Expression, Function and Targeting of the ATP-gated P2X7 receptor in epilepsy

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Signed Alba JIMENEZ PACHECO

Student Number 12140236

Date
# Table of contents

1  Candidate Thesis Declaration  
2  Table of Contents  
10  Abstract  
13  Publications & Manuscripts  
15  Acknowledgements  
17  List of Figures  
19  List of Tables  
20  Abbreviations

**Chapter I – Introduction**

1.1 Epilepsy  
1.2 Seizures  
1.3 *Status epilepticus*  
  1.3.1 Outcomes of *status epilepticus*  
  1.3.2 Etiologies of *status epilepticus*  
  1.3.3 Pharmacotherapy of *status epilepticus*  
1.4 Neurotransmission and epilepsy  
  1.4.1 Glutamate  
  1.4.2 γ-aminobutyric acid (GABA)  
1.5 Molecular pathways regulating seizure-induced cell death  
  1.5.1 Apoptosis pathway  
  1.5.2 Necrosis pathway  
1.6 Gliosis  
  1.6.1 Astrocytes  
  1.6.1 Microglia  
1.7 Inflammation  
1.8 Epileptogenesis  
1.9 Symptomatic Focal Epilepsies (Temporal Lobe Epilepsy)  
1.10 Modelling Temporal Lobe Epilepsy  

25  
27  
29  
30  
31  
31  
32  
32  
36  
37  
37  
38  
39  
39  
42  
43  
46  
48  
49
1.10.1 Traumatic brain injury model 49
1.10.2 Kindling model 51
1.10.3 Status epilepticus model 52
  1.10.3.1 Electrical stimulation 52
  1.10.3.2 Chemical stimulation 52
    1.10.3.2.1 Pilocarpine model 52
    1.10.3.2.2 Kainic acid model 53
      1.10.3.2.2.1 Systemic kainic acid 54
      1.10.3.2.2.2 Intra-cerebral kainic acid 55
        1.10.3.2.2.2.1 Intra-hippocampal kainic acid 55
        1.10.3.2.2.2.2 Intra-amygdala kainic acid 56
1.11 Seizure termination / AED treatments 58
1.12 Alternative anti-epileptic approaches 60
1.13 ATP as a neurotransmitter 61
  1.13.1 Discovery 61
  1.13.2 ATP release mechanisms 62
1.14 ATP-gated P2X receptors 62
  1.14.1 ATP involvement in seizures 70
1.15 P2X7 receptor 70
  1.15.1 P2X7 receptor activation 71
  1.15.2 P2X7 receptor expression and localization 72
  1.15.3 P2X7 receptor functions 73
1.16 P2X7 receptor expression in diseases of the CNS 74
  1.16.1 P2X7R in epilepsy 75
1.17 Pharmacology of the P2X7R receptor 77
  1.17.1 Functional evidence of P2X7R in epilepsy/SE 78
1.18 Molecular mechanisms regulating P2X7R expression 81
  1.18.1 Transcription factors 81
  1.18.2 DNA methylation 82
1.19 Hypothesis and objectives of the thesis 84

Chapter II – Materials and Methods
2.1 Materials 85

2.2 Methods 86

2.2.1 In-vivo model of experimentally induced status epilepticus and developed chronic epilepsy 86

2.2.1.1 Animals and husbandry 86
2.2.1.2 Surgery 87
2.2.1.3 Status Epilepticus induction 87
2.2.1.4 Drug injections 88

2.2.2 Behaviour analysis of seizures 90

2.2.3 EEG analysis of long term telemetry recording 90

2.2.4 Open field behaviour test 91

2.2.5 Brain microdissection 92

2.2.6 Western blotting 93

2.2.6.1 Protein extraction 93
2.2.6.2 Protein quantification 94
2.2.6.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) 94
2.2.6.4 Protein Transfer 94
2.2.6.5 Immunoblotting 95
2.2.6.6 Densitometry/quantification of protein signal 96

2.2.7 mRNA expression analysis 96

2.2.7.1 Homogenisation 96
2.2.7.2 Phase Separation 96
2.2.7.3 RNA precipitation 96
2.2.7.4 RNA purification 97
2.2.7.5 Re-dissolving the mRNA 97

2.2.8 cDNA Synthesis and qPCR 97

2.2.8.1 cDNA synthesis (Reverse transcriptase PCR) 97
2.2.8.1.1 DNA degradation 97
2.2.8.1.2 Reverse transcription to cDNA 98
2.2.8.1.3 qPCR (SYBR Green method) 98
2.2.9 Histological studies

2.2.9.1 Fresh frozen tissue

2.2.9.1.1 Brain removal and tissue processing

2.2.9.1.2 Fluoro-Jade B staining (FJB)

2.2.9.2 Immunohistochemistry and Immunofluorescence

2.2.9.2.1 Free floating sections

2.2.9.2.2 Immunofluorescence microscopy

2.2.9.2.3 DAB (3, 3′-diaminobenzidine)

2.2.10 Synaptosome preparation for epifluorescence microscopy or immunostaining

2.2.10.1 Isolation of functional synaptosomes

2.2.11 Calcium microfluorimetric analysis

2.2.12 Synaptosome isolation for Western blot analysis

2.2.13 Electrophysiological recordings

2.2.13.1 Tissue preparation

2.2.13.2 BeATP and A437980 administration and P2X7R current measurements

2.2.14 DNA methylation analysis of the P2rx7 promoter region

2.2.14 Extraction of DNA, RNA and Protein

2.2.14.1 Tissue homogenisation

2.2.14.2 Genomic DNA purification

2.2.14.3 Total RNA purification

2.2.14.4 Bisulfite conversion of DNA

2.2.14.5 PCR amplification of the P2rx7 promoter region

2.2.14.6 Purification of PCR products

2.2.14.7 Sequencing of PCR products

2.2.15 Data analysis

Chapter III – Neocortical expression of the P2X7 receptor after status epilepticus and in epilepsy

3.1 Introduction
3.1.1 Neurophysiology of the cerebral cortex 117
3.1.2 Neocortex and Temporal lobe epilepsy 118
3.1.3 P2X7 receptor localization and expression after SE 119
3.1.4 P2X7R activation after seizures 120
3.1.5 P2X7R antagonists as anti-seizure agents 120

3.2 Chapter objectives 121

3.3 Results 122

3.3.1 Intra-amygdala KA-induced status epilepticus produces injury to the neocortex 122
3.3.2 P2X7R protein levels increase in the neocortex after SE and in epilepsy 122
3.3.3 Increased neuronal transcription of P2X7R in the neocortex after SE 124
3.3.4 Increased neocortical expression of the P2X7R in experimental epilepsy 129
3.3.5 P2X7R inhibition decreases convulsive behaviour during status epilepticus and protects the neocortex against seizure-induced damage 132

3.4 Discussion 135

3.4.1 Increased expression of P2X7R in the neocortex after SE and epilepsy 135
3.4.2 Increased neuronal P2X7R transcription in the neocortex after SE and neuronal and microglial P2X7R transcription in epilepsy 136
3.4.3 P2X7R inhibition decreases convulsive behaviour during status epilepticus and protects the neocortex against damage 138

Chapter IV – Expression and transcriptional control of the P2X7 receptor in the hippocampus in epilepsy

4.1 Introduction 142

4.1.1 Active inflammatory process during epilepsy 142
4.1.2 P2X receptors expression in epilepsy 143
4.1.3 Control of P2X7R expression: Involvement of epigenetics? 144
4.1.4 Sp1 as a novel transcription factor regulating P2rx7 transcript levels 145

4.2 Chapter objectives 146

4.3 Results 147

4.3.1 Intra-amygdala KA-induced SE as a model to study epilepsy 147
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.2</td>
<td>Evidence of epileptic seizures involving the ipsilateral hippocampus</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>in the intra-amygdala KA-induced SE mouse model</td>
<td></td>
</tr>
<tr>
<td>4.3.3</td>
<td>Increased molecular markers of inflammation in the hippocampus</td>
<td>151</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Up-regulation of P2X receptors in the hippocampus in epilepsy</td>
<td>154</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Increased P2X7R expression in hippocampal subfields in epilepsy</td>
<td>157</td>
</tr>
<tr>
<td>4.3.6</td>
<td>P2rx7 transcriptional regulation by the SP1 transcription factor</td>
<td>159</td>
</tr>
<tr>
<td>4.3.7</td>
<td>Effect of SP1 inhibitor on P2X7R expression in epilepsy</td>
<td>162</td>
</tr>
<tr>
<td>4.3.8</td>
<td>Analysis of the methylation status of the P2rx7 promoter in epilepsy</td>
<td>164</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>168</td>
</tr>
<tr>
<td>4.4.1</td>
<td>Recruitment of the hippocampus during spontaneous epileptic seizures in intra-amygdala KA-induced SE mouse model</td>
<td>168</td>
</tr>
<tr>
<td>4.4.2</td>
<td>Inflammation in the hippocampus in the intra-amygdala KA model of epilepsy in mice</td>
<td>170</td>
</tr>
<tr>
<td>4.4.3</td>
<td>P2X receptors in epilepsy</td>
<td>172</td>
</tr>
<tr>
<td>4.4.4</td>
<td>P2X7 receptor expression in the hippocampus in epilepsy</td>
<td>173</td>
</tr>
<tr>
<td>4.4.5</td>
<td>P2X7R expression control by the Sp1 transcription factor in epilepsy</td>
<td>174</td>
</tr>
<tr>
<td>4.4.6</td>
<td>Epigenetic control of P2X7R expression in epilepsy</td>
<td>175</td>
</tr>
</tbody>
</table>

**Chapter V - Functional evidence of P2X7R involvement in epilepsy**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>177</td>
</tr>
<tr>
<td>5.1.1</td>
<td>Aberrant expression and function of neurotransmitters</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>receptors in epilepsy</td>
<td></td>
</tr>
<tr>
<td>5.1.2</td>
<td>P2X7R expression and localization in the hippocampus in epilepsy</td>
<td>178</td>
</tr>
<tr>
<td>5.2</td>
<td>Chapter objectives</td>
<td>179</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>180</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Increased P2rx7 induction in the hippocampus in epilepsy</td>
<td>180</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Increased neuronal transcription of the P2X7R in the hippocampus in epilepsy</td>
<td>186</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Increased microglia transcription of P2X7R in the hippocampus</td>
<td>190</td>
</tr>
<tr>
<td>5.3.4</td>
<td>Increased activated microglia in the hippocampus in epilepsy</td>
<td>190</td>
</tr>
<tr>
<td>5.3.5</td>
<td>Absence of P2rx7 transcription in astrocytes in the hippocampus</td>
<td>191</td>
</tr>
<tr>
<td>5.3.6</td>
<td>Enhanced functional responses of the P2X7 receptor in EGFP-positive hippocampal cells in EGFP-P2rx7 reporter mice</td>
<td>196</td>
</tr>
</tbody>
</table>
5.3.7 Increased P2X7R localization in synaptosomes in the hippocampus in epilepsy

5.3.8 Increased functional responses of P2X7R in synaptosomes in epilepsy

5.4 Discussion

5.4.1 Increased P2X7R expression in the hippocampus in epilepsy

5.4.2 Enhanced functional responses of P2X7R in GFP-positive hippocampal cells in EGFP-\(P2rx7\) reporter mice

5.4.3 Increased P2X7R levels in synaptosomes in epilepsy

Chapter VI - Inhibition of P2X7R reduces spontaneous seizures and has pathology-modifying effects in epilepsy

6.1 Introduction

6.1.1 What are the functional implications of increased P2X7R in the hippocampus in epilepsy?

6.1.2 JNJ-47965567; a potent brain-available P2X7R inhibitor in epilepsy

6.1.3 Impact of P2X7R inhibition on the underlying pathology in epilepsy

6.2 Chapter objectives

6.3 Results

6.3.1 Suitability of the antagonist P2X7R A438079 for long-term epilepsy studies

6.3.2 JNJ-47965567 reduces spontaneous recurrent seizures in mice

6.3.3 JNJ-47965567 effects on animal behaviour in epilepsy

6.3.4 P2X7R inhibition reduces microglia responses in the hippocampus in epilepsy

6.3.5 Effect of JNJ-47965567 on astrogliosis in epilepsy

6.4 Discussion

6.4.1 Limitations of A438079 for the treatment of epilepsy

6.4.2 Lack of effect of JNJ treatment on behaviour in epilepsy

6.4.3 P2X7R inhibition leads to a diminution of microglia and astrocyte proliferation in the hippocampus in epilepsy

Chapter VII – General discussion

7.1 General discussion

7.1.1 Neocortical expression of P2X7R in epilepsy
7.1.2 The role of P2X7R in epilepsy 241
7.1.3 Lack of effective AED for epilepsy treatment 244
7.2 Future work 247
7.3 Conclusions 249

References 250
Abstract

Epilepsy is a common and disabling neurologic disease characterized by the occurrence of recurrent, unprovoked seizures, which affects about 1 % of the population. Seizures are the result of abnormal electrical activity in the brain and can be profoundly disabling, affecting work, social activity and increasing risk of harm. In at least 30 - 40 % of patients, treatment is inadequate, with patients continuing to experience seizures; therefore, there is an important need to develop new anti-epileptogenic drugs. To this end, the application of animal models of evoked seizure, such as focal-onset status epilepticus models, allows for researchers to characterize the early molecular events associated with seizure-associated pathology and the onset of epilepsy.

