraction does not occur in a P2Y12 receptor deficient mice, indicating the dependence on P2Y12 receptor for this process. This study was done *in vivo* to confirm the previous knowns role of P2Y12 receptors in triggering microglial motility (Honda *et al*., 2001). Neuronal injury results in the release/leakage of ATP, which acts as a chemoattractant signal ("find me") (Koizumi *et al*., 2013), which could act on the P2Y12 receptors, causing this motility. On the other hand a recent study showed that adenosine, by acting on A_3 receptors, modulated the ATP induced process extension (Oshawa *et al*., 2012). So the released ATP could be stimulating both P2Y12 and A_3 receptors (as adenosine), which could mean of a possible cross talk between P2Y12 and A_3 receptors in controlling process extension (Fig. 1.12 C). However, there are other molecules which could also be responsible for this process, such as glutamate, GABA (Fontainhas *et al*., 2011) and fractalkine (Liang *et al*., 2009). Nevertheless, purinergic signals could be the primary system to be activated, as the release of ATP is the initial event following neuronal death, and P2Y12 receptors are highly expressed in both surveying and extending microglia (Fig 1.12B). Additionally, it was observed that ATP/ADP-induced process extension required activations of the phosphatidylinositol 3'-kinase (PI3K) and phospholipase (PLC) pathways, inhibition of the adenylate cyclase pathways, integrin β-1 activation and its accumulation in the tip of the extending process (Oshawa *et al*., 2010; Oshawa and Kohsaka, 2011, Koizumi et a., 2013).

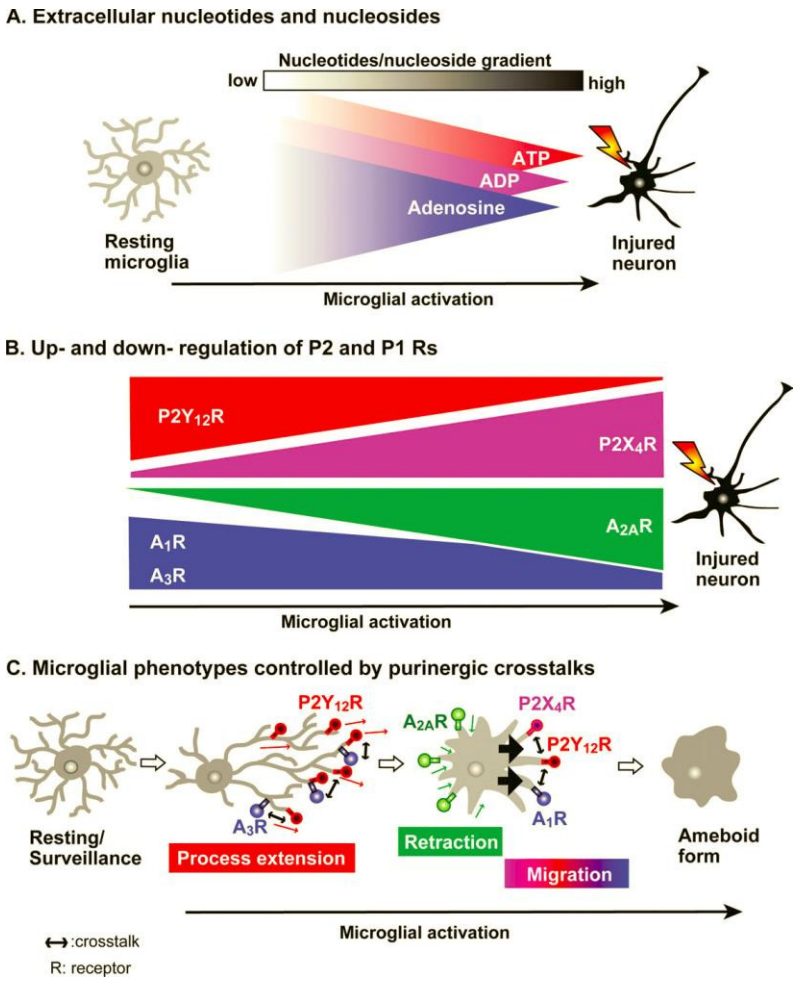


Figure 1.12 Role of purines in microglial functions: P2Y12 receptor-mediated process extension and migration in microglia, showing the effects of other P2 and P1 receptors. **A)** Release/leakage of nucleotides/nucleosides from injured neurons. When neurons or cells are injured or dead, high concentrations of ATP (mM) are leaked. The released ATP is diffused and also metabolized into ADP, AMP and adenosine. **B)** Changes in P2 and P1 receptors involved in the motility of microglia. Resting microglia in the adult healthy brains express multiple P2 and P1 receptors. Within these receptors, P2Y12, P2X4, A1, and A3 receptors are thought to be important for the dynamic motility of microglia. When microglia are activated, they upregulate P2X4 and A_{2A} receptors, whereas P2Y12 receptors are decreased (in some cases, for example spinal microglia in a neuropathic pain model, these are increased) **C)** Process extension, retraction and migration induced by P2 or P1 receptors. When resting/surveying microglia sense ATP/ADP by P2Y12 receptors, they extend their processes to the injured sites. This event is cooperatively controlled by simultaneous activation of adenosine A3 receptors by adenosine. Microglia upregulate adenosine A_{2A} receptors, by which they retract their processes and form round-shaped activated microglia. Activated microglia upregulate P2X4 receptors, whereas decrease P2Y12 receptors. Activation of P2Y12 receptors by ATP/ADP in activated microglia resulted in migration toward the injured sites. Activation of P2X4 receptors by ATP enhanced the microglial migration, suggesting positive crosstalk between P2Y12 and P2X4 receptors. The P2Y12 receptor-mediated migration requires co activation of adenosine A1 and P2Y12 receptors, suggesting cooperative and essential crosstalk between these receptors for the migration. (Koizumi et al., 2013)

1.4.2.2. Purines in process-retraction of microglia

Activated microglia retracts its processes during neurodegeneration and neuroinflammation, which is strongly co-related with the functional transformation of microglia from surveying to an activated one (Kreutzberg 1996). Adenosine has been shown to induce process retraction and the repulsion of lipopolysaccharide (LPS) treated microglia (Orr *et al*., 2006). Microglia activated by LPS has shown to retract their processes and to undergo repulsive migration in response to ATP, which is metabolized into adenosine and sensed through A_{2A} receptor (Orr *et al*., 2009). Although, initially the expression of A_{2A} receptors in microglia is very low, addition of LPS enhances it, while surprisingly decreasing the expression of P2Y12 receptor (Haynes *et al*., 2006; Orr *et al*., 2009 (Figure 1.12B). The up regulation of A_{2A} receptor is also observed in pathological condition such as in Parkinson's disease or ischemia (Pedata *et al*., 2001; Schwarzschild *et al*., 2006). The change in the expression levels of A_{2A} and P2Y12 following nerve injury is thus important in the regulation of microglial motility, including process extension and retraction as well as migration (Koizumi *et al*., 2013).

1.4.2.3. Microglia migration and Purines

On activation, microglial undergoes various morphological changes. The soma enlarges while the processes shorten and retract (Koizumi *et al*., 2013). They form an amoeboid shape, appearing as rounded cell, (Fig. 1.6) with high motility and movement around in the brain tissue (Koizumi *et al*., 2013). Different factors can influence microglial migration. Chemokines such as MCP-1 and Fractalkine (Hayashi *et al*., 1995; Lauro *et al*., 2006), neurotropic factors such as NGF and EGF (Gilad and Gilad, 1996; Nolte *et al*., 1997), amyloid-β (Du Yan *et al*., 1997), neuropeptide bradykinin (Ifuku *et al*., 2007) and neureglin-1 (Calvo *et al*., 2010) are some of the examples. However, purines also play an important role in microglial migration. A study using microglia cultured from P2Y12 receptor-deficient mice showed that ATP was unable to induce membrane ruffling and chemotaxis (Haynes *et al*., 2006). This points out to a possible role of P2Y12 receptor in the migration of microglia. As seen with the function of process extension, the activation of PI3K and PLC pathways, PI3K/Akt pathway (Oshawa *et al*., 2007; Irino *et al*., 2008), and inhibition of adenylate cyclase pathway, together with a decrease in the cyclic AMP level and protein kinase A (Nasu-Tada *et al*., 2005) are involved in the P2Y12 receptor mediated migration. Also, monocytes from P2Y2 receptor deficient mice showed decreased migration (Elliot *et al*., 2009).

Migration also involves a counteraction between P2 and P1 receptors. In animal models of neuropathic pain, the activation of spinal microglia is concurrent with the up regulation of P2X4 receptors (Tsuda *et al*., 2003, figure 1.11 B). On blockade of this receptor, using pharmacological approaches and RNA

interference based knockdown model, microglia chemotaxis was suppressed (Oshawa *et al*., 2007). ATP causes an increase in intracellular calcium concentration mainly by inducing extracellular Ca^{2+} influx through P2X4 receptor, which in turn modulates the activation of PI3K/Akt pathway, which facilitates chemotaxis (Tsuda *et al*., 2003). P2X4 receptor expression is significantly upregulated in activated microglia within the 24 hours after nerve injury or ischemia (Cavaliere *et al*., 2003; Tsuda *et al*., 2003; Schwab *et al*., 2005) (fig. 1.11 B). These studies point out that P2X4 receptor activation can modulate microglial migration under pathological conditions.

Adenosine also regulates microglial migration. ATP failed to activate P2Y12 receptor-mediated microglial migration in CD39/ENTPDase -1/ or A_1 deficient mice microglia (Farber *et al*., 2008). This failure was corrected with the addition of adenosine. Thus the ATP induced microglial migration seems to be under the control of both P2Y12 and adenosine A_1 receptor (ATP activating P2Y12 , whilst ATP converted adenosine simultaneously activating A_1 receptor). Though the precise mechanism behind this is unknown, it can be said that an intimate crosstalk among purines and their receptors modulate microglia migration. This further helps microglia to perform its other functions, namely phagocytosis.

1.4.2.4. Purines and microglia phagocytosis

On migration, microglia arrives at the injured site and determines whether to rescue or kill the damaged cells and phagocytose those (Koizumi *et al*., 2013). For this, the role of P2Y6 and UDP has been well documented. UDP is an endogenous agonist to P2Y6 receptor, released by injured and/or dead neurons (Koizumi *et al*., 2013). The activation of the P2Y6 receptor results in engulfment and phagocytosis of the target molecule (Kettenmann 2007; Inoue *et al*., 2009; Kataoka *et al*., 2011). Phagocytosis is a specialized form of endocytosis, where phagosomes uptake a cell or relatively large molecule (>1.0 µm), and is the central mechanism in tissue remodeling, inflammation and defense against foreign agents (Tjelle *et al*., 2000). Since P2Y6 is activated by UDP which is very quickly degraded by ecto-enzymes, UDP is present only temporarily and thus can restrict the targets to be phagocytosed (Koizumi *et al*., 2013). Moreover, unlike other function (process extension, retraction and migration), UDP does not stimulate any other P1 or P2 receptor or influence chemotaxis (Koizumi *et al*., 2007), but only phagocytosis. Worthy to note is that "resting" microglia show no expression of P2Y6 receptors. Upon activation by UDP, the expression levels of P2Y6 receptors increase, while those of P2Y12 decrease, causing a functional shift from migration to phagocytosis (Inoue *et al*., 2009). Though the mechanism behind this is yet to be understood, the role of P2Y6 receptor in microglial phagocytosis cannot be undermined.

Thus ATP and other nucleotides/nucleosides through their receptors are the first signals that enable microglia to sense changes in the brain environment, causing changes in their morphology, phenotype and

function. Furthermore, as described above, microglia forms a glia-neuron complex in various aspects including synaptic plasticity, although the role of purines in modulating this phenomenon has to be understood.

AIM

1.5. Aim of the project

Hypothesis

"Purines control neuron–***glia interaction during*** neuroinflammation"

Sub-hypothesis

- Microglia-derived purines impact hippocampal short term synaptic plasticity.

For this we examined step by step the following points:

- Purines and autocrine role of microglia-derived ATP and A_{2A} receptors
- Paracrine role of microglia in modulating hippocampal synaptic plasticity, with the help of purines

MATERIALS AND METHODS

2. Materials and Methods

2.1. Microglia cell culture

Murine microglial cell line, N9 (a kind gift from Professor Claudia Verderio, CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy), was grown in an RPMI medium supplemented with 30 mM glucose (Sigma, Sintra, Portugal), 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO, Invitrogen, Porto, Portugal).

Primary microglial cultures were prepared as previously described (Carreira *et al*., 2012). Briefly, primary cultures of glial cells were obtained from a postnatal (P1-P5) C57BL6 mouse and maintained for 15 days in the DMEM-F12 medium with Glutamax (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 0.25% gentamycin (Invitrogen) and 0.25 ng/mL M-CSF (murine-colony stimulating factor, Peprotech, Rocky Hill, New Jersey, USA). Microglia were then separated from the mixed primary culture by shaking (200 rpm for 2 hours), and plated in the DMEM-F12 medium with Glutamax containing 0.25% gentamycin (Invitrogen).

Cells were kept at 37°C under a humidified atmosphere with 95% O_2 and 5% CO_2. Viable cells (identified by counting tryphan-blue-excluding cellular elements) were plated at a density of 5×10^5 cells per cm^2 in 6 well trays for ATP quantification experiments, and 1×10^5 cells per cm^2 in 12 well trays for proliferation and immunocytochemistry studies.

2.2. Microglial pharmacological treatment

In order to clarify the ability of different microglial inducers - bacterial *versus* neuronal - to trigger ATP release by microglial cells, N9 cells were challenged with 100 ng/mL LPS (from *Escherichia coli*, serotype 055:B5; Sigma) or glutamate 0.5 mM (Sigma) for 6 hours. We recently reported that LPS, at this concentration and exposure time, induces changes in the density of $A_{2A}R$, which subsequently increases cell proliferation (Gomes *et al*., 2013). Glutamate at 0.5 mM induces pro-inflammatory responses of microglia, under the control of $A_{2A}R$ activation (Dai *et al*., 2010). We now aim to address the role of LPS and glutamate in regulating ATP release and mediated functions, and also pharmacologically modulate these effects by blocking $A_{2A}R$. For that, microglial cells were pre-incubated (20 min before insults) with a supra-maximal concentration of the selective $A_{2A}R$ antagonist (Canas *et al*, 2009 ; Rebola *et al*, 2003), SCH 58261 (50 nM, Tocris) which was present throughout incubation time (6h) and is known to be able to prevent LPS-induced changes in proliferation rate (Gomes *et al*., 2013).

To elucidate whether the temporal correlation between ATP variations and microglial proliferation results from a direct (mediated by ATP) or an indirect effect (mediated by ATP-derived adenosine activating $A_{2A}R$), the following modulators were added to N9 cells, 20 minutes before LPS or glutamate, in supra-maximal and selective concentrations, as reported from our previous experience in different preparations: β,γ-imATP 1 and 5 nM (adenosine-5´-(β,γ-imido)-triphosphate; a non-hydrolysable ATP analogue; Sigma) (Rodrigues *et al*, 2005); apyrase 20 U/mL (which degrades ATP; Sigma); adenosine deaminase 2 U/mL (which converts endogenous adenosine into inosine; Sigma)(Rebola *et al*, 2003); ARL 67156 100 μM (6-N,N-diethyl-D-β,γ-dibromomethylene ATP, a non-selective inhibitor of several NTPDases, a family of ectoenzymes, nucleoside triphosphate diphosphohydrolases, able to degrade ATP; Tocris) (Rebola *et al*, 2009); AOPCP 50 μM (α,β-methylene adenosine 5´-diphosphate, an inhibitor of ecto-5´-nucleotidase, the enzyme responsible for the conversion of AMP into adenosine; Sigma) (Rebola *et al*, 2009).