ATP is an essential transmitter/co-transmitter in neuron function and pathophysiology and has recently emerged as a potential contributor to prolonged seizures (status epilepticus) through the activation of the purinergic ionotropic P2X7 receptor (P2X7R). Prolonged and excessive temporal lobe seizures following experimental focal-onset status epilepticus are associated with seizure-induced neuronal cell death and sclerosis of the hippocampus, and herald the emergence of spontaneous seizures. Increased P2X7 receptor expression has been reported in the hippocampus and cortex after prolonged seizures in animal models and P2X7 receptor antagonists reduced seizure-induced damage to these brain regions. The body of work presented here in this thesis arose from the presumption that P2X7R is expressed and functional in epilepsy and its activation contributes to the development of seizures in status epilepticus and spontaneous seizures during epilepsy. The P2X7R inhibition was explored for its possible contribution in the development of spontaneous seizures and its potential disease-modifying effects in epilepsy.
First, increased P2X7R expression has been reported in the hippocampus after *status epilepticus*, and P2X7R antagonists reduced seizure-induced damage to this brain region (Engel et al., 2012b). However, *status epilepticus* also produces damage to the neocortex. The present study was designed to characterize P2X7R in the neocortex and assess effects of P2X7R antagonists on cortical injury after *status epilepticus*. *Status epilepticus* resulted in increased P2X7R protein levels in the neocortex of mice and also neocortical P2X7R levels were observed elevated in epilepsy. Immunohistochemistry determined that neurons were the major cell population transcribing the P2X7R in the neocortex within the first 8 h after *status epilepticus*. In epileptic mice, P2X7R up-regulation occurred in microglia as well as in neurons. Pretreatment of mice with the specific P2X7R inhibitor A-438079 reduced electrographic and clinical seizure severity during *status epilepticus* and reduced seizure-induced neuronal death in the neocortex.

Second, P2X7R expression and transcriptional control was assessed in the hippocampus in epilepsy, including functional evidence of the receptor. Using GFP-expressing P2X7R reporter mice we localized the increased expression mainly to neuron and microglia in epileptic animals. Isolated synaptosomes were also analysed and an increased presence of the P2X7R was also observed in the hippocampus in epilepsy. Moreover, functional studies including patch clamp technique and microfluorometric Ca$^{2+}$ measurements showed increased functional activation of P2X7R in epileptic mice. These findings support a role for P2X7R in the pathophysiology of chronic epilepsy and suggest P2X7 receptor antagonists may have therapeutic effects against recurrent seizures or progression of disease pathology.

Third, the contribution of brain inflammation in the development of spontaneous seizures has gained support over the years, with P2X7R being a potential target due to its contribution to the processing and release of IL-1β (Vezzani et al., 2011). This study assessed the effect of a novel P2X7R antagonist, JNJ-47965567, on the number of spontaneous seizures in epilepsy, which was unexplored until now, in the kainic acid-induced *status epilepticus* model. The P2X7R antagonist succeeded
to reduce the number of spontaneous seizures in epileptic mice. Additionally, microglia and astrocytes reactivity was observed to be reduced in the hippocampus of mice treated with the P2X7R antagonist. These results suggest possible disease-modifying effects through the P2X7R inhibition. Continuous administration of P2X7 receptor antagonists to epileptic mice will validate the effectiveness of P2X7 receptor inhibitors in stopping seizure occurrence and the progression of disease pathology.

In summary, this thesis shows evidence of the involvement of the P2X7R activation in the recurrence of spontaneous seizures in epilepsy and supports the development of new P2X7R inhibitors as a novel therapeutic target with disease-modifying effects to treat and prevent epilepsy.
Publications a & Manuscripts

Published


- **Bmf up-regulation through the AMP-activated protein kinase pathway may protect the brain from seizure-induced cell death.** Moran C; Sanz-Rodriguez A; Jimenez-Pacheco A; Martinez-Villareal J; McKiernan R; Jimenez-Mateos E; Woods I; Prehn J; Henshall DC and Engel T. *Cell Death Dis*. 2013 Apr 25;4:e606.

- **CHOP regulates the p53-MDM2 axis and is required for neuronal survival after seizures.** Engel T; Sanz Rodriguez A; Jimenez-Mateos E; Concannon C; Jimenez-Pacheco A; Moran C; Mesuret, G; Delanty N; Farrell M; O’Brien D; Prehn J; Lucas, Jose J.; Henshall DC. *Brain*. 2013 Feb; 136 (Pt 2):577-92. Epub 2013 Jan 29.


In submission


- **microRNA targeting of the P2X7 purinoceptor opposes a contralateral epileptogenic focus in the hippocampus**. Jimenez-Mateos E., Arribas-Blazquez M., Sanz-Rodriguez A., Concannon C., Olivos-Ore L., Reschke C., Mooney C., Lugara E., Morgan J., Langa E., Jimenez-Pacheco A., Mesuret G., Boison D., Miras-Portugal T.,...

- **P2X7 receptor inhibition as a potential therapeutical target in epilepsy.** A. Jimenez Pacheco, M. Arribas Blazquez, A. Sanz, LA. Olivos-Oré, A. Rodríguez Artalejo, MT. Miras-Portugal, M.Diaz-Hernandez, D.C.Henshall, T. Engel
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List of Figures

pg 29 1.1 GABA<sub>A</sub> receptors internalization during SE
pg 32 1.2. Mechanism of action of the benzodiazepines
pg 57 1.3 Intra-amygdala kainic acid mouse model
pg 62 1.4 ATP degradation into its different breakdown products
pg 63 1.5 The P2 purinergic receptor family
pg 66 1.6 3D crystal structure of P2X receptors
pg 67 1.7 Gating properties of P2XRs
pg 79 1.8 Structures of prototypical and novel antagonists of P2x7 receptors
pg 88 2.1 Surgical procedure for intra-amygdala injection of KA in mice
pg 90 2.2 Telemetry recordings for epilepsy monitoring
pg 91 2.3 Open field arena
pg 92 2.4 Microdissection of hippocampal subfields
pg 103 2.5 Synaptosome preparation for microfluorimetric analysis
pg 122 3.1 Cell death in neocortex after status epilepticus
pg 124 3.2 Increased P2X7R levels in the neocortex 24 h after status epilepticus induction and in chronic epilepsy
pg 125 3.3 Constitutive P2X7R transcription in cortical layers II and III
pg 126 3.4 Neuronal P2rx7 transcript after SE in the neocortex in mice
pg 127 3.5 Lack of microglia or astrocyte induction of P2X7R after SE in the neocortex of mice
pg 129 3.6 Increased neocortical GFP induction in neurons in chronic epilepsy
pg 130 3.7 Neocortical induction of GFP in microglia in EGFP-P2rx7 reporter mice with epilepsy
pg 133 3.8. P2X7R inhibition decreases seizure severity during SE and protects against neocortical damage
pg 147 4.1 Electrographic seizures and hippocampal injury following seizures evoked by intra-amygdala KA injection in mice
pg 149 4.2 Recruitment of the ipsilateral hippocampus during epileptic seizures and presence of astrogliosis and hippocampal sclerosis
pg 151 4.3 Increased molecular markers of inflammation in the hippocampus in epilepsy
pg 152 4.4 Increased microglia expression in the hippocampus in epilepsy
pg 154 4.5 Altered P2XR transcript levels in the hippocampus in epilepsy
4.6 Altered hippocampal P2XR levels in epilepsy

4.7 Increased P2rx7 mRNA and P2X7R protein levels in hippocampal subfields in epilepsy

4.8 Upregulation of Specific protein 1 (Sp1) transcription factor after SE

4.9 Increased Sp1 expression in hippocampal subfields in epilepsy

4.10 The SP1 inhibitor, Mithramycin A, reduces P2X7R expression in vivo

4.11 DNA methylation of the P2rx7 promoter region in naïve hippocampus

4.12 DNA methylation of the P2rx7 promoter in epilepsy

5.1 P2X7R induction within the brain in normal brain

5.2 Increased P2X7R inductions within the brain in epilepsy

5.3 Expression of P2X7R in the DG hippocampal subfield in control animals

5.4 Increased expression of P2X7R in CA1 and DG hippocampal subfields in epilepsy

5.5 Neuronal expression of P2X7R in the hippocampus of control mice

5.6 Neuronal expression of P2X7R in the hippocampus in epileptic mice

5.7 Increased neuronal induction of P2X7R in the hippocampus in epilepsy

5.8 Microglia expression of P2X7R in the hippocampus of control mice

5.9 Microglia expression of P2X7R in the hippocampus in epilepsy

5.10 Increased active microglia expressing GFP

5.11 Absence of P2rx7 transcription in astrocytes in the hippocampus in epilepsy

5.12 Enhanced functional responses to agonist stimulation in GFP-positive cells

5.13 Increased levels and functional responses of P2X7R in synaptosomes in the hippocampus in epilepsy

5.14 P2X7R measurement from functional synaptosome preparations

6.1 Effects of A438079 on SE and brain levels of A438079 after systemic injection

6.2 Experimental paradigm for test of JNJ-47965567 on epileptic seizures

6.3 Reduction in spontaneous seizure number after JNJ-47965567 administration

6.4 Spontaneous seizure profiles in vehicle-group mice during epilepsy recordings

6.5 Seizure profile in JNJ-47965567 treated mice during epilepsy recordings

6.6 Reduction in number of seizures after JNJ-47965567 treatment

6.7 Behaviour analyses before and after J&J treatment

6.8 Reduction in microglial cells in JNJ-47965567 treated animals

6.9 Reduction in microglia numbers in JNJ-47965567 treated animals
6.10 Reduction in microglia counts in JNJ-47965567 treated animals
6.11 Reduction in astrocytes in JNJ-47965567 treated animals
6.12 Reduction in astrocyte numbers in JNJ-47965567 treated animals
6.13 Reduction in astrocytes counts in JNJ-47965567 treated animals

List of tables

1.1 Main functions of astrocytes
1.2 P2X receptors localization and functions
1.3 P2X7 receptor antagonists
2.1 Drug injection dose
2.2 Racine Score System
2.3 RT-qPCR cycles
2.4 Sequences of primers for PCR amplification of bisulfite converted DNA
2.5 Chemicals and general reagents
2.6 Antibodies used in this study
2.7 Primers used in this study
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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Cyclooxygenase-2  Cox-2
Cysteinyl aspartate-specific proteinase  Caspase
Daltons  Da
Dentate gyrus  DG
Diacyl glycerol  DAG
Diazepam  DZP
Disease-modifying antirheumatic drug  DMARD
DNA methyltransferase  DNMT
Ecto-5'-nucleotidase  Ecto-5'-NT
Electroencephalography  EEG
Enhanced green fluorescent protein  EGFP
Electroconvulsive shock  ECS
Endoplasmic reticulum  ER
Equilibrium potential  E_{Cl}
FBJ osteosarcoma oncogene  C-fos
Fluoro-Jade B  FJB
Glucose transporter 1  GLUT1
Glutamic acid decarboxylase  GAD
Glutamine synthetase enzyme  GS
Granule cell dispersion  GCD
Green fluorescent protein  GFP
Hippocampal sclerosis  HS
High amplitude and high frequency spikes  HAHFs
High amplitude and low frequency spikes HALFs
Histone deacetylase HDAC
Hours h
Injury INJ
Intra-amygdala i.a.
Intra-cerebroventricular i.c.v.
Intraperitoneal i.p.
Interleukin-converting enzyme ICE
International league against epilepsy ILAE
Interferons IFNs
Interleukins ILs
Interleukin-6 IL-6
Interleukin-1β IL-1β
IL-1 receptor antagonist IL-1Ra
IL-1 receptor IL-1R1
Inositol trisphosphate IP3
Lipopolysaccharide LPS
Kainic acid KA
Kainate receptors KARs
Ketogenic diet KD
Lateral/neocortical temporal lobe epilepsy LTLE
Lateral fluid-percussion LFP
Long-term potentiation LTP
Long-term depression LTD
Lorazepam LZ
Mammalian target of rapamycin mTOR
Mesial temporal lobe epilepsy MTLE
microRNA miRNA
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<td>Term</td>
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<td>Reactive oxygen species</td>
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<td>Reverse transcription</td>
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<td>Vascular endothelial growth factors</td>
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<td>Voltage-gated potassium channel antibody</td>
<td>VGKC-complex antibodies</td>
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Chapter I - Introduction

1.1 Epilepsy

Epilepsy is a chronic disease of the nervous system characterized by recurrent, unprovoked seizures resulting from episodes of unusually excessive electrical activity in the brain. Recent studies estimate that epilepsy affects ~ 1 % of the general population, about 65 million people worldwide (Leonardi and Ustun, 2002; Thurman et al., 2011), among which approximately 35,000 cases are present in Ireland (Linehan et al., 2010).

Epilepsy can affect people at all stages of life, from neonates to elderly people. Over 40 distinct clinical subtypes have been described depending on the differentials of aetiology, seizure semiology and treatment response. Therefore, a classification is needed to encode the different causes and manifestations, the different seizure types and also the diverse identifiable syndromes (Duncan et al., 2006). In 1989, the International League Against Epilepsy (ILAE) created a classification system for seizures, epilepsies and epilepsy syndromes (1989). Later, in 2005, the ILAE formed a Task Force to re-formulate the operational definition of epilepsy with the aim of aiding in the clinical diagnosis. Finally, in 2014, the ILAE Executive Committee adopted a new epilepsy definition where epilepsy is a disease of the brain defined by one of the following conditions (Fisher et al., 2014): 1) suffering a minimum of 2 unprovoked (or reflex) seizures more than 24 h apart, 2) after 2 unprovoked seizures within a day, suffer another unprovoked (or reflex) seizure in the next 10 years, 3) an epilepsy syndrome has been diagnosed (Fisher et al., 2014). This classification system allows and facilitates the communication between clinicians and patients and sets the concepts for the research community. The previous system used to organise epilepsies in two main groups, the first one, according to the cause of epilepsy (aetiology) (idiopathic, symptomatic, provoked and
cryptogenic) and the second one, according to the localisation of the seizures within the brain (general or focal). The new ILAE classification organises epilepsies according to a number of underlying aetiologies:

1) Genetic. Known or presumed genetic defect(s) are the direct cause of epilepsy and seizures are the consequence of the disorder. The genetic alteration could be at a chromosomal or molecular level. Genetic alterations in the SCN1A gene coding for the neuronal voltage-gated sodium ion channel, is associated with a spectrum of seizure-related disorders in human, ranging from a relatively milder form of febrile seizures to a more severe epileptic condition (Parihar and Ganesh, 2013). The meaning of genetic is not restricted to inherited mutations as de novo mutations are not uncommon. Genetic epilepsy may also be influenced by environmental factors.