2.3.ATP quantification

Extracellular levels of ATP were assessed by the Luciferin-luciferase bioluminescence assay, which allows the determination of ATP levels ranging from $2x10^{-12}$ up to $8x10^{-5}$ M. The enzymatic oxidation of luciferin by luciferase is ATP-dependent and leads to light emission at 565 λ, proportional to ATP levels, as measured by VICTOR multilabel plate reader (PerkinElmer™), Briefly, after a period 6 hours incubation of N9 cells with different drugs/modulators, the supernatants were collected and kept at -80ºC until ATP measurements. 80 μL of cell supernatant was added to 40 μL of ATP assay mix (Sigma) designed for bioluminescence. The supernatants were then placed in white 96-well plate and kept inside the luminometer for 1 min at RT before start recording luminescence levels (5 sec of acquisition time). ATP levels were determined by extrapolation from a standard curve obtained by measuring the luminescence of solutions of different concentrations of standard ATP provided with the kit; these values were normalized by the total protein content of the respective well.

2.4. Proliferation assay

Microglia proliferation was evaluated by measuring the incorporation of 5-bromo-2´-deoxyuridine (BrdU; Sigma), a synthetic nucleoside that can be incorporated into newly synthesized DNA, replacing thymidine during cell replication. Cells were incubated with BrdU (10 μM) for the last 2 hours of pharmacological treatment, fixed in 4% PFA, washed in TBS (Tris-buffered saline) with 0.3% Triton X-100 and maintained in 1M HCl at 37ºC for 30 minutes. Non-specific binding was prevented by incubation for 1 hour in TBS with 3% BSA (bovine serum albumin) and 1% Triton X-100. Cells were incubated overnight

at 4ºC with a primary rat antibody anti-BrdU (1:100, Serotec) in a 0.1% Triton X-100 and 3% BSA solution, washed and incubated for 2 hours at RT with an Alexa Fluor 594 donkey anti-rat secondary antibody (1:200, Molecular Probes). For nuclear staining, cells were incubated for 5 minutes at RT with DAPI (Invitrogen) and mounted in Dakocytomation fluorescent medium (Dakocytomation Inc.). Fluorescent images were acquired using an Axioskop 2 Plus fluorescence microscope (Zeiss; PG-Hitec). The number of proliferating cells (BrdU-positive) was counted and expressed as a percentage of the total cells stained with DAPI (Gomes *et al*., 2013).

2.5.Slice preparation:

C57BL6/J mice (P19-P21) were used for the experiments. They were sacrificed by cervical dislocation. The brain was then quickly removed from the skull and chilled in ice cold low calcium artificial CSF (aCSF) containing the following (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 10 glucose, 25 $NaHCO_3$ and 75 sucrose with pH 7.4 adjusted by saturating with carbogen (95% 0_2 and 5% CO_2), and an osmolarity of 300-310 mOsm. Isolated brains were then glued onto a stage of a vibratome (VT 1200S Leica Microsystems, Nusslich Germany) and parasagittal hippocampal slices (320µm) were cut. Slices were then transferred to an extracellular solution containing the following (in mM): 129 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$ and 11 glucose equilibrated with 95% O2 and 5%CO2 for 30 minutes at 33°C and thereafter at room temperature, till used.

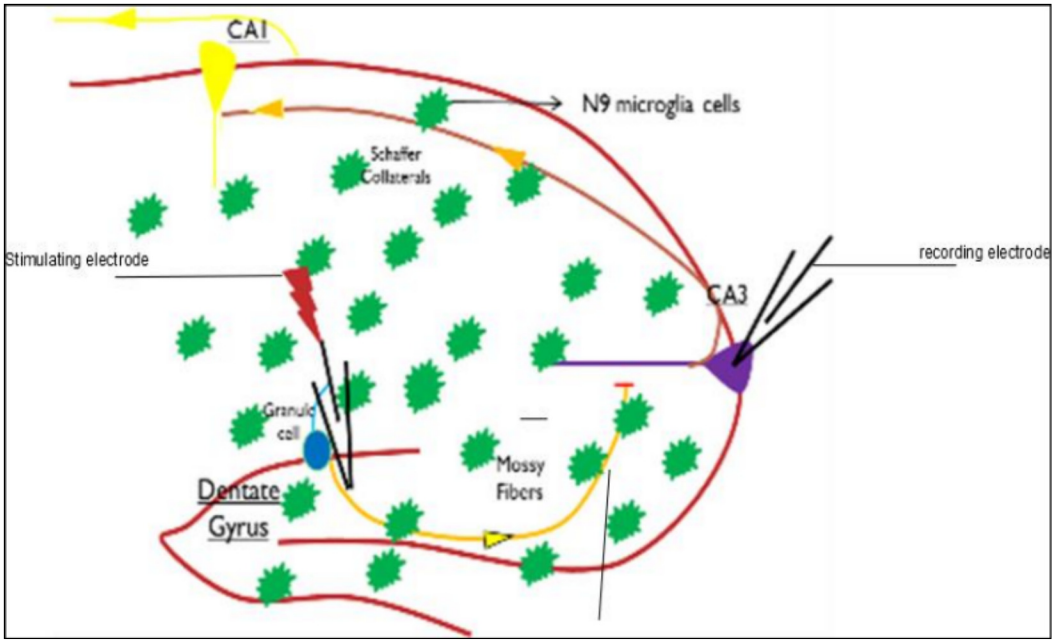
2.6. Electrophysiology

The slices were then placed onto a recording chamber, submerged and in continuous perfusion with (95% oxygen and 5% CO_2) extracellular solution at a temperature of 32-34°C. Whole cell patch clamp recordings were made at a holding potential of -70mV with electrodes of 3-4 MΩ. Prior to this, patch clamp electrodes were pulled out from a borosilicate glass (GF 150 F-10) and filled with an internal solution containing the following (in mM): 140 $CsCH_3SO_3$, 2 $MgCl_2$, 4 NaCl, 5 phospho-creatine, 2 $Na_{2A}TP$, 0.2 EGTA, 10 HEPES, and 0.33 GTP (pH 7.3) adjusted with CsOH. Bicuculline (10 µM) was added to the bath to inhibit $GABA_A$ receptors.

The voltage-clamp recordings were performed on CA3 pyramidal neurons visualized with a differential interference contrast microscope (Eclipse FN-1, Nikon, Champigny sur Marne, France) equipped with an infrared camera (VX44, till photonics, Gräfelfing, Germany) using an Axoptach -200B amplifier (Axon Instruments, Sunnydale CA, USA). Signals were filtered at 2 kHz and digitized at 5 kHz via a DigiData 132_{2A} interface (Axon Instruments). Series resistance (10-20MΩ) was monitored during the recording using a -10mV hyperpolarizing voltage step of 50ms length occurring at the beginning of each recording.

Neurons were rejected if more than a 20% change in the series resistance occurred during the experiment. Neurons with a holding current exceeding 300pA at a holding potential of -70mV were rejected. Data were collected and analysed using pClamp software 9.2 (Axon Instruments).

Before addition to slices, N9 microglia cells were first treated with LPS (100ng/ml) or not, depending on the experiment, for an hour. The cells were then trypsinized, collected and added onto a hippocampal slice at a density of about 100,000 cells per slice. N9 cells were allowed to attach to the slices for 15-20 mins and the unattached cells were washed away. Voltage clamp recordings proceeded as described before from a CA3 neuron present nearby a N9 microglia cell. A representation is given below.



Excitatory post synaptic currents from Mf-CA3 were evoked by minimal intensity stimulation (Marchal and Mulle, 2004; Sachidhanandham *et al*., 2009). A glass electrode, with a tip of about 1µm diameter, is placed on the hilus of the dentate gyrus to stimulate mossy fibers. The baseline stimulation frequency for all experiments was 0.1Hz. Mossy fiber synaptic currents were identified according to the following criteria: robust low-frequency facilitation, low release probability at low stimulation (0.1Hz), rapid rise time of individual EPSC and free of secondary peaks during decay. mGlurII agonist, LCCG-1 (10µM) was added routinely to confirm the authenticity of mossy fibers.

2.7.Immunohistochemistry

P18-P21 mice were anesthetized with intraperitoneal administration of pentobarbital (50 mg/kg body weight) and were fixed by transcardial perfusion with 50 ml of 4% paraformaldehyde (PFA). Brain was

removed and post-fixed in 4% PFA overnight and then saturated in a solution with 30% sucrose for 24h. Then brains were frozen in cold heptane and store at -80°C. Brains were cut into 40 µM-thick frontal sections on a cryostat at -20°C. Sections were collected into antifreeze medium and stored at -20°C until the staining protocol. After a thorough wash in PBST (PBS 0.1M + 0.3 %Triton X-100), free-floating sections were incubated with NDS-T (3% Normal Donkey Serum with 0.3% Triton X-100) for 20 minutes at room temperature. Then sections were incubated with primary antibody anti-P2X4 (Atlas antibodies, 1:400, rabbit) in PBST-NDS solution, overnight at 4C. Slices were washed the next day with PBS 1X three times to rinse off the excess primary antibody and incubated with the secondary antibody, Alexa 488 goat-anti- rabbit in PBST-NDS solution at 1:400 (Synaptic Systems) for two hours at room temperature. After washing three times with PBS 1X, slices were fixed using a fluoromont containing DAPI. Fluorescent images were acquired using Leica DM6000 TCS SP8X microscope, with diode laser(blue) 405nm, white laser 488nm (excitation) and 500nm-560nm (emission, green). Images were obtained by mosaic acquisition of the whole hippocampus at 20X.

2.8.Data analysis

Values are presented as mean±standard error of the mean (SEM) of *n* experiments. Either a Student´s t test for independent means or a one-way analysis of variance (ANOVA) followed by a Newman-Keuls post hoc test, was carried out to define statistical differences between absolute values, which were considered significant at $p<0.05$ unless otherwise specified. Note that although the impact of several drugs and modulators are presented as percentage values for the sake of clarity (Part 1), the statistical comparisons were always carried out by using the absolute values.

RESULTS

3. Results

Part 1: purines and autocrine role of microglia

3.1. LPS and glutamate differentially modulate microglia extracellular levels of ATP

Although it was already shown that, besides astrocytes and neurons, microglia is able to release ATP (Anderson *et al*., 2004; Fujita *et al*., 2008; Higashi *et al*., 2011; Dou *et al*., 2012), modulators of this regulated process were not identified to date.

Lipopolysaccharide (LPS), a component of the outer membrane of Gram negative bacteria, has largely been used as a microglial inducer in the analysis of cellular inflammatory responses considered important in the pathophysiology of brain disorders (e.g. release of inflammatory mediators) (Sweet MJ and Hume DA , 1996). However, the inflammatory phenotype of immune-competent cells, such as microglia, relies on the nature of the inducer, and the main phenotypic differences are expected between cells exposed to infectious agents or to intrinsic neuronal cues. Glutamate-mediated toxicity has been associated with distinct pathological conditions of the brain, including degenerative diseases (Obrenovitch and Urenjak, 1997; Haydon and Carmignoto, 2006). Accordingly, there is previous evidence that LPS and glutamate might engage different pathways to 'activate' microglia, as gauged by the different phenotype of 'activated' microglia upon exposure to these two inducers (Dai *et al*., 2010) Thus, it was considered of relevance to compare the effect of LPS (here used as an infectious inducer) and glutamate (used as an intrinsic neuronal signal) on the ability of N9 cells to release ATP.

After incubation of N9 cells with LPS (100 ng/mL) or glutamate (0.5 mM) for 6 hours, ATP levels were measured in the supernatants. In basal conditions (non-treated N9 cells), mean extracellular levels of ATP were 2.6±0.4 nM (n=6). In the presence of LPS (100 ng/ml), a decrease in extracellular ATP levels (1.1±0.2 nM, n=6, $p<0.01$ as compared with non-treated cells, Fig. 3.1) was observed, whereas glutamate (0.5 mM) induced an increase in ATP levels (5.1±0.4 nM, n=6, $p<0.001$ as compared with non-treated cells, Figure 3.1 A). When denoted in % form the values come to LPS 57.72±6, glutamate 194±22.5. Although the levels of ATP measured in the supernatants of primary microglia (in the range of 10^{-7} M) are higher than those obtained from N9 cells (in the range of 10^{-9}M) in basal conditions (lack of added drugs), the effects of LPS and glutamate were still observed: LPS decreased (80±3, n=4, $p<0.05$, as compared with non-treated cells, Figure 3. 1C), whereas glutamate (0.5 mM) increased extracellular ATP levels (184±32%, n=5, $p<0.05$, as compared with non-treated cells, Figure 3. 1C)

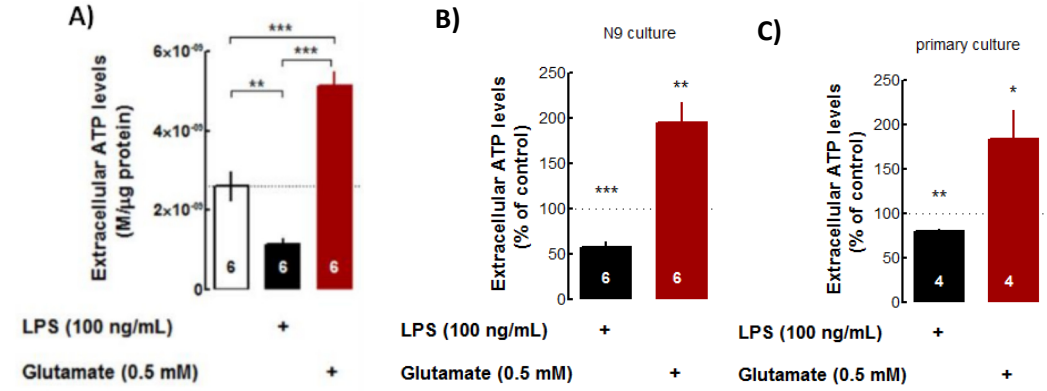


Figure 3.1: Extracellular ATP measurements from microglia cells: A) Extracellular ATP levels observed in control, as opposed to LPS(100ng/ml) and Glutamate(0.5mM) treated N9 microglia cells. As seen, LPS stimulation leads to decrease in the extracellular ATP levels, while Glutamate stimulation leads to an increase, as opposed to control condition. Control 2.6±0.4 nM , n=6, LPS 1.1±0.2 nM, n=6; glutamate 5.1±0.4 nM, n=6 **p <0.005, *** p<0.001, one way anova, Neuman Keuls m.c.t. **B)** Extracellular ATP levels of LPS and glutamate treated N9 cells denoted in % form. (LPS 57.72±6, n=6; glutamate 194±22.5, n=6) **C)** Extracellular ATP levels obtained from LPS and Glutamate treated microglia primary cultures denoted in % form (LPS 80±3, n=4, glutamate 184±32, n=4)* p<0.05, **p<0.005, one sample t test.

The present results indicate that extracellular levels of ATP, which result from a balance between the release and extracellular metabolism of ATP (Kukulski *et al.*, 2011), are oppositely regulated by LPS and glutamate (glutamate 0.5 mM, p<0.001 as compared with LPS-treated cells, Figure 3.1), as anticipated by the diverse nature of LPS and glutamate. We have previously reported that LPS, in the same experimental conditions used in the present work, induces microglial proliferation (Gomes *et al.*, 2013). Altogether, these observations led us to hypothesize that the regulation of extracellular levels of ATP may impact on cell function, in particular proliferation.

3.2. ATP levels correlate with microglia proliferation

Considering the selectivity of LPS and glutamate in regulating extracellular ATP released by microglia, we next evaluated if a correlation exists between ATP levels and microglial proliferation. In accordance to the study reported by our group (Gomes *et al.*, 2013), we show LPS induced increase in microglial proliferation (182.8±25.3%, n=4, p<0.05 as compared with non-treated cells, Figure 3.2 A, B; non-treated cells exhibit a ratio BrdU-positive nuclei/DAPI of 0.3±0.02, n=4 and this value was taken as the reference

with which the ratio obtained in the presence of LPS or glutamate was compared, in order to calculate % of effects). Microglial cells exposed to glutamate (0.5 mM) exhibited a decrease in proliferation (73.3±9.4%, n=4, p<0.01 as compared with non-treated cells, Figure 3.2 A, B).

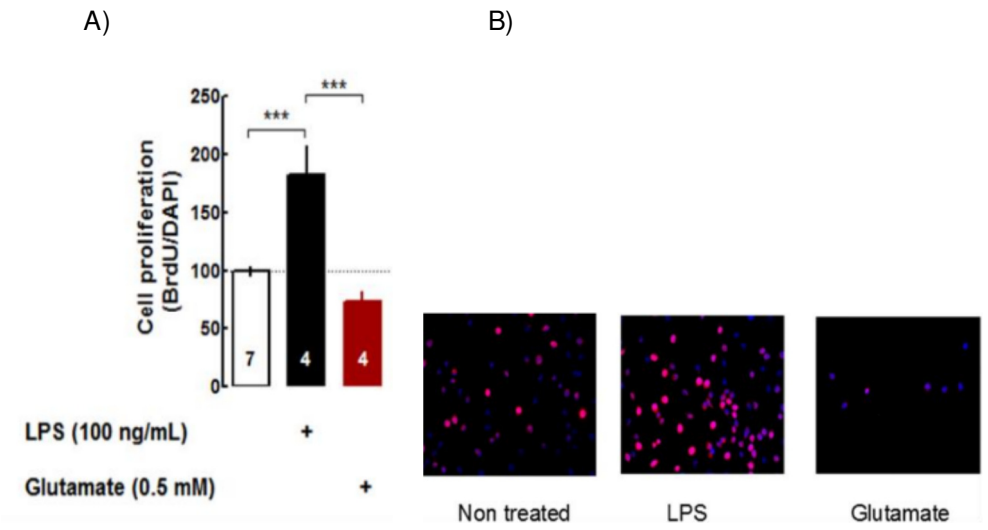


Figure 3.2: Microglia proliferation levels under LPS and glutamate conditions A) Microglia cell proliferation levels observed in LPS (100ng/ml) and Glutamate (0.5mM) treated cell. LPS stimulation causes increase in proliferation as opposed to glutamate and control conditions (LPS 182.8±25.3, n=4 glutamate 73.3±9.4,n=4 *** p<0.001, one way anova. Neumann Keuls M.C. T. All values are S.E.M **B)** Representative images of each of the conditions. All the cells are labelled blue with DAPI, while the proliferating cells are labelled pink. [BrdU(red)+DAPI(blue)].

The present results suggest that ATP levels selectively modulate proliferation: 1) extracellular ATP levels selectively modulate proliferation so that high extracellular ATP levels are associated with decreased proliferation and lower ATP levels are permissive for proliferation increments.; 2) the opposite effects of LPS and glutamate on proliferation seem to be indirectly defined by their ability to oppositely modulate the extracellular levels of ATP

Considering that, at lower ATP levels (that is, in the presence of LPS), proliferation is increased and because we previously found that $A_{2A}R$ activation is required for LPS-induced increase in proliferation (Gomes *et al*., 2013), we further investigated if $A_{2A}R$ blockade influenced the extracellular ATP levels and the proliferation of N9 cells

3.3. $A_{2A}R$ blockade normalizes LPS- and glutamate-induced changes of extracellular ATP levels, but only abrogates LPS-induced changes of proliferation

$A_{2A}R$ are important regulators of different functions, namely the reactivity to LPS *ex vivo* (Link *et al.*, 2000) the release of inflammatory mediators (Link *et al.*, 2000; Saura *et al.*, 2005), process retraction and extension (Orr *et al.*, 2009), glutamate release (Papoli *et al.*, 2003), among others. Furthermore, the neuroprotective potential of $A_{2A}R$ antagonists is, at least in part, attributed to their ability to control microglial-mediated neuroinflammation (Frau *et al.*, 2011; Rebola *et al.*, 2011;). We next tested the effect of $A_{2A}R$ blockade in the control of extracellular levels of ATP and proliferation in the presence of LPS or glutamate

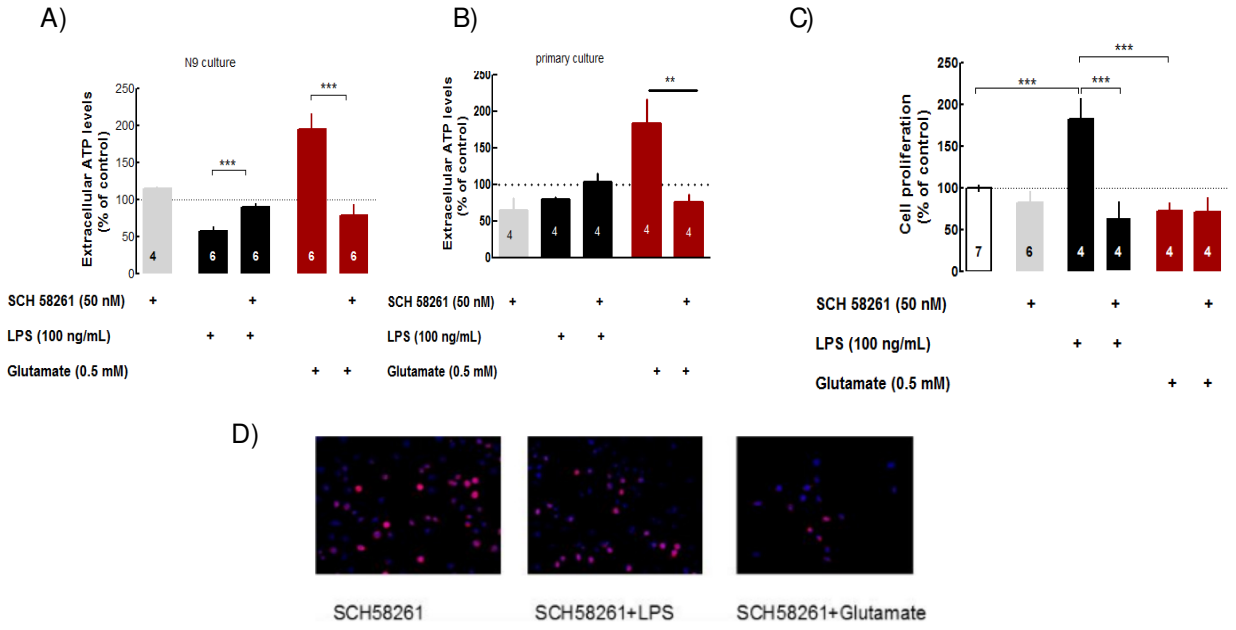


Figure 3.3. Modulatory role of $A_{2A}R$ in microglial ATP release and proliferation: A) Effect of A_{2A} receptor blockade (SCH568261 50nM) on extracellular ATP levels in N9 microglia culture. SCH58261 on its own has no effect on extracellular ATP levels, however inhibits the effect of LPS (100ng/ml) and glutamate (0.5mM) on ATP release (SCH58261-115.1±3, LPS-57.72±6, SCH58261+LPS-90.75±4.23, glutamate-194±22.5, SCH58261+glutamate 78.40±15.17). **B)** Comparative Effect of A_{2A} receptor blockade on extracellular ATP levels in primary microglial culture (SCH5826165.80±30.86, LPS 79.73±3.4, SCH58261+LPS 103.4±11.51, glutamate 184.2±31.98 SCH58261+glutamate 76.68±10.29). **C)** SCH58261 also inhibits the effect of LPS in N9 microglia cell proliferation, while having no preventive effect in glutamate stimulated cells (SCH58261 82.73±13.79, LPS 182.8±25.26, SCH58261+LPS 65.94±17.96, glutamate 73.33±9.4, SCH58261+glutamate 71.6±16.8) **D)** Representative images of each of the conditions. All the cells are labelled blue with DAPI, while the proliferating cells are labelled pink [BrdU(red)+DAPI(blue)]. All values are SEM, one way anova Neumann Keuls M.C.T

Since we have previously found that $A_{2A}R$ activation control microglia proliferation (Gomes *et al.*, 2013), we next investigated if $A_{2A}R$ controlled the LPS- or glutamate-induced changes of extracellular ATP levels and microglia proliferation. Figure 3.3A shows that the selective $A_{2A}R$ antagonist SCH58261 (50 nM) did not modify the extracellular ATP levels in basal conditions (115.1±3%, n=4, $p>0.05$, as compared with non-treated cells. However, $A_{2A}R$ blockade prevented the LPS (90.75±4.23, n=6, compared to LPS) and glutamate (78.40±15.17, n=6, compared to glutamate) induced changes in extracellular ATP levels (Figure 3.3A), suggesting that $A_{2A}R$ may function as a normalizer of altered extracellular ATP levels in the vicinity of microglial cells. Figure 3.3B shows the ability of $A_{2A}R$ blockade to modulate the effects of LPS or glutamate on the extracellular ATP levels in primary microglia cultures: as observed with N9 cells, SCH58261 (50 nM) prevented both the LPS- (103±12%, n=4, $p<0.05$, as compared with LPS-treated cells) and glutamate-induced changes (76.68±16%, n=4, $p<0.05$, as compared with glutamate-treated cells) of the extracellular ATP levels.

SCH58261 prevented the LPS-induced increase in proliferation levels (65.6±18%, n=4, $p<0.001$, compared with LPS-treated cells, Figure 3.3C, D), as previously reported (Gomes *et al*, 2013). By contrast, $A_{2A}R$ blockade did not modify the glutamate-induced decrease of microglia proliferation (71.6±16.8, n=4, $p>0.05$, compared with the absence of SCH58261, Figure 3C, D).

Altogether, our findings indicate that, although LPS-induced proliferation is prevented by $A_{2A}R$ blockade, microglial "growth arrest" by glutamate is not prevented in the same conditions. This suggests that high ATP levels are necessary, but not sufficient to induce the decrease in proliferation observed in the presence of glutamate and raises the question whether this "growth arrest" is a direct effect of ATP or, alternatively, a result from its metabolic degradation into adenosine.

3.4.Proliferation arrest in the presence of high concentrations of ATP is not a direct effect of ATP

In order to exclude the possibility of a direct effect of extracellular ATP in the glutamate-induced decrease in cell proliferation, two approaches have been taken: (1) pharmacological blockade of ATP receptors (P2) in the presence of glutamate, a condition associated with higher ATP levels; (2) incubation of N9 cells with β,γ-imATP [adenosine-5´-(β,γ-imido)-triphosphate], a non-hydrolysable ATP analogue, used in the range of concentrations of extracellular ATP measured in the presence of LPS and glutamate. ATP exerts its effects through the activation of different P2 receptors (Burnstock *et al*, 1978); among which P2X7 receptor control microglial proliferation (Monif *et al.*, 2009, 2010). Thus, ATP-mediated

effects were prevented by the non-selective pharmacological blockade of P2X receptors (PPADS 10 μM) and by selectively blocking P2X7 receptors (BBG 1 μM).

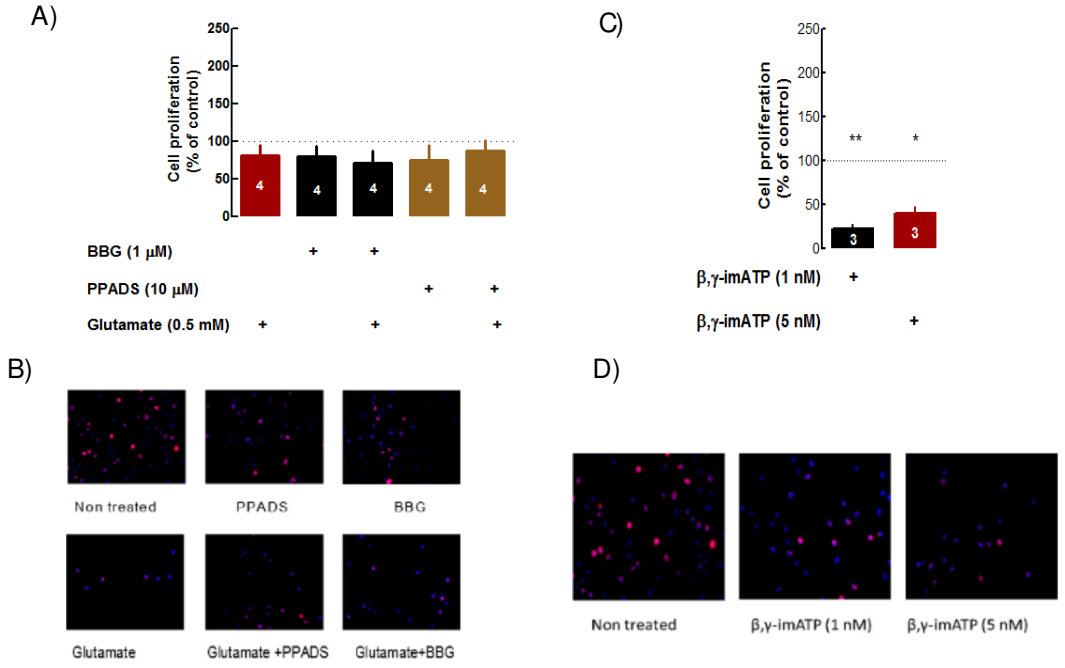


Figure 3.4 Proliferation arrest in the presence of high concentrations of ATP is not a direct effect of ATP **A)** Proliferation levels of microglia cells when subjected to inhibition of ATP receptor (BBG 1μM) and PPADS (10μM). Significant differences were observed between microglia cells with antagonists and control. However no differences were observed when comparing with glutamate stimulated cells (Glutamate 81.75±17.16, BBG 79.76±13.99, BBG+glutamate 71.52±15.75, PPADS 74.81±19.82, PPADS+glutamate 87.28±13.77, n=4 for all conditions). **B)** Representative images of microglia proliferation assay. All the cells are labelled blue with DAPI, while the proliferating cells are labelled pink [BrdU (red)+DAPI(blue)] **C)** Proliferation levels of microglia cells when subjected to ATP analogue (β,γ-imATP) in different concentrations (1nM and 5nM). Significant differences were observed between the two concentrations and control conditions in the rate of proliferation [β,γ-imATP (1nM) 22.48±4.6, β,γ-imATP (5nM) 39.94±7.6], n=3 all conditions *$p<0.05$**$p<0.005$, one sample t test, **D)** Representative images of microglia proliferation assay. All the cells are labelled blue with DAPI, while the proliferating cells are labelled pink [BrdU (red)+DAPI(blue)]. All values are SEM

Both antagonists (PPADS and BBG) did not have an effect in cell proliferation, function (BBG: 79.76±13.99, PPADS: 74.81±19.81, n=3, Fig 3.4A). Moreover, both drugs were unable to modulate glutamate-associated levels of proliferation (Fig. 3.4A) further suggesting that ATP might not be causally related with growth arrest. Supporting this hypothesis, incubation of N9 cells with the ATP analogue, β,γ-imATP, in a concentration range similar to that obtained in the presence of LPS (i.e. 1 nM) or equivalent to the maximum concentration of extracellular ATP obtained in the presence of glutamate 0.5 mM (i.e. 5 nM) decreased microglial proliferation (β,γ-imATP 1 nM: 21.48±4.6, n=3, $p < 0.001$ as compared with non-treated cells; 5 nM: 39.9±7.3, n=3, $p<0.01$ as compared with non-treated cells, Figure 3.4C). This may be explained by the fact that β,γ-imATP is not hydrolysable, further strengthening the hypothesis that increase in proliferation obtained in the presence of LPS may be related with the ATP metabolic conversion into metabolites, namely adenosine. These observations, together with the ability of the $A_{2A}R$ selective antagonist to modulate ATP levels, but not ATP-associated growth arrest (Figures 3.3 and 3.4), indicate that the temporal coincidence between high extracellular ATP levels and growth arrest (or the opposite) are not a result of a direct, receptor-mediated effect of ATP.