2) Structural. This consists of structural brain abnormalities that underlie the epilepsy or increase the risks of developing epilepsy. Acquired disorders including stroke, trauma or infection might cause structural lesions as well as different malformations of cortical development (although they are considered a disorder themselves) (Leventer et al., 2008).

3) Metabolic. This encompasses metabolic abnormalities associated with a high risk of developing epilepsy. Metabolic abnormalities are understood as a separate disorder interposed between the genetic defect and the epilepsy, for example GLUT1 deficiency (Wang et al., 1993).

4) Immune. These epilepsies have a particular immune-mediated aetiology. Examples include voltage-gated potassium channel antibody (VGKC-complex antibodies) (LGI1 or CASPR2). In these patients, auto-antibodies developed against the voltage gated potassium channels, bind to these proteins forming a complex, causing limbic encephalitis (Irani and Vincent, 2011).

5) Infectious. This is the most frequent aetiology in underdeveloped countries. Acute symptomatic seizures and epilepsy can be provoked by infections in the CNS. Examples of infectious agents include HIV, cerebral malaria, tuberculosis, cerebral toxoplasmosis, among others (Singhi, 2011).
6) Unknown. This includes those epilepsies where the nature of the underlying cause is unclear. It might be related to a genetic defect or a still unknown disorder (Fisher et al., 2014).

The ILAE also includes a classification of epilepsies based on the characteristics of seizure types most common in a given patient. 1) Generalized seizures, which begin in a particular region of the brain and are rapidly distributed through the networks of both hemispheres. The involvement of the entire cortex is not always necessary; sometimes the bilateral network could just affect cortical and subcortical areas. 2) Focal seizures, which refer to those where the origin of the seizure is limited to only one hemisphere. The ictal onset is consistent between seizures and might originate in subcortical structures (Panayiotopoulos, 2011).

1.2 Seizures

Normal brain function depends on finely balanced networks of excitatory and inhibitory neurons. Seizures are a sudden surge of electrical activity within the brain caused by hyper-synchronous discharges of a group of neurons. Seizures can manifest in many different forms, ranging from short-lived, localized events with few clinical signs through to long-lasting, bilateral electro-clinical events producing tonic-clonic convulsions of the body. Seizures normally have a beginning, middle and end, although the three stages may not be clearly divisible from each other (Schachter, 2014). Seizures are not a disease in themselves but are, instead, a symptom of an underlying imbalance between excitation and inhibition in the brain (Schachter, 2009).

Most seizures last a few seconds or minutes at most. A variety of factors have been proposed for how seizures normally (eventually) stop. This includes dysregulation of intracellular ion-activated potassium currents which, after the depolarization of the membrane, permit intracellular influx of calcium and sodium, allowing membrane repolarisation. In postsynaptic membranes, intracellular calcium levels increase as a result of the activation by glutamate of the calcium-dependent membrane
potassium channels, allowing potassium efflux and membrane hyperpolarisation, resulting in the cessation of neuronal firing. As well as calcium, a sodium current could activate a sodium-dependent potassium efflux that decreases neuronal excitability by hyperpolarizing the membrane (Alger and Nicoll, 1980). Energy failure is also a mechanism of seizure termination; higher intracellular levels of adenosine triphosphate (ATP) are used in a permanent neuronal activation to restore the ion gradients through the membrane. When intracellular levels of ATP decrease, due to different mechanisms such as the presence of ATP-gated potassium channels (K_{ATP}) or an impairment of mitochondrial function, some neurons suffer an activity reduction (Lado and Moshe, 2008). Neuronal excitation induces a decrease of pH levels within the brain, which activates the acid-sensing ion channels (ASICs), especially ASIC1a. An acidosis state terminates epileptic activity, so ASIC1a activation facilitates the function of inhibitory interneurons contributing to seizure termination (Kweon and Suh, 2013). In addition, another mechanism that might contribute as well to seizures termination due to intracellular acidification is the reduction of excitability by decreasing the gap junction function (de Curtis et al., 1998).

Some neuromodulators have also been suggested to function in seizure termination, including endocannabinoids, adenosine and neuropeptide Y (NPY) (Lado and Moshe, 2008). Seizures increase extracellular levels of adenosine, which provides inhibition of neuronal activity by inhibiting the presynaptic release of neurotransmitters (Boison, 2005). Four receptor subtypes are known for adenosine (A_1, A_2A, A_2B, A_3); however it is the type A_1 that is most abundant in the hippocampus and neocortex (Cunha, 2005). Numerous lines of evidence support a role for adenosine in seizure termination. For example, increased seizure susceptibility is observed by blocking adenosine receptors after electrical status epilepticus induction in rats (Boison, 2005). Also, homozygous A_1 receptor knockout mice show higher seizure susceptibility (Kochanek et al., 2006). Adenosine kinase (ADK) is the cytosolic protein that regulates the levels of adenosine, converting it to adenosine monophosphate (AMP); thereby, reducing adenosine levels (Boison,
Kainic acid (KA)-induced status epilepticus in mice provokes an increase of ADK as a result of hippocampal gliosis (Fedele et al., 2005). Experiments using an ADK inhibitor that blocked the removal of adenosine from the extracellular space resulted in a decrease in seizures in mice (Gouder et al., 2004).

NPY is another modulator of neuronal activity. NPY is widely expressed in the CNS especially in hippocampal mossy fibers and GABAergic inhibitory interneurons, where it can modulate the activation of hippocampal pyramidal neurons (Colmers and El Bahh, 2003). As with adenosine, there is much animal data supporting an anticonvulsant role for NPY and inhibitory effects on seizures (Vezzani et al., 1999a). For example, seizures are strongly inhibited in mice overexpressing NPY (Vezzani et al., 2002b; Lin et al., 2006).

1.3 Status epilepticus

Status epilepticus (SE) is defined as a continuous or intermittent seizure lasting at least 5 min without full recovery of consciousness between seizures (Wasterlain and Chen, 2008). While most seizures are self-limiting, and finish within a few minutes, during SE the normal mechanisms of seizure termination fail. The mechanisms underlying this transition are not fully understood. During this change from single seizures to self-sustaining SE, γ-amino butyric acid (GABA_A) receptors become internalized by endocytosis. GABA receptors then become inactive, leading to decreased GABA inhibitory effects. At the same time, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors and N-methyl-d-aspartate (NMDA) receptor subunits are externalized and recruited onto the synaptic membrane, increasing excitatory postsynaptic responses (Figure 1.1) (Mazarati and Wasterlain, 1999; Wasterlain and Chen, 2008; Jacob et al., 2009). This process enhances excitability and contributes to the maintenance of SE (Wasterlain et al., 2002; Goodkin et al., 2005). However, other mechanisms are certainly also involved in the generation of SE (Goldberg and Coulter, 2013). The Cl− equilibrium potential (E_Cl) might also change with high intracellular Cl− concentrations; meaning any
release of GABA has either no effect or the effect is actually de-polarizing (Staley et al., 1996).

**Figure 1.1 GABA\textsubscript{A} receptor internalization during SE**

Figure 1.1 showing the internalization of GABA\textsubscript{A} receptors leading from single seizures to SE. After repeated seizures, massive GABA release occurs and the GABA\textsubscript{A} receptors present in the synaptic membrane are internalized by endocytosis into clathrin-coated vesicles. These vesicles evolve into endosomes inactivating the receptors, thereby preventing inhibitory effects of GABA receptors on seizures (Adapted from Wasterlain and Chen, 2008).

**1.3.1 Outcomes of status epilepticus**

Repeated seizures and SE cause complex pathophysiological changes in the brain, leading to serious neurological complications (Wasterlain et al., 1993). These changes include alteration of ion channel expression and neurotransmitter release, neuroinflammation, gliosis, neuronal loss and up-regulation of excitatory
components or down-regulation of inhibitory circuits (Goodkin et al., 2005; Wasterlain and Chen, 2008).

SE is a clinical emergency associated with a risk of profound widespread brain damage to the hippocampus. In particular, cell loss in the cornu ammonis subfield 1 (CA1) and subfield 3 (CA3) of the hippocampus and other extrahippocampal regions has been observed after SE. SE may also lead to serious neurological complications such as hippocampal sclerosis, epilepsy, encephalopathy and focal neurological deficits (Wasterlain et al., 1993; Fountain, 2000; Fujikawa et al., 2000).

### 1.3.2 Etiologies of status epilepticus

The main etiologies for SE in adults have been reported to be symptomatic cases of an underlying structural brain injury or a toxic or metabolic problem (Riggs, 2002; Beghi et al., 2010). If the origin of the underlying medical or structural cause was provoked in a short time period, SE is referred to as acute symptomatic. SE episodes may arise due to non-compliance with antiepileptic drugs (AEDs), a previous lesion (remote symptomatic) and an overlying stressor associated with metabolism, infection, or pharmacology such as a medication change or uremia (Barry and Hauser, 1993). Patients with a recognized diagnosis of focal or generalized idiopathic epilepsy are more susceptible to develop SE (Hesdorffer et al., 1998). Febrile SE is the most common etiology in children, representing one third of the cases diagnosed (Singh et al., 2010). While in adults, approximately half of the cases are acute symptomatic etiologies, followed by remote symptomatic in identified epilepsy patients (DeLorenzo et al., 1996; Hesdorffer et al., 1998).

### 1.3.3 Pharmacotherapy of status epilepticus

The main pharmacotherapy for SE is GABA-potentiating anticonvulsants such as benzodiazepines (BDZ), with lorazepam being the most common drug and first choice when a seizure starts (Lowenstein, 2005; Wasterlain and Chen, 2008).
Benzodiazepines bind to the GABA<sub>A</sub> receptors and boost inhibitory neurotransmission by increasing the frequency of opening Cl⁻ channels (Figure 1.2) (Macdonald and Barker, 1978; Riss et al., 2008). In many cases, repeated dosing of anticonvulsant therapy is required to terminate SE, due to the reduction in sensitivity to anticonvulsants over time, with seizures becoming pharmacorefractory in 10 – 40 % of SE patients (Lowenstein, 2005). Clinically, if after 30 min from benzodiazepine administration seizures keep occurring and the patient has developed pharmacoresistance to GABAergic drugs, benzodiazepines are combined with another drug that acts at a different site. AEDs are often administered as second-line therapy, including phenytoin, valproate and levetiracetam (Wasterlain and Chen, 2008). If second line treatment fails, anaesthetic agents such as propofol are used. Using anaesthetics has shown positive effects in clinical studies such as early seizure cessation and reducing neuronal injury and pharmacoresistance; however, anaesthetic use might lead to serious complications including respiratory collapse (Riviello et al., 2013).

Thus, we still lack appropriate treatment options for SE to effectively stop seizures and protect the brain from seizure-induced damage. Novel treatments not associated or depending exclusively on GABA receptors have to be identified in order to find systems that do not de-sensitize over time.

1.4 Neurotransmission and epilepsy

1.4.1 Glutamate

The most abundant excitatory neurotransmitter found in the CNS is glutamate. In general, excitatory glutamatergic neurotransmission is responsible for most neuronal communication. Glutamate mediates its effects via two types of receptors: ionotrophic receptors and metabotropic receptors. The mechanisms of these two sub-classes of receptors differ as follows; the activation of metabotropic
Figure 1.2 Mechanism of action of the benzodiazepines

Benzodiazepines bind to the gamma sub-unit of the GABA_A receptor. Their binding causes an allosteric (structural) modification of the receptor that results in an increase in GABA_A receptor activity. BDZs do not substitute for GABA, but increase the frequency of channel opening events which leads to an increase in Cl^- conductance and inhibition of the action potential.

Receptors leads to a series of intracellular events that eventually can also open ion channels or releases intracellular Ca^{2+}, although this pathway requires secondary messengers. The activation of the ionotropic receptors directly causes channel opening, allowing the flowthrough of ions such as K^+, Na^+ and Cl^-.

Glutamate has been reported to activate three classes of ionotropic receptors, AMPA, NMDA and kainate receptors (KARs). The most abundant ionotropic receptors in the CNS are the AMPA receptors. They were first cloned in 1989 by the Hollmann group (Hollmann et al., 1989). AMPA receptors are composed of four subunits classified as GluA1 – GluA4 (known as GluR1-4), all encoded by separated genes (Lodge, 2009).

The AMPA receptor is formed by four domains; a large extracellular one, responsible for trafficking and proper receptor assembly, a ligand binding domain containing the agonist recognizing sites (i.e. glutamate), a transmembrane domain, which forms the ion channel and last, a cytoplasmic carboxy-terminal domain, that allows the proper positioning of the receptor at the synapse (Ahmed et al., 2011).
When an excitatory neuron terminal releases glutamate it diffuses across the synaptic space, binds to AMPA receptors in the postsynaptic membrane, allows depolarizing Na⁺ entry and thereby mediates fast excitatory neurotransmission (Traynelis et al., 2010).

NMDA receptors are hetero-tetrameric transmembrane proteins formed by two NR1 domains and two NR2 (NR2A-D) or two NR3 (NR3A-B) domains (Dingledine et al., 1999). Unlike AMPA receptors, NMDA receptors are calcium-permeable. This property expands the cellular effects of NMDA receptors activation beyond depolarization to include various downstream pathways mediated by a rise in intracellular Ca²⁺. Thus, NMDA receptor activation exerts numerous effects on the cells including synaptic transmission, dendritic remodelling, synaptic and neuronal maturation and plasticity (Paoletti and Neyton, 2007). NMDA receptors are mainly present postsynaptically, however, it has been shown that they might also have a presynaptical role (Xie et al., 1992; Engelman and MacDermott, 2004). Recent studies showed how presynaptical NMDARs (preNMDARs) can modulate neurotransmitter release and short term plasticity in many brain regions, including the neocortex. Nevertheless, the cellular mechanisms by which preNMDARs can affect neurotransmitter release are largely unknown (Lonchamp et al., 2012; Kunz et al., 2013; Park et al., 2014).