3.5.Microglia proliferation is dependent upon ATP catabolism into adenosine

Adenosine is the end-product of ATP catabolism (Lomax and Henderson 1973). Our results showing the requirement of $A_{2A}R$ tonic activation for proliferative events, the ability of these receptors to control ATP levels, but not the "growth arrest" observed at high extracellular ATP levels, suggest that adenosine, rather than ATP, is important to control microglial proliferation. To clarify if the metabolic conversion of ATP into adenosine is required for proliferation, we used different approaches: 1) degradation of extracellular ATP (by using apyrase) in the presence of glutamate; 2) decrease of adenosinergic endogenous tonus in the presence of LPS; 3) prevention of extracellular metabolism of ATP in the presence of LPS.

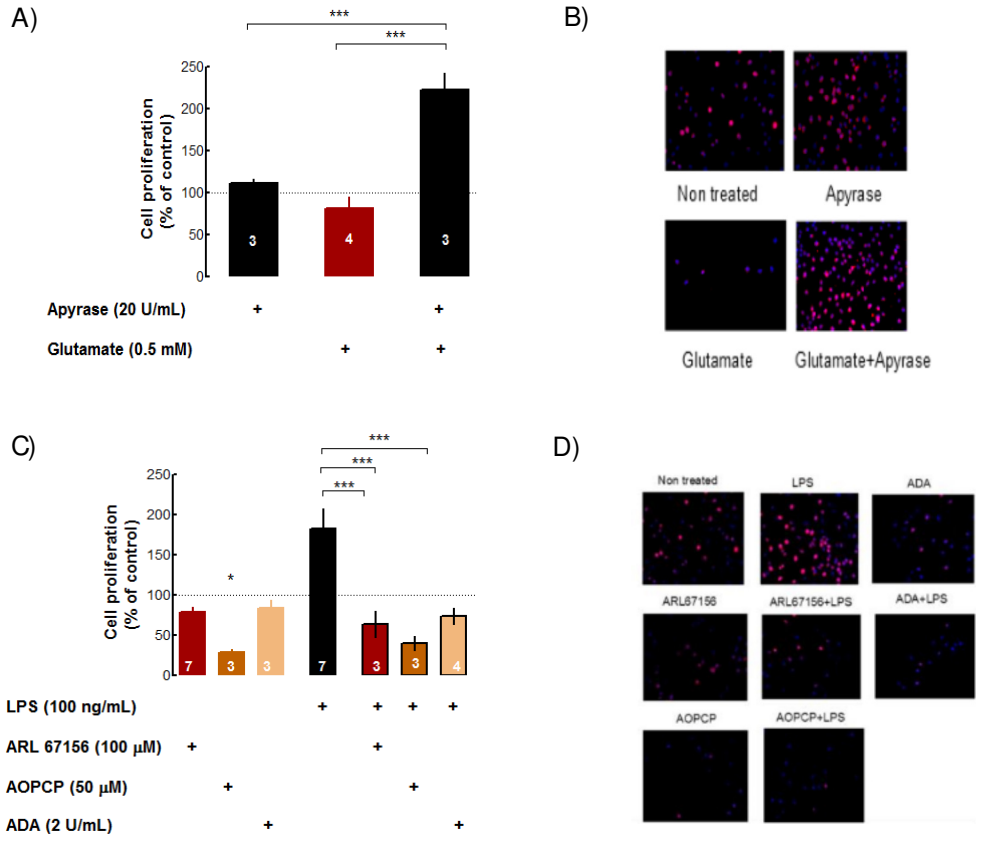


Figure 3.5: Microglia proliferation is dependent upon ATP catabolism into adenosine: A) Proliferation levels of microglia cells when in presence of apyrase (20U/ml). Addition of apyrase led to a huge increase in microglial proliferation in presence of glutamate (apyrase 111.7±4.4, glutamate 81.75±13.16, apyrase+glutamate 222.8±20.16 ***p<0.001.) **B)** Representative images of the microglia proliferation assay with respect to apyrase. All the cells are labelled blue with DAPI, while the proliferating cells are labelled pink [BrdU (red)+DAPI(blue)]. **C)** Proliferation levels of microglia cells when in treated with Ecto - 5`nucleotidase inhibitor; ARL67156(100µM) and AOPCP(50µM) ,and ADA (2U/ml) in presence and absence of LPS. Presence of each of the enzyme prevented LPS induced increase in microglial proliferation (ARL67156 78.72±6.9, AOPCP 29.5±2.6, ADA 83.9±10.1, LPS 182.8±25.26 ARL+LPS 64.11±16.39, AOPCP+LPS 39.83±8.7, ADA+LPS 79.38±9.9). **D)** Representative images of the microglia proliferation assay with respect to the various ecto-phosphatases. All the cells are labelled blue with DAPI, while the proliferating cells are labelled pink [BrdU (red)+DAPI(blue)]. All values are with respect to control in SEM ***p<0.001, one way anova , Neuman Keuls M.C.T

Although apyrase (20 U/ml) alone is not able to interfere with proliferation (111.7±4.4, n=3), it prevented the decrease of proliferation observed in the presence of glutamate (Figure 3.5 A, B). Addition of apyrase in presence of glutamate led to an huge increase in microglia proliferation (222.8±20.16, n=3). On the other hand the pharmacological blockade of the enzymatic conversion of extracellular ATP into ADP, AMP and ultimately, adenosine (by using ARL 67156, 100 μM) prevented LPS-induced proliferation (64.1±16.4, n=3, $p<0.001$ as compared with LPS-treated cells, Figure 3.5 C, D). The selective blockade of the last step of ATP catabolism to adenosine (by using AOPCP 50 μM) also prevented LPS-mediated increase in proliferation (39.8±8.7, n=3, $p<0.001$ as compared with LPS-treated cells, Figure 3.5 C, D). Finally, removing endogenous adenosine from the medium (by using adenosine deaminase, ADA 2U/ml) prevented LPS effects upon proliferation (74.4±10, n=4, $p<0.001$ as compared with LPS-treated cells, Figure 3.5 C, D).

This part of the work was the basis of the work published:

George J, Gonçalves FQ, Cristóvão G, Rodrigues L, Meyer Fernandes JR, Gonçalves T, Cunha RA, Gomes CA. Different danger signals differently impact on microglial proliferation through alterations of ATP release and extracellular metabolism **Glia**. 2015; Apr 4. doi: 10.1002/glia.22833 [Epub ahead of print]

Part 2: purines and paracrine role of activated microglia in synaptic plasticity

3.6 Addition of N9 microglia on acute hippocampal slice

After analysing the role played by purines in the autocrine role of microglia (ATP release and proliferation), we decided to look at the effect of activated microglia on synaptic transmission, and the putative role of purines. Since we had established that N9 microglia cells release ATP, modulated by $A_{2A,}$ we decided to use them. Also since N9 culture cells behave similarly to primary culture with respect to ATP release, and owing to the tiresome process to obtain and maintain primary culture cells, N9 cells were a promising candidate for the experiment. To study the effect of activated microglia on short term plasticity, we added N9 microglia cells onto acute hippocampal slices using the methodology previously seen in the studies by Centonze et al., (2009) and Nistico et al (2013). Around 100,000 microglia cells were counted and collected (using haemocytometer) and added onto the slices, placed in the recording chamber. The perfusion of the slices as stopped as the slice environment needed to be still for proper adhesion of N9 microglia cells onto the slice. However to prevent the slices from oxygen deprivation, the solution containing slice and N9 cells was directly but slowly oxygenated, making sure the environment was as still as possible. The N9 cells were thus incubated onto the slice and allowed to adhere to them. After 20 minutes the perfusion was started and the slice was washed off any unattached N9 cells. Since targeted addition (just at the CA3 of the hippocampus) of N9 cells onto the slice was not possible. The whole hippocampus was seen to be covered with N9 cells (Fig. 3.6 A, B).

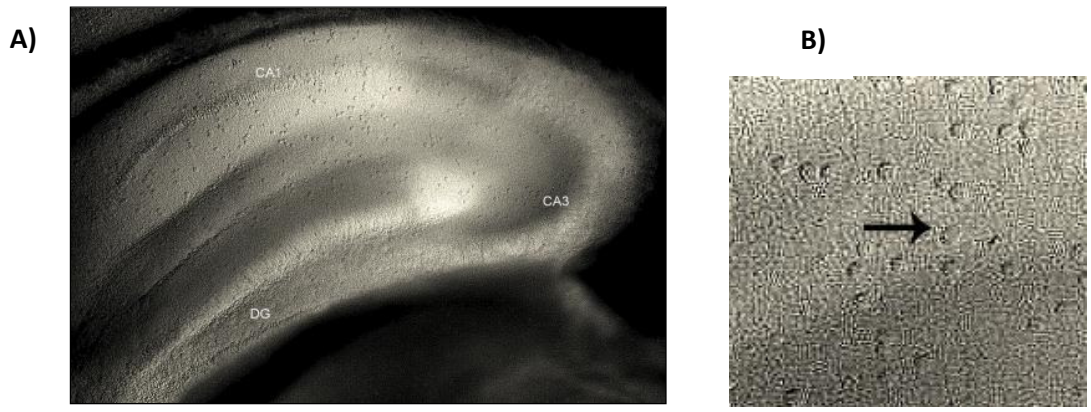


Figure 3.6 Hippocampal slice with adhered N9 microglia cells. **A)** An acute hippocampal slice with added N9 microglia cells adhered to them, as seen under 4X. **B)** A much closer (zoomed in) view of an area of the hippocampus with the N9 cells (indicated by the black arrow) adhered on top.

The amount of adhered N9 cells might differ with each experiment as quantification of them was not conducted. However, almost every pyramidal cell was of the CA3 region of the hippocampus was seen to be surrounded by N9 cells. 'Activation' of N9 microglia cells was done by pre-treatment with LPS (100ng/ml, one hour), prior to its collection and subsequent addition onto the slice. No difference in morphology between non-treated (3.6C) and LPS-treated N9 microglia (3.6D) cells were observed.

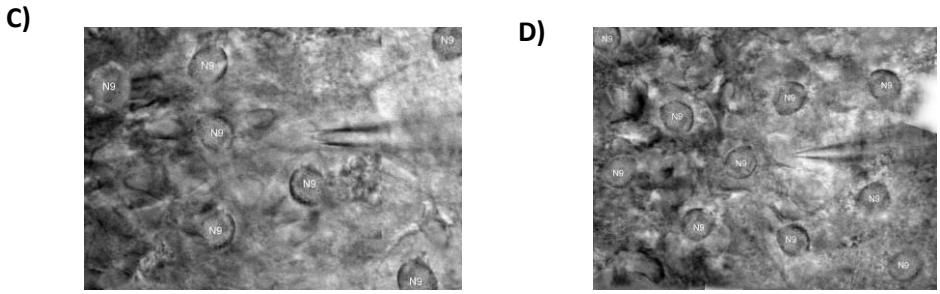


Figure 3.6 A picture showing the presence of N9 cells around a recorded CA3 pyramidal cell, with no notable difference in their morphology (Under 60X) C) Image showing non-treated microglia surrounding a CA3 pyramidal cell patched with a pipette and under recording. **D)** Image showing the presence of LPS treated N9 microglia cells surrounding the recording CA3 pyramidal cells

3.7. LPS treated microglia cause a reduction in mossy fiber frequency facilitation and paired pulse facilitation

To evaluate whether presence of an activated microglia cell can have an effect on short term plasticity, particularly frequency facilitation and paired pulse facilitation, we recorded a CA3 pyramidal cell surrounded by the added microglia, with stimulation on mossy fibers. Since previous results show functional activation of microglia with respect to ATP release and proliferation (also Gomes *et al*., 2013), the same concentration of LPS (100ng/ml) was employed to activate the N9 microglia cells. N9 microglia cells were treated with LPS (100ng/ml) for a period of one hour (Nakamura *et al*.1999) in culture, collected and added onto acute hippocampal slices, as described above (3.6). Recordings were made from a CA3 cell 'near' to a N9 microglia cell while mossy fibers were stimulated. 'Inactivated' microglia/ non-treated microglial cells (no LPS) were also added on other slices and recorded from, as control experiments. Forms of short term presynaptic plasticity, that is frequency facilitation (FF) and paired pulse facilitation (PPF), and in some cases basal transmission were studied.

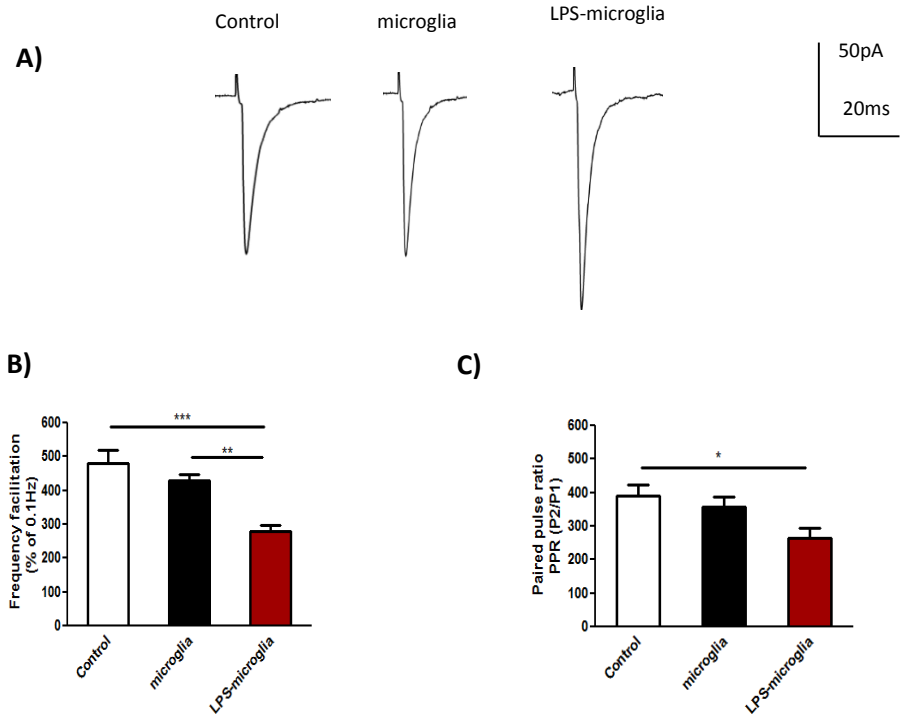


Figure 3.7. Effect of LPS treated microglia on short term synaptic plasticity. A) Representative traces of Mf-CA3 synaptic currents obtained at 0.1Hz under control and in presence of non-treated microglia and LPS (100ng/ml) treated microglial cells, in different slices. **B)** Bar graph representing the variations observed in frequency facilitation (Control 482.0±38.74, microglia 429.8±18.38, LPS-microglia 280.9±17.78; n=9 for all conditions) **C)** Bar graph representing the variations observed in paired pulse facilitation (Control 391.7±31.82, microglia 357.1±34.89, LPS-microglia 277.0±30.54; n=9 for all conditions). The frequency facilitation and paired pulse recordings were made one after another, on the same cell. The white bar represents recordings in control conditions (no N9 cells added to them), black bar represents recordings from slices with non-treated N9 cells and red bar represents recordings from slices with LPS-treated N9 cells. Data represented as mean SEM. * $p<0.05$, ** $p<0.005$, *** $p<0.001$ from one way anova, Newmann-Keuls multiple comparison test (MCT).