The third class of ionotropic glutamate receptor found widely expressed in the CNS is the Kainate receptors. Molecular cloning of KARs revealed five subtypes divided into two families, based on sequence homology and agonist binding properties. GluK1, GluK2 and GluK3 (known as GluR₅₋₇), and GluK4 and GluK5 (named as KA1 and KA2) with subunits having a 40 % similarity between families (Huettner, 2003). KA receptors have been shown to play key roles in neurotransmitter release regulation and synapse formation, contributing also to excitatory postsynaptic currents in many regions in the CNS (Bortolotto et al., 1999). Pyramidal neurons of the CA3 subfield are particularly enriched in KA receptors (Werner et al., 1991). High affinity KARs facilitate the neurotransmission by regulating glutamate release, while low affinity KARs mediate depression of synaptic activity in GABAergic
terminals (Jane et al., 2009). Moreover, the contribution of the postsynaptic KARs in postsynaptical potentials was also reported (Huettner, 2003). Providing evidence for their involvement in epilepsy, studies showed GluR6-deficient mice were less susceptible to seizures (Mulle et al., 1998).

The role of glutamate as an excitatory neurotransmitter was first observed around 1960, by Hayashi’s group. Dogs were administrated with glutamate into the brain grey matter to induce clonic convulsions (Watkins and Jane, 2006). Since then, the involvement of glutamate in epileptiform activity steadily increased and it was in the 1990s, when NMDA receptors were considered responsible for short synchronous bursts in neurons and the paroxysmal depolarising shifts recorded in the epileptic brain (Brady et al., 1991; Lee and Hablitz, 1991; Telfeian and Connors, 1999). The major excitatory input to the hippocampus comes from glutamatergic pathways from the entorhinal cortex, and a change in glutamate-mediated excitability may be the consequence of the pathogenesis of epileptic discharges (Carter et al., 2011). In addition, other studies tested the involvement of NMDARs in seizure-induced selective excitotoxic cell death and NMDAR antagonists were shown to potently protect hippocampal neurons against seizure damage (Meldrum, 1993). AMPA receptors are also implicated in seizures (Rogawski and Donevan, 1999). Supporting evidence was provided by an autoradiograph study, which showed an increased density of AMPA receptors in brain slices from patients with epilepsy (Hosford et al., 1991). Moreover, the blockade of AMPA receptors in amygdala slices from patients with medically intractable TLE showed an inhibition of interictal-like electrical activity (Graebenitz et al., 2011). Recently, perampanel, an AMPA receptor blocker has been approved for the treatment of epilepsy (Steinhoff, 2015).

Injection of kainic acid into animals triggers status epilepticus (see later section). However, the contribution of KARs to seizures is complex; both excitatory and inhibitory effects of KARs are possible. For example, studies have proposed the activation of KARs as an antiepileptic therapy approach (Khalilov et al., 2002). Recent work studied mice lacking GluK1 and GluK2 after systemic administration of
ATPA (a potent agonist of KARs) and in superfusion into amygdala slices (which contain a high number of GluK1 KARs). Results showed that selective activation of the receptors caused seizure activity and epileptiform discharges, but these experiments failed to confirm antiepileptic effects of blocking GluK1 KARs (Fritsch et al., 2014). Furthermore, GluR5 KARs have been shown to be implicated in the excitation of interneurons (Cossart et al., 1998; Wondolowski and Frerking, 2009) and in the presynaptical modulation of GABA (Christensen et al., 2004) and glutamate (Aroniadou-Anderjaska et al., 2012). Thus, KAR activation may have inhibitory as well as excitatory effects.

1.4.2 γ-amino butyric acid (GABA)

GABA is the main inhibitory neurotransmitter in the brain and it regulates the balance between the inhibitory and excitatory neuronal responses. GABA is synthesised in GABAergic axon terminals via the enzyme glutamic acid decarboxylase (GAD). When released into the synaptic space GABA acts via two kind of receptors; the ionotropic GABA_A receptors, which regulate Cl⁻ efflux into the cell and metabotropic GABA_B receptors, responsible for increasing K⁺ conductance and decreasing Ca²⁺ entry, which also inhibits neuronal excitation.

Glial cells and presynaptic nerve terminals reuptake GABA from the synaptic space. GABA taken back up into nerve terminals is available for reutilization, whilst GABA taken up by glia is first metabolized to succinic semialdehyde by GABA transaminase and cannot be resynthesized since glia lack GAD. The role of GABA in the mechanisms and treatment of epilepsy has been studied in experimental models and humans (Treiman, 2001). GABAergic functional abnormalities have been found in genetic and acquired animal models of epilepsy. For example, studies using GABA agonists including benzodiazepines and barbiturates, which increase the opening frequency and the opening time of Cl⁻ channels, observed seizure suppression and anticonvulsant effects (Study and Barker, 1981; Macdonald and McLean, 1987). Due to the powerful anticonvulsant effects observed in numerous
studies, increasing the concentration of GABA neurotransmitter within the synaptic space represents an optimal therapeutical approach to develop more effective AEDs. Indeed, many of the currently used anticonvulsants and AEDs work through increasing GABA levels in the brain (Bialer et al., 2004).

1.5 Molecular pathways regulating seizure-induced cell death

A key feature of either normal nervous system development or neuropathological conditions is neuronal cell death. In the mature animal, seizure-induce neuronal death is driven by two main regulated cell death mechanisms: apoptotic and non-apoptotic (necrosis pathways) (Akhtar et al., 2004; Vanden Berghe et al., 2014).

1.5.1 Apoptosis pathway

Apoptosis is classically described as a controlled cellular program that triggers cell death (Fujikawa, 2005). Apoptotic pathways are usually classified as the intrinsic pathway, which is triggered by the disruption of intracellular organelle function, such as because of intracellular Ca²⁺, proapoptotic B-cell lymphoma-2 (Bcl-2) protein activation, or ROS (Orrenius et al., 2003); or the extrinsic pathway, in which cell surface death receptors become activated by ligands including TNF-α, Fas ligand and TNF receptor apoptosis-including ligand (TRAIL) (Henshall and Simon, 2005). During apoptosis, demembranation and condensation of the nucleus into particular chromatin masses occurs and nuclear masses get dispersed within the cell in numerous membrane-enclosed bodies (Niquet et al., 2012).

Analysis of brain regions susceptible to cell degeneration following SE and in chronic epileptic animals have revealed the modification of the expression and activity of apoptosis-associated proteins, including Bcl-2 family proteins and the cysteiny1 aspartate-specific proteinase (caspase) family (Bengzon et al., 2002). In the setting of seizures, the triggers for apoptosis pathways are thought to include DNA damage, endoplasmic reticulum (ER) stress, changes to mitochondrial
functions and others. There is evidence for each of these processes in the brain after seizures (Henshall and Meldrum, 2012). Bcl-2 homology 3 (BH3)-only proteins act and block the anti-apoptotic Bcl-2 protein family, activating the pro-apoptotic family members which trigger the mitochondria membrane permeabilization, and ultimately cytochrome-c release and other pro-apoptotic effectors (Kluck et al., 1997; Steckley et al., 2007). Cytoplasmatic cytochrome-c triggers the activation of the apoptotic protease activating factor-1 (Apaf-1)/Caspase-9 complex and activates caspase-3, which subsequently will result in DNA fragmentation and eventually cell death (Kluck et al., 1997; Cregan et al., 2002; Hector et al., 2012). SE results in the release of cytochrome-c from mitochondria (Henshall et al., 2000a) and formation of the Apaf-1/cytochrome c complex (Henshall et al., 2001b). Caspase inhibitors administrated before and after experimentally induced SE result in a decrease in cell death, suggesting the involvement of caspases in neuronal degeneration after seizures (Henshall et al., 2000a; Henshall et al., 2001a; Engel and Henshall, 2009).

### 1.5.2 Necrosis pathway

Necrosis is thought to be the predominant mechanisms of cell death after prolonged seizures (Niquet et al., 2012). Traditionally, necrosis has been considered as a passive process where the activation of the cell death program was not necessary. During necrosis the plasma membrane is ruptured due to mitochondrial and organelle swelling (Niquet et al., 2012). However, data now suggest various signalling pathways are required for necrosis, giving rise to the concept of ‘programmed necrosis’. Among these is the molecular pathway of necroptosis, which stimuli promotes the interaction of the receptor-interacting protein kinase 1 (RIPK1) and RIPK3 under conditions in which caspase-8 is not active (Pasparakis and Vandenabeele, 2015). This RIP1/RIP3 complex form an heterodimeric amyloid structure through their RHIMs, which is a functional signalling complex that mediates programmed necrosis (Li et al., 2012).
1.6 Gliosis

Under normal physiological conditions glial cells, including microglia and macroglia (astrocytes, oligodendrocytes, ependymal cells and radial glia), support neurons to maintain tissue homeostasis in the CNS. Oligodendrocytes insulate axons allowing the propagation of the electrical signals; Astrocytes are involved in the modulation of synaptic function and plasticity, regulating the release of neurotransmitters; Radial astroglia help guide neuronal migration during development and microglia support cellular immunity in the brain, restoring functions and promoting healing (Hanisch and Kettenmann, 2007; de Lanerolle et al., 2010). The nonspecific reactive modification of glial cells due to an injury in the CNS is known as gliosis. This process involves the proliferation or hypertrophy of ependymal cells, astrocytes, microglia and oligodendrocytes (Fawcett and Asher, 1999). Over the years, gliosis has been documented in many CNS diseases and has always been considered a process with negative implications (Zhang et al., 2010). Additionally, a characteristic of the epileptic human brain and experimental epilepsy models is an abnormal glial function. These abnormalities may lead to seizure induction and epileptogenesis, promoting an increase of excitability and inflammation (Wetherington et al., 2008). However, the perception of reactive gliosis in the pathogenesis of different neurological disorders has shifted with the emergence of more experimental data supporting beneficial effects of astrocytes and microglia activation on CNS pathologies (Pekny et al., 2014).

1.6.1 Astrocytes

Astrocytes are in charge of many functions that allow the optimal function of neurons and the brain in general. Table 1.1 lists the normal physiological functions of astrocytes.
Table 1.1 Main functions of astrocytes

<table>
<thead>
<tr>
<th>Astrocyte functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>• K⁺ buffering in the extracellular space</td>
</tr>
<tr>
<td>• control of extracellular pH due to H⁺ and HCO₃⁻ transporting enzymes</td>
</tr>
<tr>
<td>• release or uptake of transmitters such as glutamate, ATP and GABA from synapses</td>
</tr>
<tr>
<td>• modulation of synaptic transmission</td>
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<tr>
<td>• metabolic support, providing nutrients to neurons</td>
</tr>
<tr>
<td>• vasomodulator as intermediaries in neuronal regulation of blood flow</td>
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<tr>
<td>• promotion of the myelinating activity of oligodendrocytes</td>
</tr>
<tr>
<td>• water transport and specific expression of Aquaporin 4</td>
</tr>
<tr>
<td>• antioxidant</td>
</tr>
<tr>
<td>• glial scar formation for nervous system repair</td>
</tr>
<tr>
<td>• long-term potentiating roles (integrated in learning and memory processes in the hippocampus)</td>
</tr>
</tbody>
</table>

(Ishibashi et al., 2006; Kimelberg and Nedergaard, 2010).

Additionally, astrocytes are interconnected by gap junctions which allow electrical synchronization during an action potential forming a syncytium (Bennett et al., 2003). These gap junctions are important for Ca²⁺ waves generated by constant influx of Ca²⁺ into the astrocytes. This in turn leads also to increase extracellular ATP signalling (Newman, 2001). Indeed, ATP has been proposed as the extracellular molecule involved in Ca²⁺ wave propagation between astrocytes. Confirming this, studies using pharmacological compounds which degrade ATP or block ATP gated-purine receptors block of inter-astrocytic Ca⁺² waves (Guthrie et al., 1999). The elevation of astrocytic Ca⁺² concentration promotes the activation of mechanisms that release gliotransmitters including glutamate, D-serine, nitric oxide (NO), acetylcholine and thrombospondin (Ben Achour and Pascual, 2010).
Furthermore, glial cells have been proposed as the main contributors to synaptic scaling within the hippocampus. Synaptic scaling allows the balance between excitatory and inhibitory inputs, which in contrast to long-term potentiation (LTP) and long-term depression (LTD), is non-associative and its homeostatic mechanisms adjust the strength of all the synapses of a neuron and maintain constant firing rates (Haydon, 2001). Studies showed how astrocytes-release cytokine TNF-α elevates the expression of AMPA receptors provoking an increase in synaptic strength (Beattie et al., 2002) and synaptic scaling (Stellwagen and Malenka, 2006).

Given the numerous homeostatic functions of astrocytes, it is not surprising that proliferation and hypertrophy of astrocytes profoundly alters brain excitability. Reactive astrocytes show characteristic molecular and physiological changes under pathophysiological conditions. K⁺ homeostasis is disturbed due to the reduction of Kir channel expression, the main K⁺ channel subunit of astrocytes in the rodent and human hippocampus, enhancing seizure susceptibility (Olsen and Sontheimer, 2008). Studies show that knockout (KO) of Kir interferes with the ability of astrocytes to remove glutamate and K⁺ from the extracellular space. Additionally, mice lacking Kir also showed enhanced short-term synaptic potentiation (Djukic et al., 2007).