As seen in Fig. 3.7 B and C, Mf-CA3 synaptic currents recorded from slices with LPS treated N9 cells show decrease in frequency facilitation (280.9±17.78, n=9, $p<0.001$; fig 3.7 B) and paired pulse facilitation (277.0±30.54, n=9, $p<0.05$; fig 3.7C) compared to slices with no added cells (control condition; 482.0±38.74, n=9, fig.3.6B), and 391.7±31.82, n=9; fig 3.7 C) respectively, and slices with non-treated microglial cells (429.8±18.38, n=9; fig 3.7 B) and 357.1±34.89, n=9; fig 3.7 C) respectively. Since no change was seen from control slices and slices with non-treated microglia cells, the decrease in frequency facilitation and paired pulse facilitation seen in Mf-CA3 synaptic currents from slices with LPS-treated microglia cells was attributed to the activation state of microglia, caused by LPS treatment. Slices with

LPS treated N9 cells also showed increase in baseline recording (0.1Hz) as opposed to control and non-treated microglia conditions (fig 3.7A).

3.8. A_{2A} receptor inhibition prevents changes observed in short term plasticity.

Since LPS treated cells can release ATP (Fig3.1), albeit under low concentrations, we hypothesized its role in the decrease observed in synaptic transmission. Moreover, since blockade of A_{2A} receptor on N9 microglia cells with the use of its antagonist (SCH58261, 50nM) inhibited the effect of LPS in ATP release (Fig.3.2) we decided to block A_{2A} receptors on N9 microglia before its treatment with LPS and further addition onto acute slices. The cells were pre-incubated with the A_{2A} receptor antagonist for 20 min prior to the addition of LPS (100ng/ml). The rest of the methodology is the same as mentioned in § 3.6.

As observed (Fig. 3.8B), when recorded from slices with LPS treated microglia pre-incubated with SCH58261 (50nM), no decrease was observed in frequency facilitation (373.3±26.84, n=9, $p<0.05$) as opposed to LPS treated conditions. The decrease in paired pulse facilitation was also slightly inhibited (324.5±31.0, n=9), albeit not significantly (Fig 3.8C). We also observed reduction in basal transmission levels recorded at 0.1Hz (shown by representative traces) at Mf-CA3 synapse, between LPS treated and SCH-LPS treated microglial cells (Fig 3.8A). Thus the ability of $A_{2A}R$ blockade to inhibit the changes observed in short term synaptic plasticity, further centres our hypothesis that LPS treated microglia cells release ATP which could impact Mf-CA3 synaptic currents, leading to changes in short term synaptic plasticity.

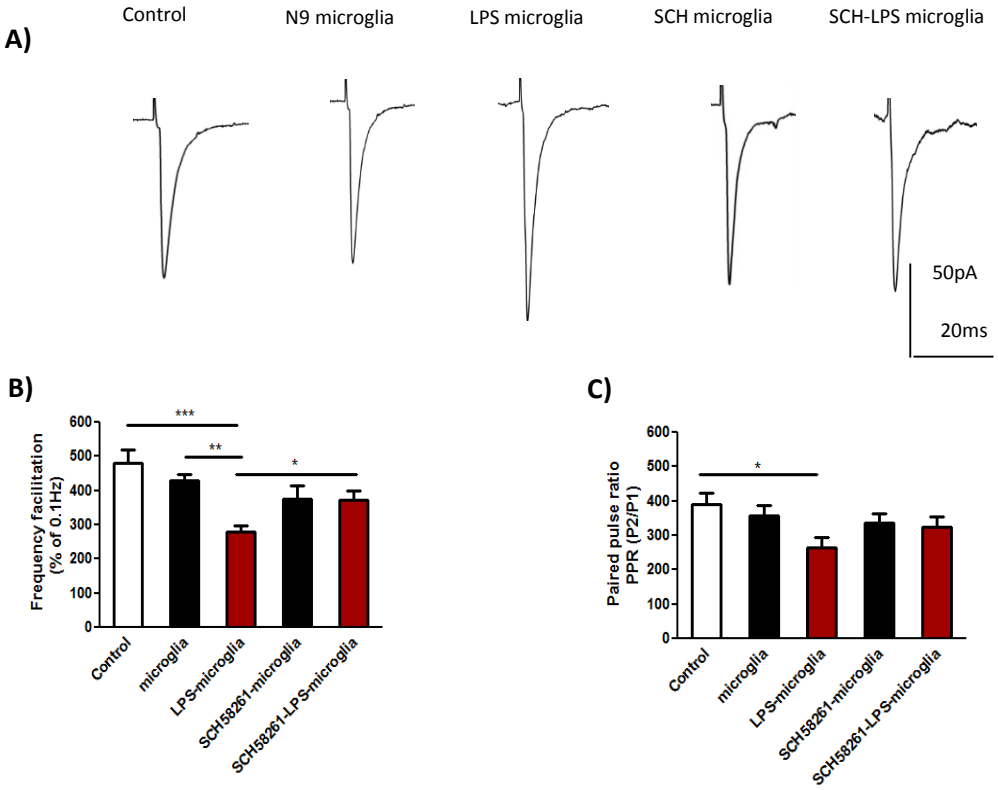


Figure 3.8. Microglial A_{2A} receptor inhibition prevents changes in short term plasticity. **A)** Representative traces of basal transmission obtained at 0.1Hz under different conditions. **B)** Bar graph representing the difference in frequency facilitation recorded from slices with differently treated N9 microglia on them (Control 482.0±38.74, microglia 429.8±18.38, LPS-microglia 280.9±17.78, SCH-microglia 374.6±9.26, SCH-LPS-microglia 373.3±26.84; n=9 for all conditions). **C)** Graph representation of the paired pulse facilitation observed from Mf-CA3 synaptic currents on slices with differently treated N9 microglia cells on them (Control 391.7±31.82, microglia 357.1±34.89, LPS-microglia 277.0±30.54, SCH-microglia 336.4±27.96, SCH-LPS-microglia 324.5±31.00; n=9 for all conditions). The frequency facilitation and paired pulse recordings were made one after another, on the same cell. Data represented as mean SEM. * $p<0.05$, ** $p<0.005$, *** $p<0.001$ from one way anova, Newmann –Keuls multiple comparison test (MCT).

3.9.P2X4 receptor modulates short term synaptic plasticity.

Along with ATP release, A_{2A} receptors also modulate BDNF release (Gomes *et al*., 2013). Thus to validate the hypothesis of a role of ATP, but not BDNF, in the change in synaptic plasticity, we investigated the effects of specific ligands of subtypes of P2X receptors. ATP can bind to metabotropic purinoreceptors (P2Y receptors) and ionotropic purinoreceptors (P2X receptors). The role of P2X receptors has been investigated in the context of synaptic plasticity (Pougnet *et al*, 2014). Within the P2X receptor family, the expression of P2X7 receptors on CA3 neurons has been reported (Yu *et al*., 2008) whereas controversial results have been documented for a presynaptic expression, that is by mossy fibers (Armstrong *et al*., 2002; Sperlagh *et al*., 2002; Kukley *et al*., 2004). On the other hand, the putative role of P2X4 receptors in the modulation of synaptic strength (Baxter *et al*., 2011) and/or plasticity has been well documented (Sim *et al*., 2006). Using knockout mice, Sim *et al*, (2006) showed the importance of the P2X4 receptor in modulating LTP at the CA1-CA3 synapse. However, the expression of P2X4 receptor at this synapse has not been documented so far. So, to investigate if P2X4 receptor was involved in the alteration seen in frequency facilitation and paired pulse facilitation, we used its selective antagonist, 5-BDBD; 10µM (Donnelly Roberts *et al*., 2008).

As shown in Fig. 3.9A, 5-BDBD (10 µM) decreased basal synaptic transmission, as opposed to in control. This decrease was observed within 10 minutes of 5-BDBD addition [5-BDBD (red), control (black), Fig. 3.9A,]. This decrease was found to be significant when compared with control conditions (Fig. 3.9B, $p<0.0005$) Conversely, Ivermectin (3 µM), which specifically potentiates P2X4 receptor currents (Khakh et al., 1999; Sim *et al*., 2006), increased basal synaptic transmission significantly as opposed to Control [Ivermectin (blue), control (black), Fig. 3.9C,D, $p<0.05$]. This increase was observed within 10 min of addition of Ivermectin. LCCG, a group II mGluR agonist (10µM) was added at the end of the experiment to ensure the authenticity of mossy fibers (Rebola *et al*., 2008).

We then investigated the possible involvement of P2X4 receptor in the modulation of frequency facilitation and paired pulse facilitation. As shown, prior blockade of the P2X4 receptor before the addition of LPS-treated microglia prevented the decrease in frequency facilitation (378.7±28.03, n=9, Fig 3.9F). However it failed to inhibit the decrease seen in paired pulse facilitation (242.3±4.55, n=9 Fig3.9G). Moreover, use of 5-BDBD on slice alone, itself caused reduction in paired pulse facilitation (227.5±19.49, n=6, Fig 3.9G), and also when in presence of non-treated microglia cells (212±39.66, n=6, Fig 3.9G).

Because P2X4 receptors are also expressed by endogenous microglia (Toulme and Khakh, 2012) but also by added N9 microglia cells (Xiang and Burnstock, 2005), we had to investigate if the effect of 5-BDBD

was due to P2X4 receptors at the Mf-CA3 synapse or expressed by N9 microglia cells added on the slice. To do so, we treated slices obtained from P2X4 receptor KO mice with LPS activated N9 cells. As shown in Fig. 3.9H, the decrease of frequency facilitation induced by LPS activated N9 cells was blunted in slices obtained from P2X4 receptor KO mice (337.1±23.04, n=6, as opposed to LPS-microglia, Fig.3.9H). Furthermore just as observed with the antagonist of P2X4 receptor, the knockout also failed the inhibit the reduction in paired pulse facilitation (264.7±40.68, n=6 as opposed to LPS-microglia, Fig 3.9I)

Last we wanted to validate the expression of P2X4 receptors at Mf-CA3 synapse as the electrophysiological data strongly suggested. For this purpose, we used a specific antibody raised against P2X4 receptors. As shown in Fig. 3.9J, the hippocampal mossy fiber tract displayed a strong immunostaining for P2X4 receptors (green) which was absent in slices obtained from P2X4 receptor KO mice. (Figures on the next page)

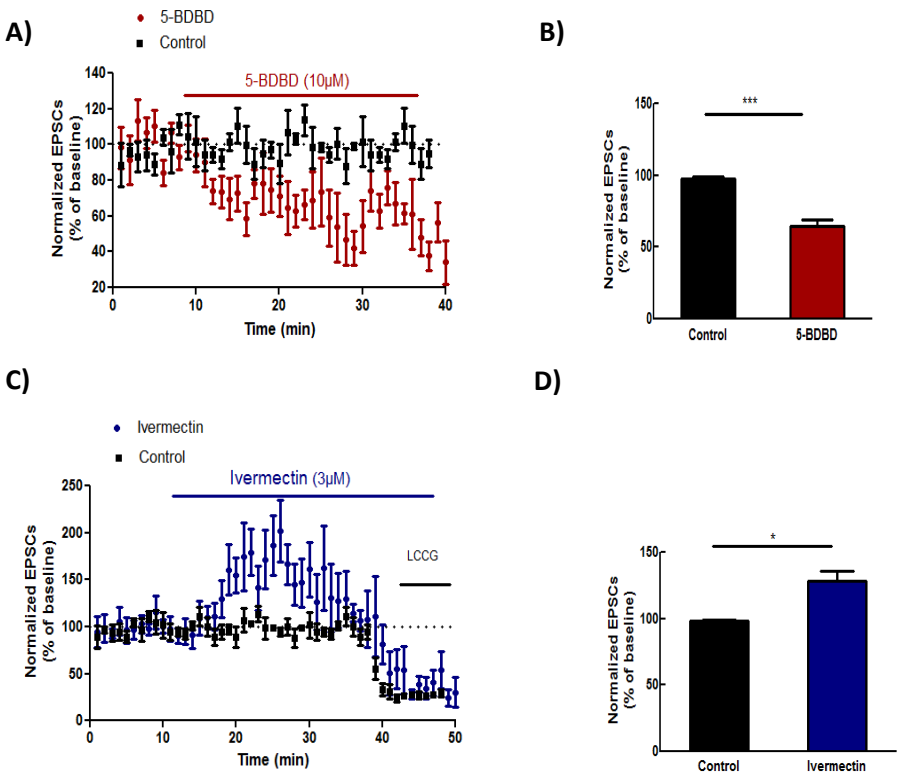


Figure 3.9 P2X4 Receptor regulates Mf-CA3 synaptic transmission and short term plasticity. A) Summary plot of the effect of 5-BDBD (P2X4R antagonist) on synaptic basal transmission. 5-BDBD (10 μM,) induced a decrease of synaptic transmission, within 5 min, measured by the amplitude of excitatory post synaptic currents (EPSCs) **B)**.Graph representation of the significant difference in basal transmission levels between control conditions and us of 5-BDBD (Control 97.96±1.3, 5-BDBD 64.7±4.5) n=9 for both conditions **C)** Summary plot of the effect of ivermectin (P2X4 potentiator, 3 μM,(n=6) on basal synaptic transmission. Ivermectin increased basal synaptic transmission within 10 minutes of addition which was later abolished by the LCCG, indicating the stimulation to be of mossy fibers**. D)** Graph representation o f the differences in basal transmission levels between Ivermectin and Control conditions (Control 97.96±1.3, Ivermectin 128.3±7.3). Values represented are SEM *p<0.05, ***p>0.0005 unpaired t test

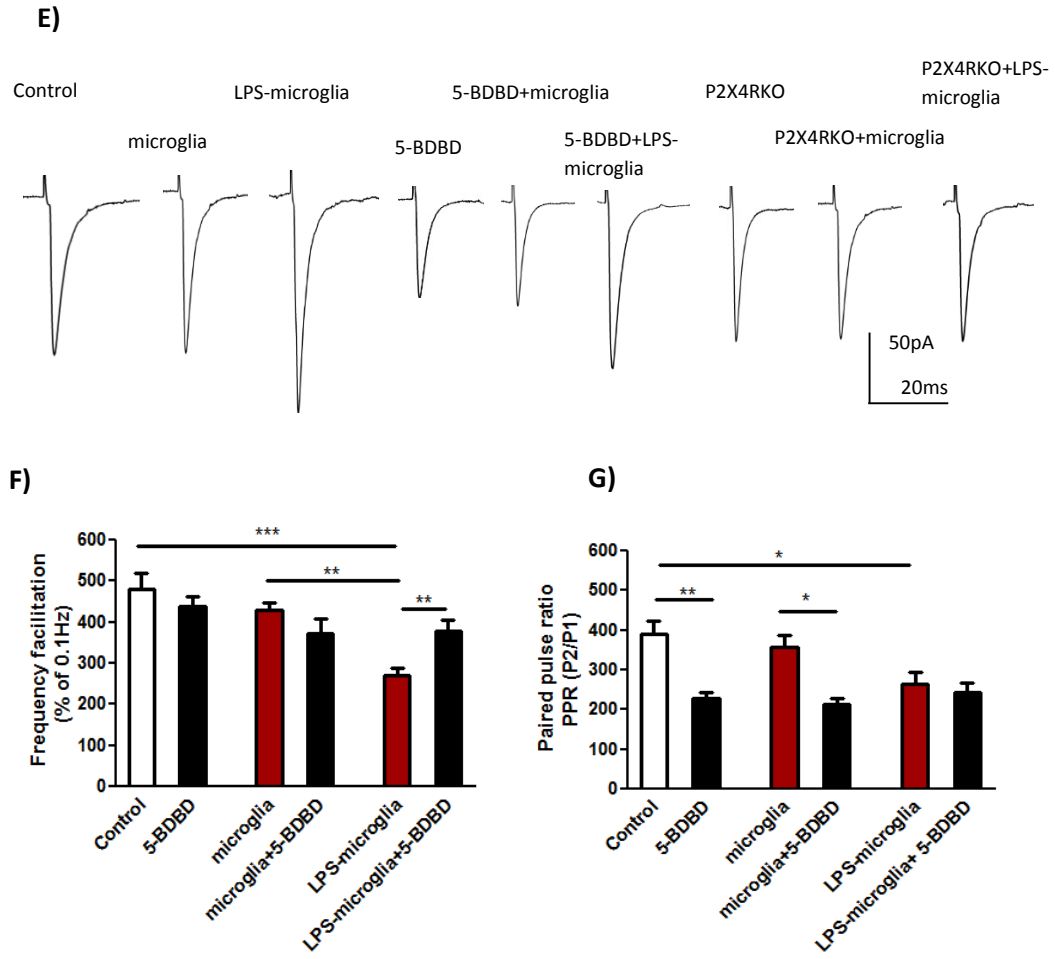


Figure 3.9 P2X4 Receptor regulates Mf-CA3 synaptic transmission and short term plasticity(Continued). E) Average representative traces of EPSCs triggered at a low frequency of stimulation (0.1Hz). **F)** Ratio of the frequency facilitation observed in different conditions. Blockade of P2X4 receptor by 5-BDBD inhibits the effect of LPS-activated microglia on frequency facilitation (Control 482.0±38.74, microglia 429.8±18.38, LPS-microglia 280.9±17.78, 5-BDBD 438.34±24.59, 5-BDBD+microglia 373.7±35.03, 5-BDBD+LPS-microglia 378.07±28.03). **G)** Graph representation of paired pulse facilitation observed in different conditions. Blockade of P2X4 R has no effect on the reduction of paired pulse, while on its own causes reduced paired pulse facilitation (Control 391.7±31.82, microglia 357.1±34.89, LPS-microglia 277.0±30.54, 5-BDBD 227.5±16.49, 5-BDBD+microglia 212.0±17.74, 5-BDBD, 242.3±24.55) Values represented are SEM *p<0.05,**p<0.005,***p<0.0001 from one way anova, Newman Keuls M.C.T

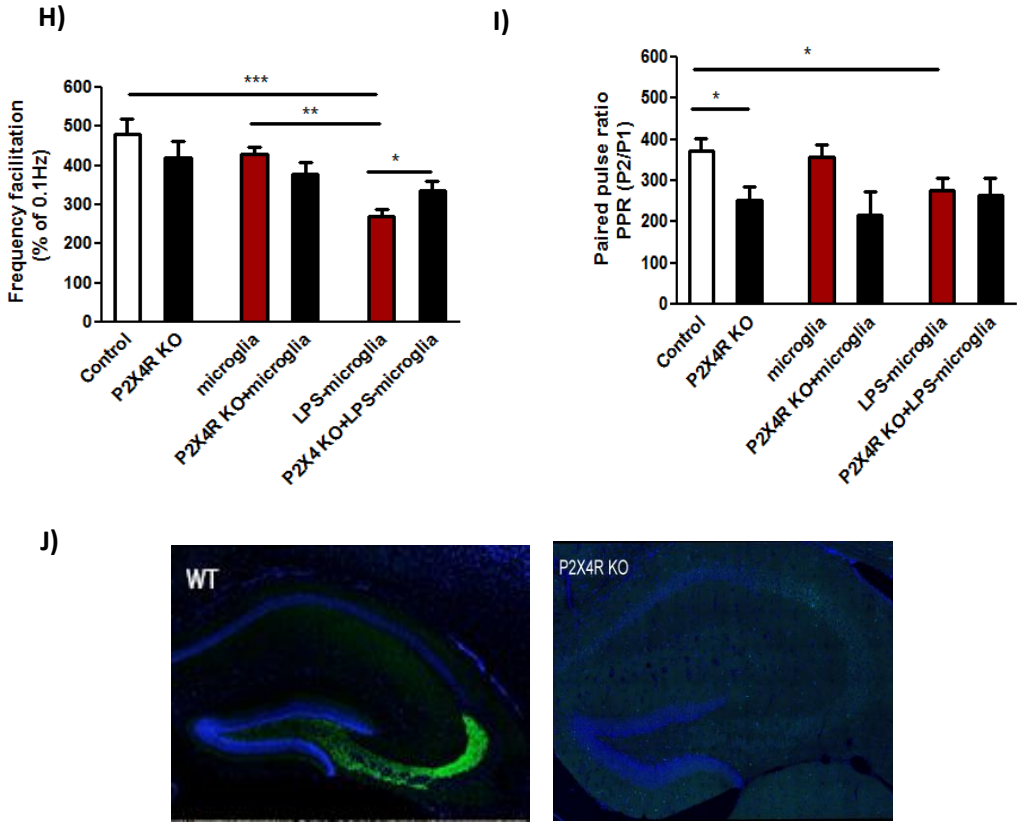


Figure 3.9 P2X4 Receptor regulates Mf-CA3 synaptic transmission and short term plasticity (Continued). H) Graph representing the effect of genetic deletion of P2X4 receptor (P2X4 KO) on LPS-activated microglia decrease of frequency facilitation (Control 482.0±38.74, microglia 429.8±18.38, LPS-microglia 280.9±17.78, P2X4R KO 419.1±42.70, P2X4 KO+microglia 377.±30.70, P2X4 KO+LPS-microglia 337.1±23.04). **I)** Ratio of the paired pulse facilitation observed between control and P2X4 KO in presence and absence of non-treated and LPS treated microglia cells (Control 391.7±31.82, microglia 357.1±34.89, LPS-microglia 277.0±30.54, P2X4 KO 253.0±31.72, P2X4 KO+microglia 216.5±56.9, P2X4 KO+LPS-microglia 264.7±40.68). Values represented in all graphs are SEM *p<0.05, **p<0.005,***p<0.001 from one way anova, Neumann Keuls M.C.T. **J)** The hippocampal mossy fibers exhibit a strong immune-staining for the $P2X_4$ receptor (green) in wild type mouse (left), DAPI staining for nuclei is shown in blue, while seen to be absent in slice from P2X4 R KO mouse (right).

3.10. LPS-microglia derived ATP regulates synaptic transmission

According to the results so far, ATP was the main candidate for the resulting decrease in frequency facilitation. So to further validate its role in observed synaptic change, two pharmacological strategies were employed, namely apyrase and ARL67156. Apyrase is an ATP degrading enzyme (George *et al.*, 2015) which is widely used to decrease the amount of extracellular ATP, while ARL67156 is an ecto-nucleotidase (NTPDase1) inhibitor which blocks the degradation of extracellular ATP (Rebola *et al.,* 2008). By increasing the rate of extracellular ATP catabolism through apyrase, and inhibiting endogenous extracellular ATP conversion into its metabolites (through ARL67156), the amount of extracellular ATP was increased and decreased respectively, to study its effect independently, on basal synaptic transmission and short term synaptic plasticity.

As shown in Figure 3.10A, addition of apyrase (10U/ml) decreased basal synaptic transmission at low frequency (0.1Hz) within 5 minutes of its addition. This decrease was found to be significant ($p<0.0005$) on being compared with control (Fig. 3.10B). Furthermore, consistent with our hypothesis, LPS-microglia induced decrease in frequency facilitation was totally abrogated (392.9±24.43, n=4, $p>0.005$, as opposed to LPS-microglia) in the presence of apyrase (Fig. 3.10F). However, the reduction in paired pulse observed in LPS-microglia conditions was also abolished in the presence of apyrase (466.3±8.61, n=4, $p<0.05$, as opposed to LPS-microglia; Fig 3.10G).

On the other hand, ARL67156 was used in control slices to examine if increased levels endogenous ATP in control conditions, could mimic the alterations induced by LPS-microglia derived ATP. As shown in Fig. 3.10 C,D, use of ARL67156 (100µM), caused a slight increase, although non-significant, in the basal transmission levels recorded at 0.1Hz, an effect which was seen to be abolished in the presence of 5-BDBD (10 µM). The addition of ARL67156 (100µM) on control slices (no microglia; LPS or otherwise) mimicked the response observed on slices (183.5±28.65, n=4, $p<0.05$; Fig 3.10H, as opposed to control) with LPS-microglia with respect to frequency facilitation (Fig. 3.10H). These data are consistent with ATP being the key molecule. Additionally, prior blockade of P2X4 receptor with 5-BDBD prevented ARL67156 induced (LPS-microglia like) decrease in frequency facilitation (502.7±107.5, n=3, $p<0.05$; Fig 3.10 H, as opposed to ARL67156) observed with ARL67156, further supporting a modulatory role of P2X4 receptor in synaptic transmission. On the opposite, ARL6716 did not affect paired pulse facilitation (323.7±71.47, n=3, Fig 3.10 I, as opposed to control). In addition, prior blockade of P2X4 receptor with 5-BDBD had no further effect on paired pulse facilitation strengthening the lack of involvement of this receptor in this short term plasticity.

In summary, removal of extracellular ATP abrogated the LPS-microglia induced decrease in frequency facilitation while increased endogenous ATP mimicked it.

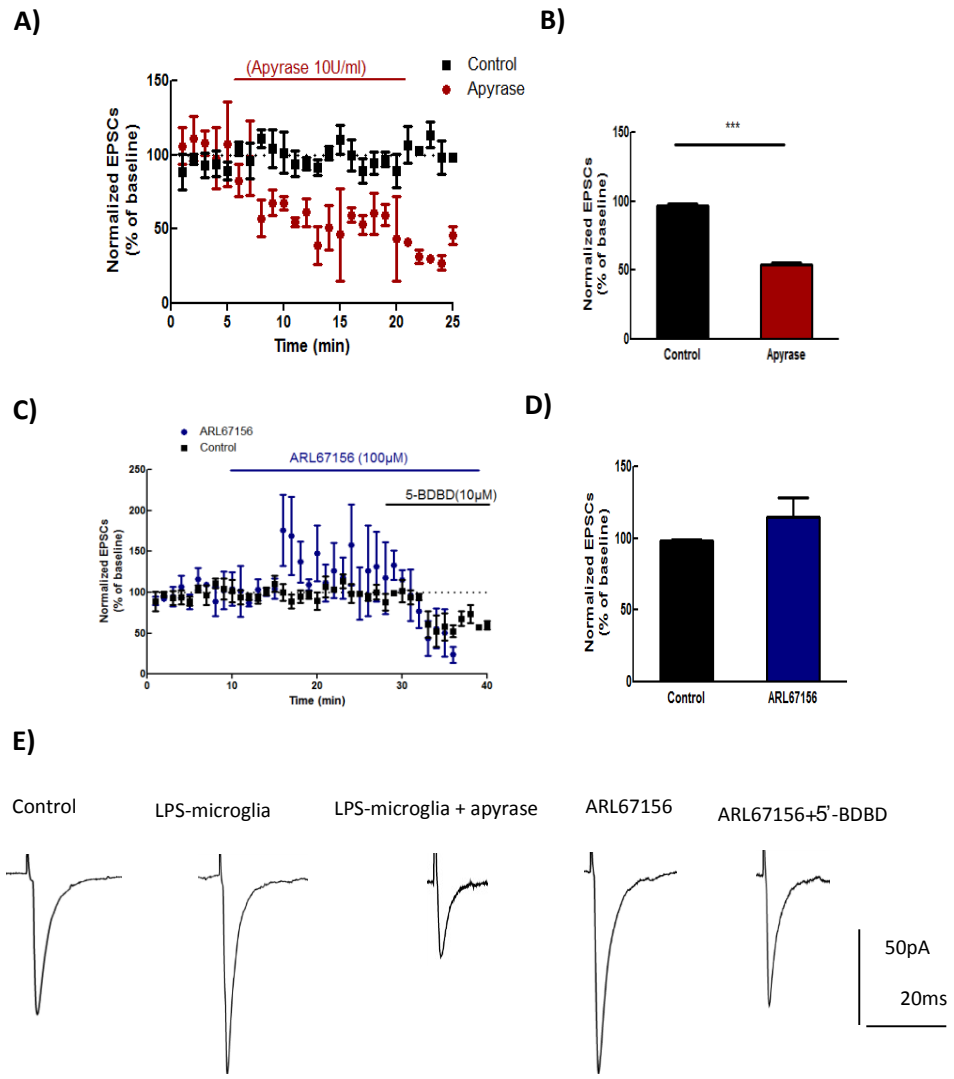


Fig 3.10 LPS-microglia derived ATP regulates synaptic transmission: A) Summary plot of the effect of apyrase (ATP degrading enzyme) on synaptic basal transmission. Apyrase (10 U/ml)(n=4) resulted in a decrease of basal synaptic transmission, within minutes, measured by the amplitude of excitatory post synaptic currents (EPSCs).B) bar graph representation of the basal transmission levels of Control and Apyrase seen in A, (Control 97.96±1.3, Apyrase 54±1.3) **C)** Summary plot of the effect of ARL67156 (10 μM) (Ecto-5' nucleotidase inhibitor) on basal synaptic activity. ARL67156 slightly increased synaptic basal transmission, within 10 minutes of its addition, which was later diminished by the addition of 5-BDBD (P2X4 R antagonist) **D)** Bar graph representation of the basal transmission levels between Control and ARL67156(n=4) seen in C (Control 97.96±1.3, ARL67156 114.6±13.46). Values represented are SEM ***p<0.0001 by unpaired t test. **E)** Average representative traces of EPSCs triggered at a low frequency of stimulation (0.1Hz, under various conditions).

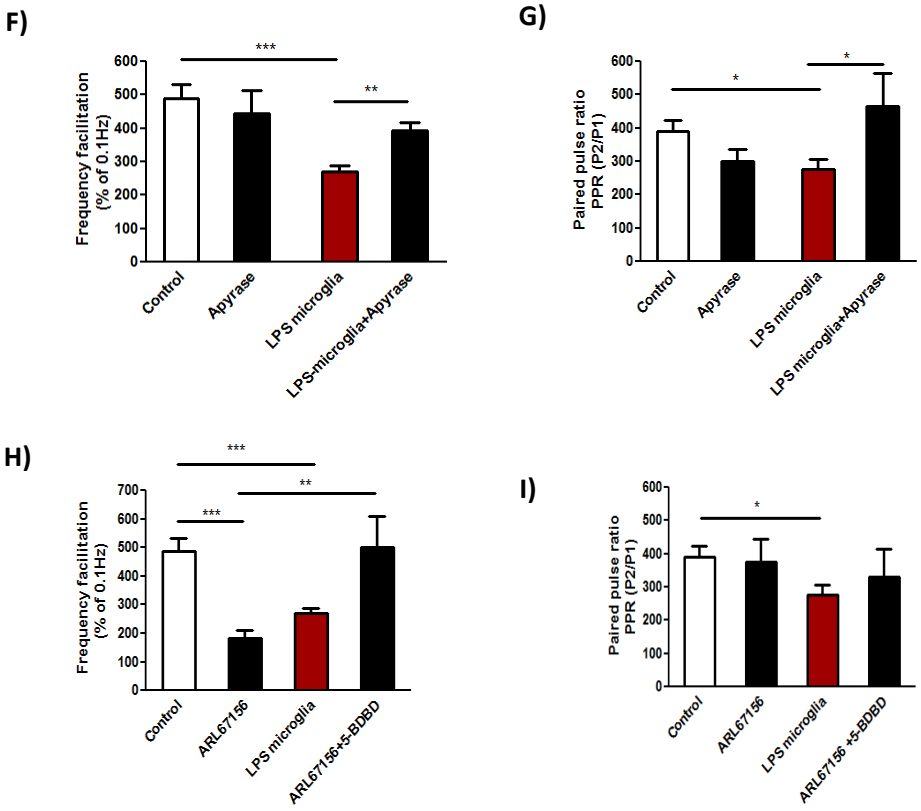


Fig 3.10 LPS-microglia derived ATP regulates synaptic transmission (Continued).F) Ratio of the frequency facilitation observed in different conditions. Addition of apyrase abolished the effect of LPS-activated microglia on frequency facilitation (Control 482.0±38.74, LPS-microglia 280.9±17.78, apyrase + LPS-microglia 392.9±24.43, n=4 all conditions). **G)** Graph representation of paired pulse facilitation observed in presence of apyrase. Apyrase abolished the reduction of the paired pulse ratio induced by LPS-microglia (Control 391.7±31.82, LPS-microglia 277.0±30.54, apyrase + LPS-microglia, 466.3±8.61, n=4 all conditions**). H)** Graph representing the effect of ecto-5'nucleotidase inhibitor (ARL67156, 100 µM) on frequency facilitation. Addition of ARL67156, mimics LPS-microglia induced decreased in frequency facilitation (Control 482.0±38.74, LPS-microglia 280.9±17.78, ARL67156 183.5±28.6, ARL67156 + 5-BDBD 502.7±107.5, n=3 all conditions). **I)** Graph representing the effect of ecto-5'nucleotidase inhibitor (ARL67156, 100 µM) on paired pulse ratio. Increasing endogenous ATP has no effect on paired pulse facilitation (Control 391.7±31.82, LPS-microglia 277.0±30.54, ARL6715 6323.7±71.47, ARL67156 + 5-BDBD 331.8±81.98, n=3 all conditions) Values represented in all graphs are SEM *p<0.05, **p<0.005, ***p<0.001 from one way anova, Neumann Keuls M.C.T.