Furthermore, changes in glutamate transporters (GLAST and GLT-1) or enzyme systems that lead to hyperexcitability also accompany astrogliosis (Sofroniew, 2009; Devinsky et al., 2013). Loss of the glutamate transporter GLT-1 leads to increased synaptic glutamate levels and seizures in response to the administration of subconvulsive doses of pentylenetetrazol (PTZ) (Tanaka et al., 1997). Recent data has emerged suggesting that astrocytes acquire stem cell-like properties after injury. This fact opens the door to consider the manipulation of post-injury astrocytic response to generate new neuronal circuits (Buffo et al., 2008; Colak et al., 2008).
1.6.2 Microglia

Microglia are normally present within the brain in a resting or ramified state. Microglia fulfill a variety of different functions within the CNS mainly related to both immune response and maintaining homeostasis. The main role is phagocytosis of foreign material including cellular debris, lipids, damaged and apoptotic cells, virus and bacteria among others. Microglia can also release a variety of cytotoxic substances, proteases, cytokines and neurotransmitters such as glutamate (Gehrmann et al., 1995). Moreover, microglia promotes tissue reparation after inflammation. These functions include synaptic stripping, secretion of anti-inflammatory cytokines, recruitment of neurons and astrocytes to the damaged area and formation of gitter cells (Davis et al., 1994).

Microglial activation occurs following an injury or presence of pathogens in the CNS (Dheen et al., 2007). Characteristic morphological changes accompany microglial activation, including retraction of their processes and the adoption of a rounded amoeboid morphology (Eder, 1998; Ducharme et al., 2007; Skaper, 2011). In vitro and animal studies of microglia have identified two activation states: M1, classical activation with pro-inflammatory responses and cytokines release, and M2, normally associated with anti-inflammatory responses, growth factor and anti-inflammatory cytokine release. M2 activation will in turn promote the inhibition of M1 activated microglia (Boche et al., 2013). M1 activated microglia release pro-inflammatory mediators, including cyclooxygenase-2 (Cox-2), interleukin-6 (IL-6), interleukin-1β (IL-1β), TNF-α, prostaglandins (PGs) and vascular endothelial growth factors (VEGF) among others. A strong microglial activation leads to the release of large quantities of these molecules and other alterations including the production of reactive oxygen species (ROS) through the function of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), TNF-α and IL-1β, leading to changes such as the impairment of the blood-brain barrier (BBB) function. Studies testing the effect of a NADPH oxidase inhibitors in lipopolysaccharide (LPS)-activated microglia from rat microvascular endothelial cells co-cultured with
microglia showed functional blockage of injury to the BBB (Sumi et al., 2010). Prolonged microglial activation ultimately contributes to BBB damage (Devinsky et al., 2013; da Fonseca et al., 2014).

Microglial activation has been linked to many negative effects including neuronal injury and seizure induction (Gallic et al., 2012). The release of a variety of cytotoxic substances including H$_2$O$_2$ and NO can directly damage cells and lead to neuronal cell death (Wienfeldt et al., 2011). Moreover, microglia secrete proteases that catabolise specific proteins causing direct cellular damage, and cytokines such as IL-1β, which promotes the demyelisation of neuronal axons. Finally, microglia can injure neurons through NMDA receptor-mediated processes by secreting glutamate and aspartate (Gehrmann et al., 1995). The release of pro-inflammatory mediators by activated microglia has also been proposed to inhibit mossy fiber sprouting (MFS), a form of synaptic reorganization in the dentate gyrus (DG) (Shu et al., 2011).

1.7 Inflammation

Inflammatory processes have emerged as important contributors to acute and chronic neurodegenerative disorders including Alzheimer’s disease (AD), ischemic stroke and traumatic brain injury (TBI) among others (Allan et al., 2005). Clinical studies and experimental animal work support a major role for inflammation in epilepsy, contributing to the etiopathogenesis of seizures and eventually development of a chronic epileptic focus (Vezzani et al., 2011).

The development of inflammation involves the activation of innate and adaptative immunity, a process that provokes the production of an array of inflammatory mediators such as cytokines (Nguyen et al., 2002). Interleukins, interferons (IFNs), TNFs and growth factors are the main cytokines released in the CNS by glia, neurons, and immunocompetent and endothelial cells (Bartfai and Schultzberg, 1993). These molecules drive the communication between cells during an immune response or after an injury, interacting once released in the extracellular space,
with one or more receptors. There are several mechanisms regulating cytokine activity, including cellular release, gene transcription, cleavage of cytokine precursors (e.g. pro-IL-1β or pro-TNF) and receptor signalling (Allan and Rothwell, 2001). The most studied cytokines in the CNS are IL-1β, TNF-α and IL-6 (Bartfai and Schultzberg, 1993; Allan and Rothwell, 2001). Chemokines, are a further important class of molecule, the release of which is controlled by pro-inflammatory cytokines such as IL-1β (Allan et al., 2005). Chemokines act as chemoattractants, guiding cell migration in inflammatory and homeostatic processes. The main chemokines formed under pathological conditions are CXCL-8, CCL2, CCL3, CCL4, CCL5, CCL11 and CXCL10 (Cardona et al., 2013). They are known as inflammation-specific because their activation leads to leukocyte migration from the blood to the site of infection or injury through the endothelial barrier (Wilson et al., 2010). This process initiates angiogenesis, neurogenesis and synaptogenesis. During brain development, chemokines provide axon guidance and regulate microglial and neuronal stem cell migration (Szekanecz and Koch, 2001; Semple et al., 2010). Chemokine expression has been reported in experimental models of epilepsy (Fabene et al., 2010). CCL2 has been implicated in the development of BBB permeability and the recruitment of immune cells at the injury area after SE (Manley et al., 2007; Fabene et al., 2010). In addition, increased production of chemokines and its receptors have been observed in neurons and astrocytes in the hippocampus of mice after pilocarpine and KA induced SE (Manley et al., 2007; Foresti et al., 2009; Xu et al., 2009). These results are in agreement with chemokine studies in human tissue, where the upregulation of CXCR4 expression was observed in microglia and astrocytes in the hippocampus of TLE patients (Lee et al., 2007).

Experimental models have consistently shown that prolonged seizures result in an increase of inflammatory mediators in the tissue at the site of seizure activity onset and within regions of the brain to which the seizure propagate (Aronica et al., 2007; Ravizza et al., 2008; Fabene et al., 2010). Seizures induce the production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in activated microglia and astrocytes, followed by an increase of cytokine receptor activation in glia cells and
neurons in the hippocampus (Vezzani and Granata, 2005). When IL-1β is released, it binds with high affinity to the type 1 IL-1 receptor (IL-1R1), expressed by hippocampal neurons (Takao et al., 1990). IL-1R1 activation results in the production of ceramide, which activates tyrosine kinase protein (Src). This in turn mediates the phosphorylation of the NMDA receptor, which differentially controls the trafficking and surface expression of NMDA receptors (Chen and Roche, 2007). This mechanism is thought to underlie the proconvulsant actions of this cytokine in the hippocampus (Vezzani et al., 1999b). IL-1β may have other pro-convulsive effects and has been linked to an increase in glutamate release from astrocytes via TNF-α induction (Bezzi et al., 2001) and to the inhibition of glutamate reuptake from astrocytes (Ye and Sontheimer, 1996). In addition, as a secondary effect of TNF-α induction from IL-1β activation, AMPA-receptor density and activity increases in neurons, which results in an increase in Ca^{2+} influx (Stellwagen et al., 2005).

The production of IL-1β is accompanied by the synthesis of the endogenous IL-1R antagonist (IL-1Ra), which occludes the activation site of the IL-1R1 (Dinarello, 1996). However, during seizures the production of IL-1Ra is much lower within the brain suggesting a failure in terminating the production of endogenous IL-1Ra (De Simoni et al., 2000). The anti-convulsant effects of IL-1Ra have been repeatedly demonstrated in different models of SE (Vezzani et al., 2002a). In addition, genetically modified mice that overexpress IL-1Ra in astrocytes display reduced seizure susceptibility (Vezzani et al., 2000; Vezzani et al., 2002a). Moreover, work in acute models and chronic epileptic mice, in which the enzyme that produces the active form of IL-1β, interleukin-converting enzyme (ICE) or caspase-1, is blocked pharmacologically or genetically have shown a 50 % reduction in seizure duration and 70 % reduction in the number of seizures (Ravizza et al., 2006; Vezzani et al., 2010).

IL-1β is also known for inducing the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) (Gilmore, 2006). NFkB activates the transcription of inflammatory cytokines contributing to the activation of astrocytes and microglia (Lawrence, 2009). Clinically, over-expression of NFkB in astrocytes
and neurons has been observed in resected tissue from the hippocampus of epileptic patients with hippocampal sclerosis (HS) (Crespel et al., 2002). Hence, NFκB might be another contributor to the glio-neuronal reorganization in epilepsy (Crespel et al., 2002; van Gassen et al., 2008).

1.8 Epileptogenesis

Epileptogenesis is commonly defined as the process by which a normal brain is transformed into one capable of generating seizures. There is normally an inciting event followed by a period of time known as the ‘latent period’ during which various changes occur culminating in the onset of seizures (Bragin et al., 2000; Walker et al., 2002). During this latent period different structural and functional changes occur in the brain leading to the development of epilepsy (Walker et al., 2002). Epileptogenesis can be triggered by febrile seizures, SE, hypoxic-ischemic injury or head injury (Hart et al., 1990; Annegers et al., 1998; Walker et al., 2002). In acquired epilepsy in humans, this seizure-free period is highly variable in duration, ranging from weeks to years (French et al., 1993; Annegers et al., 1998).

Currently, there are numerous experimental animal models to understand the pathophysiology of epileptogenesis and to simulate or provoke the epileptogenesis process. Differences between models are based on the method of seizure induction, including systemic (Olney et al., 1979; Nadler, 1981) and intra-cerebral (Ben-Ari, 1985) administrations of chemoconvulsants such as KA or pilocarpine (Cavalheiro et al., 1996) or electrical stimulation (Sloviter, 1987). Despite individual differences, all models involve seizure propagation and cause damage to limbic structures, particularly the hippocampus. Each model is associated with a varying duration latent period after the initial injury and ultimately emergence of spontaneous seizures (White, 2002). This can occur as soon as 2 - 3 days after the initial insult. SE models have the advantage of provoking epilepsy in up to 100 % of animals but SE is not a common cause of epilepsy in humans (Mazarati et al., 2002).
The experimental evidence supports the existence of several primary mechanisms that underlie epileptogenesis. They include cell death, gliosis, axonal sprouting, alterations in neurotransmitter receptor expression, reorganization of neuronal networks and neurogenesis (Armijo et al., 2002; Herman, 2006). Some of the mechanisms mentioned previously have been discussed before but it is important to mention that the loss of CA3 pyramidal neurons as an indication of epileptogenesis have been reported in the intra-amygdala KA model (Li et al., 2008b; Jimenez-Mateos et al., 2010). Conversely, cell death has been reported to be located in the DG after perforant path stimulation (Sloviter, 1991). The loss of GABAergic interneurons in the hilus, as well as the loss of excitatory mossy neurons has also been suggested to underlie the development of epilepsy (Ribak et al., 1982).

Efforts to prevent epileptogenesis using AEDs, either clinically or experimentally, have generally failed (Pitkanen, 2010; Langer et al., 2011) and it is still uncertain if the progression can be stopped pharmacologically (Sloviter, 2011). As the epileptogenic period can have different timeframes and the processes involved develops differently between animals and patients it is very difficult to identify potential translatable treatments (Sloviter and Bumanglag, 2013). Efforts to minimize cell death during this period might be the only possible therapeutic option (Langer et al., 2011; Sloviter, 2011; Sloviter and Bumanglag, 2013). An alternative therapeutic strategy is to focus on modifying epilepsy development instead of trying to completely prevent the disease. Numerous different antiepileptogenic therapeutic approaches have been proposed to interrupt the process of epileptogenesis by targeting secondary processes that occur during the state of epilepsy maturation after the first focal seizures (Sloviter and Bumanglag, 2013). They include suppression of seizure spread (Sloviter and Bumanglag, 2013), surgical excision of the seizure focus (Gotzsche et al., 2012), regularization of ion channel function (Richichi et al., 2008), averting aberrant neurogenesis (Parent and Murphy, 2008) and avoiding synaptic reorganisation (Zeng et al., 2007), among others.
1.9 Symptomatic Focal Epilepsies (Temporal Lobe Epilepsy)

Temporal lobe epilepsy (TLE) is divided in mesial temporal lobe epilepsy (MTLE), where the seizure focus is within limbic areas such as the hippocampus, and lateral/neocortical temporal lobe epilepsy (LTLE). Commonly, the site of ictogenesis (origin of seizures) in TLE is localized in different brain areas such as the amygdala, the hippocampus and the parahippocampal gyrus, including as well other temporal structures. MTLE is the most common form of focal epilepsy in adults, and it is generally thought to be an acquired epilepsy, a result of a CNS injury such as infection, head trauma or perinatal injury (Halasz and Fogarasi, 2006). MTLE is often distinguished pathologically by selective morphological alterations in the hippocampus, although the relationship between hippocampal injury and MTLE is complex. Injury to the hippocampus may precipitate epilepsy, and seizures themselves may further damage the hippocampus. Furthermore, pathology can be highly variable, from almost no obvious temporal lobe damage through to severe hippocampal sclerosis (Mathern et al., 1996).

The most common alteration observed in surgical and autopsy MTLE patients is hippocampal sclerosis (Engel, 1996; Zhang et al., 2002; Thom et al., 2009), with the hippocampus displaying reactive gliosis, synaptic rearrangement and neuron loss. HS is defined as a neuropathological condition with severe neuronal cell loss and gliosis in the hippocampus, particularly loss of the neurons from CA1, CA3, subiculum and hilus of the DG. In contrast, dentate granule neurons are typically spared along with neurons of the CA2 hippocampal region (VanLandingham et al., 1998; Cavazos et al., 2004). HS has been suggested to contribute to the development of chronic seizures in patients with diagnosed epilepsy (Mathern et al., 1995; Cavazos et al., 2004; Thom et al., 2009). Mossy fiber sprouting is another frequent result of HS. The axons of DG granule cells develop collaterals that grow into an abnormal location and expand upwards into the molecular layer of the DG. It has been reported that mossy fiber sprouting may contribute to epileptogenesis by creating potentially self-excitatory connections within the DG (Dudek and Shao,
However, the exact role of MFS remains controversial. Other groups have argued that MFS in fact helps inhibit excitability (Sloviter, 1992). MSF may also have no significant function in epilepsy. Pitkänen and colleagues showed no relation between mossy fiber sprouting density in the hippocampus and the frequency of epileptic seizures in experimental or human TLE (Pitkanen et al., 2000).