3.11. Adenosine is involved in LPS-microglia mediated regulation of paired pulse facilitation.

Since our data strongly suggest that ATP is the key molecule responsible for the changes in frequency facilitation, its end product, that is adenosine, could also probably influence short term synaptic plasticity. Rebola *et al* (2008) showed the expression of A_{2A} receptor on CA3 neurons while A_1 receptor was expressed on the presynaptic side on mossy fibers. As LPS treated microglia reduced paired pulse facilitation, which is known to be a presynaptic phenomenon (Schulz *et al.*, 1994), we decided to determine if A_1 receptor was involved in this phenomenon. To do so, we used DPCPX (A_1 receptor antagonist) and CCPA (A_1 receptor agonist). Whereas, DPCPX and CCPA increased and decreased basal synaptic transmission respectively at the Mf-CA3 synapse, both had no effect on the frequency facilitation (Rebola *et al.*, 2008)

We first studied the effects of DPCPX and CCPA on frequency facilitation. DPCPX alone did not produce any significant changes in frequency facilitation when compared to control (426.3±86.71, n=9 Fig 3.11B), supportive to the study done by Rebola *et al*, (2008), nor did it in the presence of unstimulated microglia (367.33±41.17, n=9, compared to non-treated microglia control, Fig 3.11 B) or LPS-microglia cells (249.9±38.58, n=9, compared to LPS-microglia control, Fig. 3.11 B).

However surprisingly, addition of DPCPX was able to prevent LPS treated microglia induced change in paired pulse facilitation (326.4±17.10, n=9, $p>0.05$, as opposed to LPS-microglia; Fig 3.11 C), while, no significant change was observed in the presence of non-treated microglia (332.9±9.21, n=9, as opposed to non-treated microglia Fig 3.11C) and slice alone (326.4±17.70, n=9, compared to control Fig 3.11 C).

Addition of A_1 receptor agonist, CCPA (229.8±42.11, n=7, as opposed to LPS-microglia, Fig 3.11 D) also have no effect on LPS treated microglia induced decrease in frequency facilitation. Conversely, the A_1 receptor agonist did reduce paired pulse facilitation when added on slice alone (268.8±38, n=7, $p>0.05$, opposed to control, Fig 3.11 E). A slight decrease is also seen in presence of non-treated microglia (270.1±25.44, n=7, opposed to non-treated control counterpart Fig. 3.11E) albeit not significant, and, also having no effect in the LPS treated microglia induced decrease (258.9±38.69, n=7, compared to LPS-microglia condition, Fig. 3.11E) indicating a possible role of adenosine in the paired pulse facilitation.

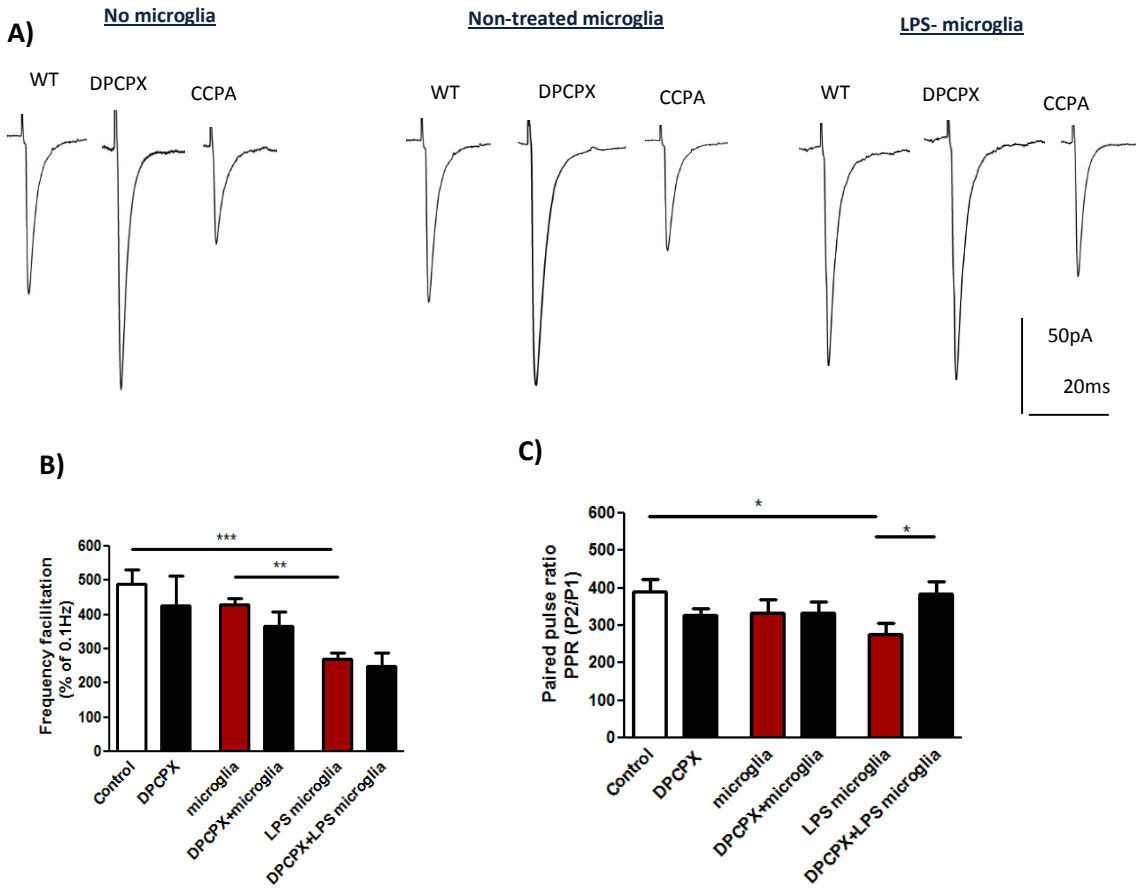


Fig 3.11 Adenosine is involved in LPS-microglia mediated regulation of paired pulse facilitation: A) Average representative traces of EPSC's triggered at a low frequency of stimulation (0.1Hz). **B)** Ratio of the frequency facilitation observed in different conditions. Blockade of A1 receptor by DPCPX has no effect in LPS-microglia induced change in frequency facilitation (Control 482.0±38.74, microglia 429.8±18.38, LPS-microglia 280.9±17.78, DPCPX 326.4±17.70 DPCPX+microglia 367.33±41.17, DPCPX+LPS-microglia 249.9±38.5, n=9 for all conditions. **C)** Graph representation of paired pulse facilitation observed in different conditions. Blockade of A1 inhibits the reduction of paired pulse facilitation by LPS-microglia (Control 391.7±31.82, microglia 357.1±34.89, LPS-microglia 277.0±30.54, DPCPX 326.4±17.70, DPCPX+microglia 332.9±9.21, DPCPX+LPS-microglia 326.4±17.10, n=9 for all conditions) Values represented in all graphs are SEM *p<0.05, **p<0.005,***p<0.001 from one way anova, Neumann-Keuls M.C.T.

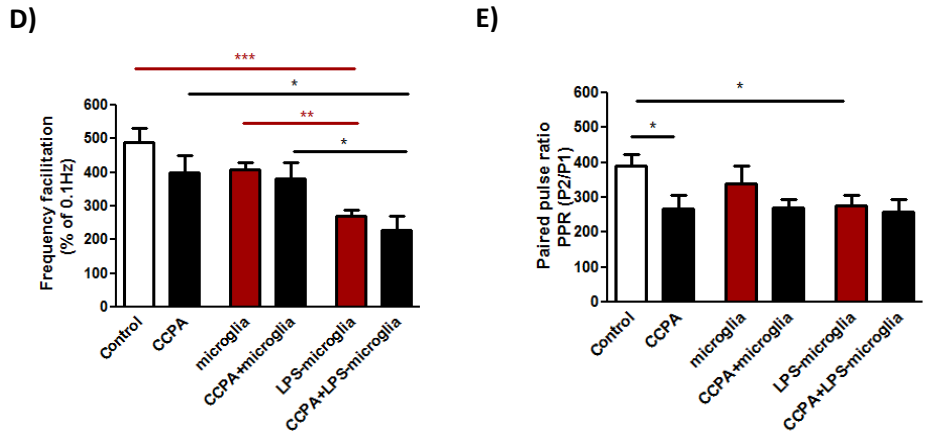


Fig 3.11 Adenosine is involved in LPS-microglia mediated regulation of paired pulse facilitation (continued) D) Graph representing the effect of A1 receptor agonist (CCPA (100µM) on LPS-activated microglia decrease of frequency facilitation (Control 482.0±38.74, microglia 429.8±18.38, LPS-microglia 280.9±17.78, CCPA 398.7±52.36, CCPA+microglia 381.7±48.17 CCPA+LPS-microglia 229.8±42.11, n=9 for all conditions). **E)** Ratio of the paired pulse facilitation observed between control and A1 R agonist in presence and absence of non-treated and LPS treated microglia cells. A1R agonist decreases paired pulse facilitation; mimicking LPS induced decrease in paired pulse facilitation (Control 391.7±31.82, microglia 357.1±34.89, LPS-microglia 277.0±30.54, CCPA 268.8±38, CCPA+microglia 270.1±25.44, CCPA+LPS-microglia 258.9±38.69, n=9 for all conditions). Values represented in all graphs are SEM *p<0.05, **p<0.005,***p<0.001 from one way anova, Neumann -Keuls M.C.T.

3.12ATP and Adenosine act independently on P2X4 receptor and A_1 receptor respectively to modulate synaptic plasticity

Our data strongly suggest that extracellular ATP acts on P2X4R and adenosine catabolized from extracellular ATP acts on A_1R to modulate frequency facilitation and paired pulse facilitation respectively. To decipher between a possible dual effect of ATP-adenosine and separate effects of ATP and adenosine, we investigated frequency facilitation and paired pulse facilitation in the presence of DPCPX (A1 receptor antagonist) on slices obtained from P2X4 receptor KO mice.

As shown in Fig 3.12 B, the LPS-microglia induced decrease in frequency facilitation occluded by the genetic deletion of P2X4 receptor was preserved in the presence of DPCPX [402±38,n=5 as opposed to 337.1±23.04(P2X4R+LPS-microglia), Fig 3.12A] confirming that the blockade of A1R has no effect on the role of P2X4R in modulating frequency facilitation.

Furthermore on analysing the paired pulse facilitation levels, it was observed that knockout of P2X4R couldn't inhibit the effect of DPCPX in modulating paired pulse facilitation [416.6±35.96, n=5 as opposed to 383.3±34.84 (DPCPX+LPS-microglia), Fig. 3.12 C]. In other words, addition of DPCPX also inhibited the decrease of paired pulse facilitation seen in P2XRKO+LPS-microglia, and LPS-microglia (Fig. 3.9 I)

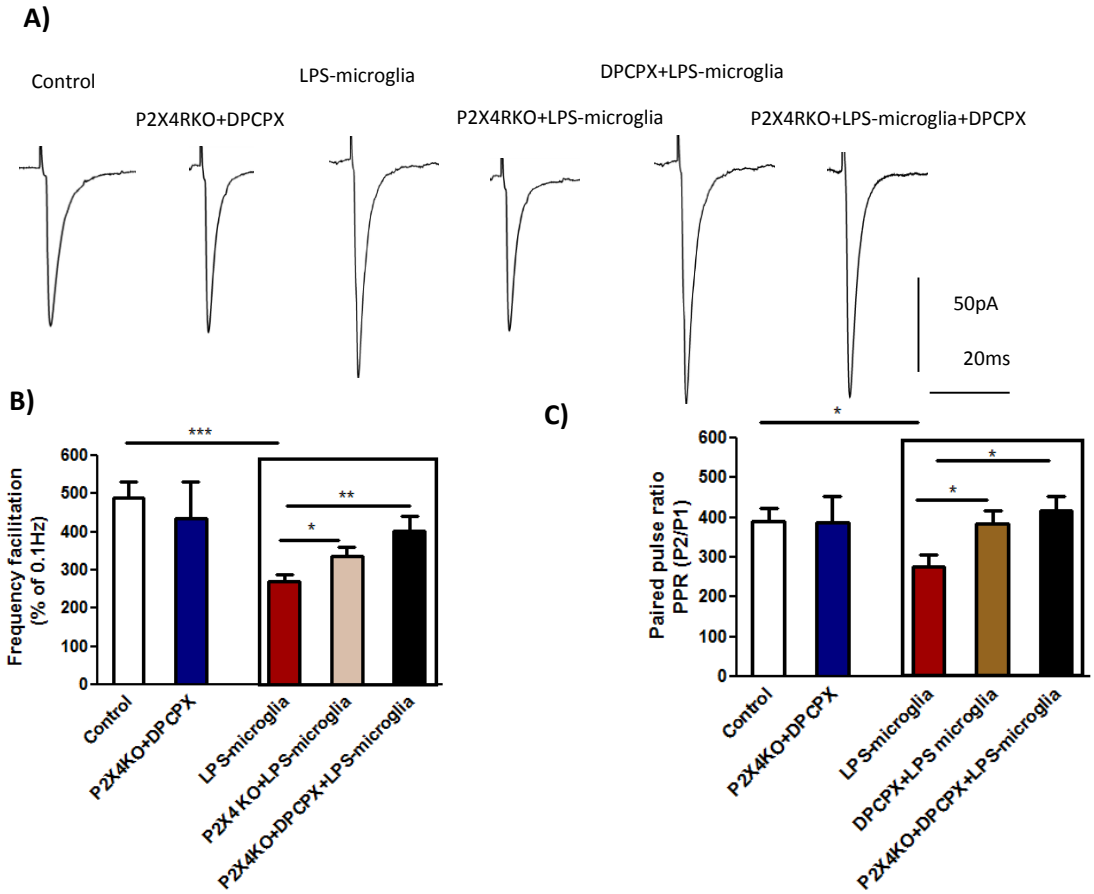


Figure 3.12. ATP and Adenosine act independently on P2X4R and A_1R. A) Average representative traces of EPSC's triggered at a low frequency of stimulation (0.1Hz). **B)** Ratio of the frequency facilitation observed on P2X4R-/- slices in presence of A1R antagonist (DPCPX 100 nM). Blockade of A1 receptor by DPCPX has no effect in the inhibitory effect of P2X4R knockout in LPS-microglia induced change in frequency facilitation (Control 482.0±38.74, LPS-microglia 280.9±17.78, P2X4RKO+DPCPX 434.4±98.62, P2X4RKO+LPS-microglia 337.1±23.04, P2X4RKO+DPCPX+LPS-microglia 402±38.09 **C)** Graph representation of paired pulse facilitation observed in P2X4R KO in presence of DPCPX. Knockout of P2X4R has no effect on the effect of DPCPX to inhibit LPS-microglia induced reduction in paired pulse facilitation (Control 391.7±31.82, LPS-microglia 277.0±30.54, DPCPX+LPS-microglia 383.3±34.84, P2X4RKO+DPCPX 388.5±66.48, P2X4RKO+DPCPX+LPS-microglia 416.6±35.96**).** Values represented in all graphs are SEM *p<0.05, **p<0.005,***p<0.001 from one way anova, Neumann Keuls M.C.T.