A long-standing controversy in the field is whether HS is the cause or an effect of seizures. Studies in animal models certainly show that HS can be caused by repeated temporal lobe seizures (Jefferys, 1999), but few animal data show that spontaneous seizures cause neuronal cell death (Gorter et al., 2003; Sutula et al., 2003; Drexel et al., 2012). Clinical data are consistent with the animal data; the major cell/morphological changes arise from an initial precipitating injury, with relatively minor contribution of repeated seizures (Mathern et al., 1995; Thom et al., 2009).

1.10 Modelling Temporal Lobe Epilepsy

Temporal lobe epilepsy is usually considered as an acquired epilepsy, typically developed after brain injury. It is the most widely modelled form of epilepsy. Animal models of TLE have made a significant contribution to our knowledge of the epileptogenic process and in the identification of novel treatments (Buckmaster, 2004).

1.10.1 Traumatic brain injury model

One of the most common risk factors for developing temporal lobe epilepsy is head trauma (Shorvon and Neligan, 2009). Any damage to the brain caused by an external mechanical force such as accidents, impacts, or penetrations by projectiles is defined as traumatic brain injury (TBI) (Buckmaster, 2004). Between 10 - 20 % of symptomatic epilepsies are estimated to be due to trauma (Pitkanen and McIntosh, 2006). More than 20 % of adults develop a spontaneous seizure within 2 years of
TBI (Weiss et al., 1983). TBI can result in temporary or permanent impairment of cognitive, physical and psychosocial functions (Xiong et al., 2013). Metabolic, cellular and molecular changes occur over minutes to months after the injury, leading to local and remote neuronal cell loss and tissue damage and, in a significant proportion, development of post-traumatic epilepsy (Pitkanen and McIntosh, 2006; Xiong et al., 2013).

Many different animal models have been used to study cellular and molecular changes caused after TBI, and studies have revealed that a significant number of injured animals develop epileptiform activity including recurrent spontaneous seizures (Pitkanen et al., 2011). Rates of epilepsy development vary considerably among the different TBI models. In the controlled cortical impact (CCI) model in both rats and mice a proportion of animals develop recurrent spontaneous seizures (Hunt et al., 2009; Statler et al., 2009; Pitkanen et al., 2011). However, only increased seizure susceptibility has been shown in the closed skull traumatic brain injury model (Chrzaszcz et al., 2010); These animals do not develop epilepsy in this model (Pitkanen et al., 2011). The parasagittal and lateral fluid percussion injury model has been reported to produce epilepsy in significant numbers of animals (D’Ambrosio et al., 2005; Kharatishvili et al., 2006; Kharatishvili et al., 2007). Lateral fluid-percussion (LFP) injury has been performed in cats, rats, mice and rabbits pro-epileptic results depending on the severity and the location of the insult (Lindgren and Rinder, 1969; Dixon et al., 1987; Hayes et al., 1987; Kharatishvili and Pitkanen, 2010; Bolkvadze and Pitkanen, 2012). LFP injury causes a lesion to the neocortex, including the perirhinal, postrhinal and entorhinal cortex, and is considered one of the most relevant to post-traumatic epilepsy (PTE) (Buckmaster, 2004). The injury also affects the hippocampus, despite the distance from the neocortex, and induces neuronal loss, granule cell synaptic reorganization and ultimately hyperexcitability in the hippocampus (Buckmaster, 2004). There is no evidence that spontaneous seizures are developed by a moderate impact injury, although it has been reported that severe injuries in rats result in the development of spontaneous behavioural seizures (D’Ambrosio et al., 2004; Nissinen and Pitkanen, 2007).
TBI models are useful to model temporal lobe epilepsy because of their relevance as a risk factor, but the low proportion of animals that develop epilepsy remains a limiting factor in their application (Buckmaster, 2004).

### 1.10.2 Kindling Model

Repeated, mild, electrical stimulation in different parts of the brain such as the amygdala, the hippocampus or the olfactory lobe, eventually leads to a progressive and permanent state of hyperexcitability (Goddard et al., 1969). This process is termed ‘kindling’ and the kindling model has been a useful tool in understanding the pathogenesis of TLE. One important mechanism by which kindling is thought to occur is via an increase in excitatory transmission through NMDA-type glutamate receptors in the dentate granule cells of the hippocampus (Kohr et al., 1993). Kindling causes an enhancement of NMDA receptor channel opening. This modifications result in membrane depolarization and activation of a calcium-dependent second-messenger signal transduction cascade (Mody and Heinemann, 1987). Kindled animals are more susceptible to the activation of NMDA receptors, which makes excitatory inputs capable of triggering action potentials and seizure activity (Mody and Heinemann, 1987; Kohr et al., 1993). However, studies have shown that NMDA receptor conductance decreases to control values within a month after kindling stimulations cease, while the kindling-induced increase in seizure susceptibility is permanent. Therefore, NMDA receptors may be involved in the induction of kindling but probably do not account for the preservation of the seizure-sensitive condition (Buckmaster, 2004). Another limitation of the kindling model is that seizures are evoked rather than spontaneous; kindled animals normally do not display recurrent spontaneous seizures. HS is not present in kindled animals either. Thus, while kindling model is helpful in understanding mechanisms and treatment, the applicability of this model to simulate temporal lobe epilepsy patients remains uncertain (Sayin et al., 2003).
1.10.3 Status epilepticus models

The most effective trigger of epilepsy in animals is an episode of SE. A number of different techniques have been used to induce SE, which are reviewed briefly below.

1.10.3.1 Electrical stimulation

For this technique, electrodes are placed in different brain areas such as the hippocampus and the amygdala. Animals then receive pulses of currents at different frequencies which are continued until they develop SE (Buckmaster, 2004). The technique is readily applied to rodents, cats and monkeys, among others and the advantage of this model is that the average number of animals developing self-sustaining SE is almost 90 %. Also, the stimulation is local and non-chemical, avoiding widespread effects on regions outside the area of interest. Electrical stimulation produces patterns of damage in the hippocampus that correspond to human TLE and other pathological hallmarks such as granule cell synaptic reorganization. Spontaneous seizures develop with a high percentage (~ 80 %) after electrical stimulation (Mangan and Bertram, 1998; Nissinen et al., 2000).

1.10.3.2 Chemical stimulation

1.10.3.2.1 Pilocarpine model

The pilocarpine model is among the most widely used for the study of TLE (Turski et al., 1983; Buckmaster and Haney, 2012). Pilocarpine acts as a non-selective muscarinic acetylcholine receptor agonist. These receptors are metabotropic acetylcholine receptors that signal via intracellular G-protein-receptor complexes. Activation of these receptors has a number of different effects in the brain that together, result in increased neuronal excitability and the eventual development of synchronous firing and seizures (Turski et al., 1989; Eglen, 2006).
The most common procedure to induce SE is by a systemic injection of pilocarpine. Animals are often also given a pretreatment with lithium, which acts to reduce the amount of pilocarpine needed to trigger SE. Animals must also receive atropine or scopolamine before pilocarpine administration to block peripheral cholinergic side effects (Jope et al., 1986; Buckmaster and Haney, 2012). Pilocarpine-induced SE typically develops within 30 min and lasts for many hours. Mortality is usually high in this model. After SE, animals show a seizure-free latent period varying from a few days to two-weeks after which they develop lifelong recurrent spontaneous seizures (Furman, 2013; Toyoda et al., 2013). Neuropathological changes and brain damage after pilocarpine treatment can be found in different brain regions including the hippocampus, amygdala and cortex. These include preferential neuronal loss in the CA1 and hilus as well as CA3 hippocampal regions, accompanied with gliosis, synaptic reorganisation and changes in ion channels and neurotransmitters (Cavalheiro et al., 1987). The model does have limitations. Induction of SE is thought to be secondary to activation of peripheral inflammation and BBB opening (Marchi et al., 2007). Also, Fabene’s group showed that animals do not need to develop SE to become epileptic after pilocarpine (Navarro Mora et al., 2009).

1.10.3.2.2 Kainic acid model

Kainic acid, a powerful neuroexcitatory amino acid, is another commonly used substance for the induction of SE. To elicit SE, KA can be injected either directly into the ventricle, cortex, hippocampus or amygdala, or given systemically. This makes the KA model a highly versatile model of focal seizures, as well as a model for epileptogenesis after SE (Nadler et al., 1980; Bragin et al., 1999; Furtado Mde et al., 2002).
1.10.3.2.2.1 Systemic kainic acid

The simplest method to trigger SE is by a systemic injection of KA. The dose of KA required to elicit SE after systemic injection depends on the age and species of the animal. Rats were the first species tested (Scerrati et al., 1986). Early protocols suggested the use of a high intra-peritoneal (i.p.) KA dose (12 to 18 mg/kg) to induce SE. Results showed induction of SE lasting a few hours, but this was accompanied by high mortality and a low number of animals developed spontaneous recurrent seizures (Olney et al., 1979; Schwob et al., 1980; Nadler, 1981; Cronin and Dudek, 1988). Further modifications of dose and route have been applied to improve reproducibility (Glien et al., 2001). Systemic KA produces characteristic EEG patterns comprising high amplitude and high frequency spikes (HAHFs) and polyspikes with high amplitude and low frequency spikes (HALFs), with hour-long persisting electrographic seizures (Giorgi et al., 2005). A multiple low-dose protocol was also developed, where several systemic injections of low doses of KA were administered to the point of eliciting SE. This had the effect of lowering mortality while also producing a chronic epileptic state 1 - 3 months later. However, animals display a highly variable epileptic seizure frequency (Hellier et al., 1998). Neuropathologically, systemic KA results in development of features of HS including significant cell loss and granule cell synaptic reorganization. Pyramidal neuronal loss in the rat hippocampus is found in both CA3 and CA1 regions (Balchen et al., 1993). However, prevalent extrahippocampal damage is evident in the systemic KA model, a pattern dissimilar to the hippocampus restricted damage in most TLE patients (Schwob et al., 1980; Sloviter, 1991). The systemic KA model is more problematic in mice. Not all mice strains exhibit seizure-induced neuronal death when using systemic KA (Schauwecker and Steward, 1997). Schauwecker and Steward showed that C57BL/6 mice, the most common strain in genetic studies, do not display hippocampal damage after systemic KA. This has limited the use of systemic KA model.
1.10.3.2.2 Intra-cerebral kainic acid

There are a number of disadvantages with the systemic KA model such as highly variable pathology and bi-lateral damage that is often extensive in the cortex (Mouri et al., 2008a). Some of these problems can be avoided by intra-cerebral local injection of KA, including intra-hippocampal KA and intra-amygdala KA (Schwarcz et al., 1978). However, there are some disadvantages to this approach, in particular KA diffusion can lead to degeneration of cells distant from the injection (Wuerthele et al., 1978). Many studies have shown that local injection of KA generates extensive pyramidal cell loss and severe granule cell dispersion in the hippocampus (Schwarcz et al., 1978). This marked loss of neurons and the limbic pathological alterations observed in the brains of KA treated rodents reflect the pathological features observed in human TLE. Therefore, the intra-cerebral KA animal models can be used as an isomorphic model of human TLE.

1.10.3.2.2.1 Intra-hippocampal kainic acid

Many groups have used this model to induce SE in rodents (Schwarcz et al., 1978; Cavalheiro et al., 1982; French et al., 1982; Bouilleret et al., 1999). Doses of KA vary from 0.1 to 3.0 μg. Studies by Schwarcz and colleagues showed that 2 μg injection of KA into the rat hippocampus provokes a unilateral fast and complete degeneration of neurons in the hippocampus followed by gliosis and atrophy of the hippocampus (Schwarcz et al., 1978). After KA injection, animals display recurrent paroxysmal discharges in the EEG and typical behavioural features such as immobility, staring and clonic components. Bouilleret and colleagues performed intra-hippocampal KA injection in mice and they observed seizures began rapidly following KA injection and were accompanied with electroencephalographic changes, histopathology and synaptic reorganization (Bouilleret et al., 1999). Loss of pyramidal and hilar neurons was observed along with a dramatic dispersion of the granule cells. A particular enlargement of the dentate gyrus was also seen with mossy fiber sprouting in the supragranular molecular layer of CA3. This model has
been used by other groups in rodents, cats (Griffith et al., 1991), macaques (Chen et al., 2013), guinea pig (Carriero et al., 2012) among others, with a high reliability and similar features. The main limitation of the model is the near complete lesion of the hippocampus. This is common in human TLE.

1.10.3.2.2.2 Intra-amygdala kainic acid

Early studies by Ben-Ari and colleagues found that local injection of KA in the amygdala produced SE and accompanying pathological changes (Ben-Ari and Lagowska, 1978; Ben-Ari et al., 1978; Ben-Ari et al., 1980; Ben-Ari, 1985). This model was later adopted by our group and used to explore the role of DNA fragmentation and apoptosis signalling (Henshall et al., 1999; Henshall et al., 2002). Within minutes of KA injection, seizures propagate from the amygdala to the entorhinal cortex, and then via the perforant pathway to the hippocampus; seizures then spread to the contralateral hippocampus (Araki et al., 2002; Shinoda et al., 2004). The main site of damage in the rat model is the ipsilateral CA3 subfield. Damage is also present in the hilus and the injected amygdala. Relatively few studies have investigated the later development of recurrent spontaneous seizures in this model (Araki et al., 2002; Shinoda et al., 2004; Mouri et al., 2008a).

Intra-amygdala KA also leads to typical behavioural features that follow the typical pattern of the Racine score scale. There are five cumulative stages (classes) of seizures development: 1) mouth and facial movements, 2) head nodding, 3) forelimb clonus, 4) rearing and 5) rearing and falling. The Racine score scale is frequently used in research to classified and describe seizure intensity behaviour in rodent epilepsy models (Racine, 1972). A modified Racine score scale is used by our group for the seizure behaviour analyses (Jimenez-Mateos et al., 2012). Seizures are often suppressed after KA by the injection of an anticonvulsant, such as Lorazepam, to minimize mortality and morbidity.

A model in rats alone is limiting, therefore in 2001 the model was transposed to C57BL/6 mice (Araki et al., 2002). Neuropathology and SE were comparable,
although the CA1 region was more vulnerable in mice than rats, a feature that better mimics human TLE. This also contrasts with the systemic KA model in which hippocampal damage is not reliably evoked (Schauwecker and Steward, 1997). Importantly, the model has been shown to work in multiple mouse strains including BALBC (Shinoda et al., 2004), SJL (Tanaka et al., 2010) and FVB making it attractive and versatile for genetic work. The model has also been used to characterize the electrographic seizure profile and histopathology in multiple genetically modified mice including Bclw−/−, Bim−/−, Puma−/−, Chop−/−, 14-3-3ζ+/−, bmf+/−, among others (Murphy et al., 2007; Engel et al., 2010; Murphy et al., 2010; Engel et al., 2013; Moran et al., 2013).

The characteristic pathological features of the intra-amygdala KA model include select neuronal cell death and astrogliosis, with the dorsal hippocampus presenting more damage than the ventral hippocampus (Li et al., 2008a; Mouri et al., 2008a). The model presents neuronal loss mainly in the CA3 region of the hippocampus with some spare neuronal cell death in CA1. Moreover, some cell death can also be observed in the hilar region of the DG (Mouri et al., 2008a). Therefore, the damage caused in this model is the most comparable to the damage found in the human epileptic brain (Figure 1.3). Extra-hippocampal damage can be observed in the temporal, entorhinal and perirhinal cortex, as well as thalamus.

In 2008, our group reported the course of epilepsy development in mice after intra-amygdala KA (Mouri et al., 2008a). Mice were fitted with EEG telemetry units and followed for two weeks after SE. Spontaneous seizures emerged after a short latency period of 3 – 4 days. After day 5, all mice displayed epileptic seizures, with an average of 1 – 4 seizures per day and an electrographic seizure duration averaging 20.9 ± 15.6 seconds (Mouri et al., 2008a). The rates of spontaneous seizures were quite consistent over time, but increased between the second to third week (Jimenez-Mateos et al., 2010). The model has been successfully adapted by other research groups (Li et al., 2008b; Liu et al., 2013).
Figure. 1.3 Intra-amygdala kainic acid mouse model

Photomicrographs showing nissl-stained images of the C57BL/6 mouse hippocampus 24 h after intra-amygdala KA induced SE. Note, severe neuronal loss in CA3 as well as in CA1 hippocampal subfields. Scale bar = 180 μm (top); 22 μm (bottom). (Adapted from Shinoda et al., 2004).

1.11 Seizure termination / AED treatments

There is no cure for epilepsy, but for 60% of patients, seizures are controllable with medication (Eadie, 2012). The choice of treatment of epilepsy patients is based on seizure type, epilepsy syndrome, other medications used, health problems and the patient’s age and lifestyle (Duncan et al., 2006). However, up to 40% of patients fail to respond to the available AEDs or have unacceptable side effects (Kwan and Brodie, 2000; Duncan et al., 2006). TLE is one of the most pharmacoresistant of the epilepsies, with patients failing to respond inspite of multiple treatment efforts. For these patients, if a lesion is visible, surgical resection of the temporal focus may be the only remaining option. However, patients without a discernible lesion or seizure focus may be unsuitable for surgery. Uncontrolled seizures have devastating effects on patients including personal health risks. For this reason, it remains a major research priority to better understand the cell and molecular processes underlying
epilepsy and use this knowledge to find and exploit new treatment targets (Curia et al., 2014; Zhao et al., 2014).

Frontline treatments for epilepsy patients are based on anticonvulsant drugs that decrease the frequency and/or severity of seizures. Those drugs are known as AEDs and they are administered to prevent the occurrence of seizures. They do not, however, alter the underlying epileptic condition (O’Dell et al., 2012). The first effective drug to prevent seizures, potassium bromide, was identified in 1857 (Ernst et al., 1988; Lane and Bunch, 1990; Podell and Fenner, 1993; Oguni et al., 1994). It was in the early 1900’s when a barbiturate drug called phenobarbital (PB), was discovered as a less toxic and more effective AED. It remains in use today as the first option for neonatal and childhood seizures in many low-income countries and for convulsive and nonconvulsive drug-resistant SE (Kwan and Brodie, 2004). Phenobarbital works by promoting hyperpolarisation of neuronal cell membranes by prolonging the opening of the GABA_A channels on the post-synaptic cell membrane (Shorvon, 1978). The next AED discovered in the late 1930s was phenytoin (PHT), by Merritt and Putnum. The authors discovered the anticonvulsant effects of phenytoin using the electroshock seizure model. Its mechanism of action is thought to be via reducing the repetitive firing of action potentials in neurons. Phenytoin remains in use today for convulsive seizures, both in idiopathic generalised and focal epilepsy with secondarily generalised tonic-clonic seizures (Friedlander, 1986).

Carbamazepine (CBZ) was introduced in the early 1960s as an antiepileptic agent. It is used as a treatment for focal epilepsies, although it is not successful for absence and myoclonic seizures (Vajda and Eadie, 2014). The main mechanism of carbamazepine is by blocking voltage-dependent sodium channels (Granger et al., 1995; Tudur Smith et al., 2002), although other actions are known (Vajda and Eadie, 2014). Around the same time, sodium valproate (VPA) was discovered to have anticonvulsant effects and it is used for the treatment of generalized epilepsies. The exact mechanism of VPA is unknown, but studies show it increases GABAergic inhibition and blocks voltage-gated sodium channels. VPA also has histone
deacetylase (HDAC) inhibitory effects but these are not thought to underlie the anti-convulsant mechanism (Nolan et al., 2013).

In recent years, new AEDs have been licensed for clinical use and they are classified into ‘second- and third- generation’ AEDs. The second-generation AEDs include felbamate, gabapentin, levetiracetam, oxcarbazepine, among others and the third generation AEDs comprises eslicarbazepine acetate and lacosamide. If administrated together, first generation AEDs show a high tendency of pharmacokinetic and pharmacodynamic interactions, related to protein-binding displacement from albumin in the blood and metabolic inhibitory and induction interactions in liver. Second and third generation drugs show more favourable pharmacokinetic actions and many are minimally or not bound to blood albumin and are rapidly excreted or metabolized. As a result, second and third generation AEDs have more favourable side effects profiles (Tudur Smith et al., 2002; Johannessen and Landmark, 2010). However, the efficacy of the newer generated AEDs is generally not superior to older AEDs.

1.12 Alternative anti-epileptic approaches

An epilepsy patient is deemed drug resistant if they do not respond to two or more AEDs. There are few alternative treatment options for this significant patient subgroup. If the site/area of seizure can be identified, surgical intervention, consisting of epileptogenic area removal, is a possibility (Wieser, 1998). The most common structure to be removed is the hippocampus and improvements in epilepsy are seen in ~ 80 % of patients. However, many patients are not suitable candidates for surgery.

A different antiepileptic treatment approach developed in the 1920s was the ketogenic diet (KD). This diet was used mainly for children with refractory epilepsy. It is based on a high-fat, regular protein and low carbohydrate diet. By altering the diet this reduces glucose availability in the body, forcing the liver to convert fat into fatty acids and ketone bodies. Glucose is then replaced by ketone bodies as the
energy source within the brain. Studies have shown that a ketosis situation, which is a high level of ketone bodies in the blood, decreases the frequency of epileptic seizures (Freeman et al., 2007). However, KD rarely stops all seizures and it is difficult to predict who will benefit. Compliance also requires close monitoring with a dietician and strong family support.

Brain stimulation devices have emerged also as potential alternative treatments for intractable epilepsy. The most popular is vagus nerve stimulation (VNS). The vagus nerve arises from the medulla connecting, by the afferent vagal fibers, the nucleus of the solitary tract, communicating as well to other regions of the CNS. How vagal nerve stimulation controls seizures is still unclear, but some studies suggest diverse ways of action such as, alteration of norepinephrine release in projections of the solitary tract, increasing GABA neurotransmitter levels and inhibition of abnormal cortical activity through the activation of the reticular system (Ghanem and Early, 2006).

1.13 ATP as a neurotransmitter

Existing treatments for epilepsy are often ineffective and the majority of AEDs act on one of three targets: sodium channels, GABA and glutamate. There remains a need for AEDs/novel drugs that act on alternate transmitter systems.

1.13.1 Discovery

ATP is known as the main source of cellular energy since 1929. However, a novel discovery in 1972 demonstrated ATP also functions as a neurotransmitter. Burnstock proved that ATP was the non-adrenergic, noncholinergic (NANC) transmitter released by inhibitory nerves in the taenia of the guinea-pig caecum, rabbit ileum, frog stomach and turkey gizzard (Burnstock et al., 1970; Burnstock, 1972). ATP has since been shown to be released from neurons and astrocytes acting either as a sole transmitter or as a co-transmitter with other neurotransmitters such as
glutamate, GABA, acetylcholine and noradrenaline, triggering a wide array of physiological effects in the peripheral and central nervous system (Burnstock, 2007b). ATP might be present in almost every synaptic and secretory vesicle, either co-stored with other classical neurotransmitters or in ATP-only vesicles (Rudnick, 2008; Sawada et al., 2008; Abbracchio et al., 2009).

1.13.2 ATP release mechanisms

More than one potential mechanism has been proposed to contribute to ATP release into the extracellular space. It is not very likely that ATP is released via exocytosis from damaged and dying cells. Rather, a leakage or a transporter or channel-mediated release is a potential mechanism of the release from dying cells (Abbracchio et al., 2009). Under normal conditions, once it is released, ATP and other nucleotides are rapidly degraded by ectonucleotidases into different breakdown products, including adenosine (Burnstock, 2007b, 2008)(Figure 1.4). Ectonucleotidases are nucleotide metabolizing enzymes localized and expressed on the plasma membranes of cells. They present external oriented active sites which metabolize nucleotides into nucleosides (glycosylamines). The action of these ectonucleotidases is important due to the modulation of purinergic signalling depending on the availability and preference of substrates (Beldi et al., 2008). ATP availability depends on the balance of the amount released and the enzymatic degradation in the extracellular space.

1.14 ATP-gated P2X receptors

In 1972, the concept of purinergic co-transmission was starting to be recognized in the scientific community and a few years later, two families of purine receptors were identified as sensitive to ATP and adenosine diphosphate (ADP); the P1 receptors (sensitive to adenosine) and P2 receptors (sensitive to ATP) (Burnstock, 2009) (Figure 1.5). P1 receptors are all metabotropic purinergic receptors which are G-protein coupled and activated by adenosine. There are four types named A1,
A2A, A2B and A3 and they mediate a variety of cellular responses including neurotransmitter release regulation (Prince and Stevens, 1992). Adenosine acts as

![Figure 1.4 ATP degradation after extracellular release](image)

The metabolism of extracellular ATP is regulated by several ectonucleotidases, including members of the Ecto-NTPases family. Ecto-5'-nucleotidase (Ecto-5'-NT) catalyses the nucleotide degradation to adenosine.

An endogenous homeostatic regulator of network activity and its deficit has been associated with epilepsy (Boison, 2013). As mentioned before, adenosine is eliminated from the extracellular space by the astrocytic enzyme ADK phosphorylating adenosine into AMP, controlling therefore, the levels of adenosine in the brain (Boison, 2012). The A1 receptor is mainly found at presynaptic sites in excitatory neurons where, once activated, they reduce neurotransmitter release such as glutamate and facilitate the stabilization of the postsynaptic membrane potential. Thus, A1 receptor activation has a powerful anti-excitative effect (Dunwiddie and Masino, 2001; Boison, 2005). The inhibition of ADK or an overexpression of A1 receptors in
experimental models including kindled and post-SE models of epilepsy produces potent reductions in seizures (Boison, 2009). Likewise, mice with genetically enhanced expression of ADK showed higher seizure activity during SE than wildtype mice and increased neuronal injury in the hippocampus (Li et al., 2008a).

![Diagram of P1 and P2 purinergic receptor family](image)

**Figure 1.5 The P1 and P2 purinergic receptor family**

P1 receptors are activated by adenosine while P2 receptor families respond to ATP. P2X are ATP-gated ionotropic channels while P2Y and P1 are GPCRs receptors.

Other lines of work showed that increased adenosine levels and consequently, the down-regulation of ADK after SE block DNA methylation in the brain leading to suppression of genes that drive epileptogenesis (Boison et al., 2002; Williams-Karnesky et al., 2013).

A2A receptor activation has been seen to be also involved in enhancing cAMP accumulation and PKA activity and it may lead to the inhibition of A1 receptor. Therefore, its activation is involved in excitatory responses (Cunha et al., 1994). The A2B receptor has been found at a low density in many parts of the brain and is
implicated in neuron-glial excitatory interaction (Murthy et al., 1995). The A3 receptors are low-affinity inhibitory receptors and are activated by high levels of adenosine under pathophysiologic conditions (Ralevic and Burnstock, 1998).

The P2 family act as ATP-recognizing purinergic receptors and are subdivided into P2X ionotropic (ion channel receptors) and P2Y metabotropic (G protein-coupled) receptors based on their mechanism of action, pharmacology and molecular cloning (Burnstock, 2007a, b). Both families of purinergic receptors are widely distributed in the CNS and they have been shown to be expressed by neurons and glia, including astrocytes, oligodendrocytes and microglia (Abbracchio et al., 2009).