DISCUSSION

4 Discussion:

The discussion of the present work is divided into two parts:

4.1. Purines modulate microglial ATP release and proliferation

This part of the work showed that different danger signals in the brain cause an opposite impact on microglia proliferation through an opposite alteration of the release of ATP and of its extracellular metabolism into adenosine. Thus, it was proven that: 1) glutamate decreases, whereas LPS increases the activity of enzymes able to metabolize ATP into adenosine; 2) glutamate decreases, whereas LPS increases microglia proliferation; 3) microglia proliferation requires ATP conversion into adenosine and subsequent activation of $A_{2A}R$. Also, this places the ecto-enzymes converting extracellular ATP into adenosine at the heart of the mechanism underlying the control of microglia proliferation by environmental factors.

In the brain, extracellular levels of ATP result from a regulated balance between ATP released by different cells, and its extracellular catabolism (Kukulski *et al*., 2011). Neurons and astrocytes are considered to be the main cellular source of extracellular ATP (Burnstock *et al*., 2011), whereas microglia was mostly identified as an important responsive cellular element to ATP (Honda *et al*., 2001, Anderson *et al*., 2004; Davalos *et al*., 2005). Although it is now accepted that microglia can release ATP (Liu *et al*., 2006, Kim *et al*., 2007; Fujita *et al*., 2008; Higashi *et al*., 2011; Dou *et al*., 2012), the role of ATP release from microglia had not been clarified. The present observation that a bacterial antigen (LPS) and an internally generated danger signal (glutamate, in case of excitotoxicity) trigger opposite effects on ATP release from microglia provides the first evidence that environmental factors control ATP release. Furthermore, the additional demonstration that bolstering ATP catabolism, inhibiting ecto-nucleotidases and removing extracellular adenosine critically affect the different proliferative response of microglia to LPS and glutamate provides the first evidence of an autocrine role for ATP in microglia, with a particular

novel emphasis on the balanced conversion of extracellular ATP into adenosine (see also Färber *et al.*, 2008).

It was previously reported that LPS, in the same experimental conditions used in the present work, increased microglia proliferation, an effect dependent on the activation of adenosine A_{2A} receptors (Gomes *et al.*, 2013). This observation, and the known ability of $A_{2A}R$ to modulate several microglia functions, led us to test if $A_{2A}R$ were able to control the extracellular levels of ATP and their eventual impact upon microglial proliferation in the presence of LPS and glutamate. $A_{2A}R$ blockade in control conditions did not affect extracellular ATP levels, but it did prevent the changes of extracellular ATP levels induced by LPS and glutamate (Fig. 3.3). This normalization of the extracellular ATP levels by $A_{2A}R$ blockade allows anticipating that $A_{2A}R$ blockade should also normalize microglia proliferation in both conditions, which would be in agreement with the general ability of $A_{2A}R$ blockade to restore brain function upon different brain disorders (reviewed in Cunha, 2005; Gomes *et al.*, 2011). Interestingly, although $A_{2A}R$ blockade prevented the LPS-induced increase of microglia proliferation, it did not interfere with the microglia proliferation "arrest" caused by its exposure to glutamate. This indicates that changes in extracellular ATP levels, although temporally correlated (inverse correlation) with microglia proliferation, might not directly control microglia proliferation, implicating that this role may instead depend on ATP metabolites, namely adenosine. Indeed, different pharmacological approaches confirmed that it was the extracellular conversion of ATP into adenosine, rather than ATP itself that drives the modifications of microglia proliferation observed in the presence of LPS and glutamate, namely: 1) LPS increased microglia proliferation (Figure 3.2) 2) LPS-induced increase of microglia proliferation was prevented by the removal of endogenous extracellular adenosine, by the blockade of several steps of the extracellular metabolism of ATP into adenosine (Figure 3.5) and by $A_{2A}R$ blockade (Figure 3.3); 3) a stable ATP analogue, not degradable into metabolites (Cunha *et al.*, 1998), induced a concentration-independent "arrest" of microglia proliferation (Figure 3.4A); 4) glutamate decreased microglia

proliferation (Figure 3.2) and finally, 5) bolstering ATP catabolism (with exogenously added apyrase) in the presence of glutamate triggered an increase in proliferation (Figure 3.4B).

The physiopathological relevance of the present observations resides in the key role of purines in the control of microglia proliferation to sustain neuroinflammatory responses (Kettenmann *et al.*, 2011; Gomez-Nicola and Perry, 2014). In fact, in the region surrounding focal brain lesions where ATP levels are high, microglia cells are unable to proliferate (Wang *et al.*, 2004). Thus, the recruitment of microglia from areas far from the lesion is critical to cope with damage over time. Accordingly, it has been shown that a proliferative event precedes the migratory wave of microglia (Wang *et al.*, 2004). Therefore, the dual purinergic control of proliferation and migration is of critical importance to mount a spatio-temporally organized microglia response (Koizumi *et al.*, 2013). In this respect, it is tempting to note the key role of $A_{2A}R$, which blockade re-establishes microglia motility towards an injury site that is impaired in inflammatory conditions (Gyoneva *et al.*, 2014a,b), while simultaneously refraining microglia proliferation (Gomes *et al.*, 2013). This heralds a working hypothesis where the currently proposed ATP-derived adenosine, acting through $A_{2A}R$, would play a key switching role between proliferation and migration of microglia. Future *ex vivo* and *in vivo* studies are required to clarify if the proliferative peak preceding migration towards damaged areas also requires $A_{2A}R$ activation by ATP-derived adenosine, as suggested by our *in vitro* study. Thus, the purinergic modulation system is ideally positioned to control the context-dependent shifts in microglial-mediated inflammatory responses. Indeed a similar stimulus-specific regulation of purinergic metabolism was also observed in astrocytes (Brisevac *et al.*, 2012), bone marrow stromal cells (Costa *et al.*, 2010), macrophages (Zanin *et al.*, 2012) and nerve terminals (reviewed in Cunha *et al.*, 2001).

A final relevant finding is the functional association between ATP-derived adenosine and the activation of $A_{2A}R$. This is in notable agreement with a recent study showing that ecto-5´-nucleotidase-mediated formation of extracellular adenosine is responsible for the selective activation of striatal $A_{2A}R$ (Augusto *et al.*, 2013), prompting the suggestion that the manipulation of ecto-5´-nucleotidase activity would be a

novel strategy to regulate $A_{2A}R$ activity in physiological and pathological conditions of the brain (Augusto *et al*., 2013). These current findings linking the extracellular catabolism of ATP with $A_{2A}R$ function in microglia lends further support to this proposal and prompts the possibility that a similar rationale may be used to manipulate the purinergic control of microglia proliferation.

4.2. Purines regulate microglia-synapse interaction with respect to synaptic transmission and short term plasticity.

Though there have been many studies implicating purines and microglia independently in synaptic plasticity, there never had been a study combining both, and examining if together, they have an effect in synaptic transmission and short term plasticity. For this we decided to employ the same experimental paradigm used in the first part of this work, i.e. N9 microglial cell line. N9 microglial cells were added onto hippocampal slices as previously described (Centonze *et al*, 2009, Nistico et al., 2013).

As demonstrated in our work, the exogenous addition of LPS activated microglia on hippocampal slices induced a reduction in both frequency facilitation and paired pulse facilitation together with a slight increase in basal transmission (Fig. 3.7). Since, non-treated N9 microglia cells did not modify either the frequency facilitation or the paired pulse facilitation; we inferred that the reduction observed was due to the presence of LPS-treated microglia. We hypothesized that microglia as a result to its treatment with LPS, released some compound(s) acting on neurons and thus causing the reduction. Because of our work presented in the first part (George *et al*., 2015), we considered ATP as one of the stronger suspects. Since A_{2A} receptor antagonism blocked ATP release from microglia (Fig 3.3A, George *et al*. 2015), it was further utilized to investigate the modulatory effect of microglial A_{2A} receptor in the process. By doing so, the change in frequency facilitation was abolished (Fig. 3.8), making the case for ATP even stronger.

Although ATP has been shown to induce LTP in CA1 mouse (Fuji S, 2004) and guinea pig neurons (Yamazaki *et al*, 2003), its effects at the Mf-CA3 synapse and those of its receptors were poorly known. Therefore we decided to investigate if and how ATP through P2X receptors was modulating Mf-CA3

synaptic transmission, the chief candidate being P2X4 receptor. P2X4 receptor has been a centre of various studies with respect to synaptic transmission and plasticity. Increased surface P2X4 receptor expression was shown to significantly decrease the frequency and amplitude of GABAergic postsynaptic currents of SF-1 GFP positive neurons (Jo et al, 2011). Baxter *et al* (2011) reported that P2X4 receptor knockout mice had decreased synaptic potentiation and a reduced incorporation of NR_{2B} subunits into NMDA receptors. P2X4 receptor has been shown to modulate LTP at the CA1 neurons has previously been shown (Sim et al., 2006). Though P2X4 is expressed in the hippocampus, its expression on mossy fibers and its associated changes in synaptic transmission were unknown.

We observed that P2X4 receptor antagonist (5-BDBD) inhibited the reduction of frequency facilitation triggered by LPS-treated microglia (Fig 3.9 F) whereas it had no effect on paired pulse facilitation. This is good evidence that extracellular ATP released from LPS-treated microglia modulated frequency facilitation at Mf-CA3 synapse through neuronal P2X4 receptor. This was further corroborated with the use of P2X4 KO mice, where it was found the same effect (Fig. 3.9 H). Use of a P2X4 antagonist and potentiator (ivermectin), reduced (Fig. 3.9 A,B) and increased (Fig. 3.9 C, D) basal synaptic transmission respectively, hinting at the expression of presynaptic P2X4 receptors. Although inhibition of P2X4 receptor did not have an effect on LPS-treated microglia induced change in paired pulse facilitation, it did however on its own reduced paired pulse facilitation in control conditions (Fig. 3.9 G and I), further suggesting a presynaptic locus for the expression of the P2X4 receptor. Although exact location of the receptor would need electron microscopy experiments, we did however, using immuno-histochemical experiments, to show for the first time, a high and exclusive staining for P2X4 receptor on mossy fibers (Fig. 3.9 J). Though, as previously mentioned, P2X4 receptor has been shown to have a modulatory effect in synaptic plasticity (Sim et al., 2006, Pougnet et al., 2014) this work sheds light on its role in governing a form of short term plasticity at the Mf-CA3 synapses, a first of its kind.

To further validate the involvement of extracellular ATP in such modulation, apyrase and ARL67156 (ecto nucleotidase inhibitor) were employed. Apyrase and ARL67156 were able to inhibit and mimic

respectively LPS-treated microglia effects on synaptic transmission (Fig. 3.10 F and H). By prior addition of 5-BDBD (P2X4R antagonist) we were able to inhibit the effect of ARL67156 on frequency facilitation. Thus by degrading extracellular ATP (via LPS-microglia) we inhibited the decrease of frequency facilitation. Also through increase of endogenously released ATP (via ARL67156) we mimicked LPS-treated decrease of frequency facilitation and not paired pulse facilitation. This meant that frequenct facilitation was modulated by extracellular ATP, while paired pulse facilitation was not. Thus combining both these results we could further cement ATP's role on P2XR in frequency facilitation at CA3-mossy fibers. Furthermore, although Vroman *et al* (2014) demonstrated how extracellular ATP hydrolysis slowed presynaptic release of neurotransmitters further reducing synaptic transmission, the reduction of basal transmission observed with apyrase (Fig 3.10A,B) is another first of a kind effect described on mossy fibers.

Adenosine is an end product of ATP catabolism (Rebola et al., 2008). So to eliminate adenosine as the causative agent in the changes seen in frequency facilitation and paired pulse facilitation, we used DPCPX and CCPA, selective A_1 receptor antagonist and agonist respectively. Rebola *et al* (2008) previously showed how post synaptic adenosine receptors (A_{2A}) were necessary in NMDA associated LTP, seen at the mossy fibers. They also demonstrated the presynaptic expression of A1 receptor (that is on mossy fibers) and stated the modulatory role of A_1 receptor in basal synaptic transmission. In addition they also showed that A1 receptor was not modulating frequency facilitation at the Mf-CA3 synapse.

As shown in Fig 3.11B, addition of DPCPX on slices prior to that of LPS-activated microglia cells had no additional effect on frequency facilitation, suggesting that the effect was not due to ATP catabolised adenosine. However, it did inhibit the effect of LPS-treated microglia in paired pulse facilitation (Fig. 3.11 C), hinting at a possible role of adenosine in paired pulse facilitation. This result was further supported by the reduction of the paired pulse facilitation induced by CCPA, an A_1 receptor agonist (Fig. 3.11 E).

However since adenosine is an end product of ATP catabolism, there was a possibility of an ATP-adenosine effect in both frequency facilitation and paired pulse facilitation. So to rule out this dual effect, we took advantage of P2X4 receptor KO mice. On the one hand, the addition of DPCPX on P2X4 receptor KO slices had no additional effect on its ability to modulate LPS-microglia-resulting decrease in frequency facilitation (Fig 3.12 B), ruling out the involvement of A1 receptor in the modulation of frequency facilitation. Also, the inhibitory action of DPCPX on the paired pulse facilitation was not modified on P2X4 receptor KO slices (Fig. 3.12 C). In other words, A1 receptor and through that adenosine, has no effect in the reduction seen on frequency facilitation, and P2X4R and through that ATP has no role in paired pulse facilitation. Therefore ATP and adenosine modulate separately both frequency facilitation and paired pulse facilitation: ATP having an effect on frequency facilitation through P2X4 receptor and ATP converted adenosine modulating paired pulse facilitation through presynaptic A_1 receptors.

In summary, this study shows that **1)** the nature of the "danger" signal (in the present study, glutamate and LPS) differently modifies purinergic metabolism (in particular ATP conversion into adenosine), subsequently shaping microglia proliferation. Furthermore with the use of this model, **2)** the role of 'presynaptic' P2X4 receptor and A_1 receptor in modulating Mf-CA3 short term plasticity was shown for the first time. Moreover just like how ATP-adenosine regulation governed microglial extracellular ATP levels and proliferation (Part 1), its role in possibly regulating both frequency facilitation and paired pulse facilitation, independently of each other (Part 2), is also shown. This prompts a new concept that the extracellular purinergic metabolism, by balancing ATP and adenosine signalling through microglial cells, regulates some of its functions along with influencing synaptic transmission to form an eventual microglia-neuron crosstalk.

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