P2Y receptors are coupled to G proteins resulting in a stimulation or inhibition of diverse intracellular signalling pathways. The metabotropic receptor family comprises 8 different P2Y receptors (P2Y_{1,2,4,6,11-14}) and their activation is based on the diverse structural differences of the nucleotide agonists, such as ATP, ADP, UDP, among others (Weisman et al., 2012). P2YR activation causes the GDP-GTP exchange through the heterotrimeric G protein, resulting in the dissociation of GTP-Gα, which modulates the activity of intracellular enzymes (Weisman et al., 2012).

P2Y receptors coupled via G_{i} inhibit the production of cAMP (Birnbaumer, 2007). Secondly, via G_{s} alpha subunit, this is a heterotrimeric G protein subunit that activates adenylate cyclase and in consequence activates the cAMP-dependent PKA pathway. Thirdly, via G_{q} protein, also a heterotrimeric G protein subunit that enhances the function of phospholipase C (PLC) activating the signal transduction pathways of IP_{3} and diacyl glycerol (DAG) through the hydrolysis of PIP_{2}. Protein Kinase C (PKC) is activated by the second messenger DAG, and IP_{3} triggering intracellular Ca^{2+} release (Birnbaumer, 2007; Qin et al., 2011; Weisman et al., 2012).

P2YR activation modulates a diverse set of cascades in many physiological and pathophysiologic cell responses, including neurotransmission regulation, cell growth, inflammatory responses and apoptosis in the CNS (Fischer and Krugel, 2007; Burnstock and Verkhratsky, 2010; Weisman et al., 2012).
The P2X receptors comprise a subfamily of seven ATP-gated nonselective cation channels named P2X1R to P2X7R. Structurally, each P2X receptor contains two transmembrane domains, a large extracellular loop (ectodomain) and intracellular N and C termini (North, 2002) (Figure 1.6). Functional P2X receptors form as either homomeric or heteromeric trimers with each subunit containing binding sites for ATP (orthosteric) and regulation (allosteric). Three highly conserved binding sites for ATP are present in the ectodomain subunit, essential for conformational changes to allow opening of the channels (gating). However, only two molecules of ATP are required to activate channel opening.

Normally, P2XR have a 3 stage pattern of gating: in the activation phase, application of the agonist causes a rapid increase of inward current. Following this, a desensitization phase, where the current in the continuing presence of the agonist declines. The final phase, the deactivation, begins after ATP removal and results in a decrease of fast currents. The principal distinctions between receptors are the difference in these three phases, diverse depolarizing responses and sensitivity to different agonists (Surprenant, 1996; North, 2002; Surprenant and North, 2009; Coddou et al., 2011). Depolarizing responses are the result of rapid nonselective passage of cations across the cell membrane gated by P2X receptors. All P2X receptors are permeable to small cations, monovalents or divalent, including permeability to Ca$^{2+}$. Although P2X receptors gate depolarizing sodium entry this is not thought to account for the major effects of P2XR. Instead, it is the increase in Ca$^{2+}$ concentration that is believed to be the major physiological effector of the P2XRs that activates the cellular changes downstream of P2XRs (Evans et al., 1996; Surprenant, 1996; He et al., 2003; Surprenant and North, 2009).

After ATP is released into the extracellular space, only a few milliseconds are necessary to bind and open the P2XR. Homomeric P2X1 and P2X3 receptors display rapidly desensitizing currents, while P2X4 and P2X2 receptor currents are more lasting (North, 2002; Coddou et al., 2011). The most prolonged channel opening is seen after application of ATP onto P2X7 receptors, which generates long-lasting inward ion currents lasting up to a few minutes (North and Jarvis, 2013). Sensitivity
Figure 1.6 3D crystal structure of P2X receptors

A) Schematic 3D crystal structure of a rat P2X2 receptor showing the characteristic three receptor subunits inserted in the plasma membrane. B) Different subunit states of the receptor; open and closed state conformation. Adapted from (Khakh and North, 2012).

to ATP also differs between receptors. For example, P2X7 receptor responds solely to high concentrations of ATP (EC$_{50}$ 500 μM), while the rest of the P2X family respond at a much lower concentration (EC$_{50}$ 10 μM) (North, 2002) (Figure 1.7).

P2X receptors have been described to be localized both presynaptically and postsynaptically in neurons and glia. Evidence that the release of endogenous ATP mediates postsynaptic currents in the hippocampus was provided in a study where P2X receptor antagonists suramin or PPADS inhibited the currents in the CA1 and
CA3 pyramidal cells in rat hippocampal slices (Pankratov et al., 1998; Mori et al., 2001).

Other cellular functions attributed to P2X receptors include protein synthesis, transcriptional regulation and hormone secretion (Ralevic and Burnstock, 1998) (Table 1.2). All types of P2X receptors are present within the CNS and have been found to be implicated in pathological conditions such as brain trauma and ischemia, neurodegenerative and neuroimmune disorders, neuroinflammatory reactions and also in neuropsychiatric diseases such as depression and schizophrenia and epilepsy (Burnstock, 2008). One of the P2X receptors which has been most linked to epilepsy is the P2X7 receptor (P2X7R) (Rappold et al., 2006; Dona et al., 2009; Kim et al., 2011b; Engel et al., 2012b).

**Figure 1.7 Gating properties of P2XRs**
Diagram showing the different desensitizing current profiles of rat P2XRs during the administration of the agonist ATP (10 μM for P2X1R and P2X3R, 100 μM for P2X2R and P2X4R, and 3 mM for P2X7R) in transfected HEK293 cells. $\tau_{\text{des}}$ indicates the desensitization time constant derived from monoexponential fitting (mean ± S.E.M.; values from at least five records per channel). Graph adapted from (Coddou et al., 2011).
Table 1.2 P2X receptor localization and functions

<table>
<thead>
<tr>
<th>Receptor</th>
<th>First described</th>
<th>ATP EC&lt;sub&gt;50&lt;/sub&gt; (μM) &amp; desensitization</th>
<th>Localization</th>
<th>Function</th>
<th>Relevance to disease</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1</td>
<td>Rat vas deferens (1994)</td>
<td>0.07 Fast (&lt;1 S)</td>
<td>Prominent in platelets. Smooth muscle, CNS, vas deferens, spinal cord, sensory ganglia</td>
<td>Smooth muscle contraction, platelet activation aggregation, renal autoregulation</td>
<td>Thrombosis, possible role in male infertility</td>
<td>(Hu and Hoylaerts, 2010)</td>
</tr>
<tr>
<td>P2X2</td>
<td>Rat pheochromocytoma cells (1994)</td>
<td>1.2 Slow (&gt;20 s)</td>
<td>Smooth muscle, widely CNS distributed, retina, gastrointestinal and bladder tissue, autonomic and sensory ganglia</td>
<td>Modulation of Synaptic function and sensory transmission (pain and taste perception)</td>
<td>-</td>
<td>(Keceli and Kubo, 2009)</td>
</tr>
<tr>
<td>P2X3</td>
<td>Rat dorsal root ganglia (1995)</td>
<td>0.5 Fast</td>
<td>Sensory neurones and ganglia</td>
<td>Sensory neurotransmission</td>
<td>Possible role in chronic inflammation and neuropathic pain, bladder hyper-reactivity</td>
<td>(Jarvis, 2003)</td>
</tr>
<tr>
<td>P2X4</td>
<td>Rat ganglia, brain and endocrine tissue (1996)</td>
<td>10 Slow</td>
<td>Widely expressed: CNS, bladder, kidney, gastrointestinal tract, uterus, testis, lung, etc.</td>
<td>Modulation of cytokine, BDNF release from microglia, control of hemodynamic responses</td>
<td>Ischemia, muscular dystrophy, chronic inflammatory and neuropathic pain</td>
<td>(Tsuda et al., 2009)</td>
</tr>
<tr>
<td>P2X5</td>
<td>Rat sympathetic ganglia and heart (1996)</td>
<td>10 Slow</td>
<td>Skeletal muscle, gut, bladder, thymus, skin, spin cord</td>
<td>Proliferation inhibition and differentiation</td>
<td>-</td>
<td>(Ruan and Burnstock, 2005)</td>
</tr>
<tr>
<td>P2X6</td>
<td>Rat cervical ganglia brain (1996)</td>
<td>12 -</td>
<td>CNS, testis, colon</td>
<td>-</td>
<td>-</td>
<td>(Jones et al., 2004)</td>
</tr>
<tr>
<td>P2X7</td>
<td>Rat brain (1996)</td>
<td>100 Slow</td>
<td>Immune cells, CNS (glia, astrocytes and neurons), epithelia, endothelia</td>
<td>Inflammation response: cytokine secretion, cell proliferation, programmed cell death. Regulation of neurotransmitter release.</td>
<td>Chronic inflammatory and neuropathic pain</td>
<td>(Sperlagh et al., 2006)</td>
</tr>
</tbody>
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(Brass et al., 2012; Khakh and North, 2012)
1.14.1 ATP involvement in seizures

Many different stimuli could increase extracellular ATP release, including pathological events such as inflammation, cellular damage, mechanical or metabolic stress or even changes in the ionic environment (Sperlagh et al., 2006). Extracellular ATP has been proposed to be released and functional in neurotransmission and gliotransmission during epileptic seizures (Dale and Frenguelli, 2009). An extraneuronal signalling pathway in the CNS is the glial Ca\textsuperscript{2+} waves, which propagation is carried out by ATP and gap junctions. Astrocyte intercellular calcium waves have been observed to underlie seizures (Burnstock et al., 2011). Other proposed roles for ATP signalling in the induction and spread of seizures involve synaptic plasticity and synchronization of synaptic networks (Dale and Frenguelli, 2009). Studies have shown ATP release after electrical in vivo stimulation of the somatosensory cortex using a luciferin-luciferase technique. Large increases of ATP were seen in response to direct cortex stimulation (Wu and Phillis, 1978). Moreover, direct release of ATP was observed by Schaffer pathway stimulation with luciferin-luciferase technique in hippocampal slices, where the release of ATP was Ca\textsuperscript{2+} dependent (Wieraszko et al., 1989).

1.15 P2X7 receptor

P2X7R is found throughout the CNS and PNS and its activation causes many different responses in neurons including excitation and cell survival mechanisms, as well as in activation responses of microglia and astrocytes. The P2X7R is encoded in humans by the P2rx7 gene located on chromosome 12q24 and comprising 13 exons (Buell et al., 1998). It was first cloned from a rat brain cDNA library in 1996 (Brake et al., 1994; Valera et al., 1994). The P2rx7 gene encodes a protein of 595 amino-acids long in human and rodents, and shares 80 % sequence homology with the other P2X receptor family (Surprenant, 1996). Its subunit structure is mainly typical of the P2XR family with 2 transmembrane domains, a large extracellular loop containing the ATP-binding sites and the intracellular N- and C- terminal domains. The P2X7R
has a unique structure, however, with a much longer intracellular carboxy terminal domain, comprising 239 amino acids, in comparison with the other P2X receptor family that contain 27 – 129 amino acids. This is understood to be important in mediating interactions with intracellular structural and signalling intermediates (Sperlagh et al., 2006; Khakh and North, 2012).

1.15.1 P2X7 receptor activation

ATP binds to an extracellular site leading to the activation of the P2X7R and a non-desensitising channel opening, allowing the flux of sodium (Na\(^+\)), calcium (Ca\(^{2+}\)) and potassium (K\(^+\)) ions. P2X7R activation results in two different responses depending on the exposure time to ATP. Upon brief ATP stimulation, an inward cationic current is produced, similar to other P2X subunit responses but with different deactivation kinetics. Prolonged ATP application/exposure leads to sensitizing responses and increased membrane permeability. If ATP stimulation persists longer than 15 min a pore is formed, which allows entry of high molecular weight molecules and ions up to 900 Daltons (Da), such as dyes and organic cations (Surprenant, 1996; Virginio et al., 1997; Virginio et al., 1999). The formation of the dilated pore increases the sensitivity of the receptor to agonists (Roger et al., 2008; Jindrichova et al., 2015). It has been suggested that P2X7R pore formation requires the involvement of another transmembrane molecule called Pannexin-1. The long-lasting pore formation is also thought to function as a cytolytic event, leading to cell death (Pelegrin and Surprenant, 2006; Locovei et al., 2007). Ectopic expression of Pannexin-1 cDNA constitutes a hemichannel-like membrane current that allows the intracellular release of ATP in oocytes (Barbe et al., 2006). Moreover, studies have shown that Pannexin-1 can be open under mechanical stress and form Ca\(^{2+}\)-activated channel that opens at physiological extracellular Ca\(^{2+}\) concentration (Barbe et al., 2006). The necessity of pannexin-1 to pore formation is controversial. There are also supporting data for the pore dilation theory which proposes that the P2X7 channel pore itself dilates without subsequent activation of pannexin-1.
1.15.2 P2X7 receptor expression and localization

P2X7R can be found in cells of hematopoietic origin including lymphocytes, monocyte-macrophages and microglia, and in intrinsic cells of the nervous system, including neurons, astrocytes, oligodendrocytes and endothelial cells. Originally, it was thought to be expressed in the brain only in activated microglia after inflammation or damage (Collo et al., 1997). In situ hybridization and immunohistochemistry in rat and mouse brain tissue supported by studies in NTW8 cells, a mouse microglial cell line, showed a strong expression of P2X7R in microglia cells. P2rx7 mRNA and protein was also seen in bone marrow cells including granulocytes, monocyte/macrophages and B lymphocytes (Collo et al., 1997). Other lines of work showed specific immunoreactivity of P2X7R in microglia cell type in the rat hippocampus (Rappold et al., 2006).

P2X7R expression in neurons was confirmed by in situ hybridization, where P2rx7 mRNA was found in the cell body layers of granule neurons in the hippocampus and also, by immunoreactivity, where P2X7R was observed expressed in the excitatory synaptic terminals of the CA1 and CA3 hippocampal regions (Sperlagh et al., 2002; Yu et al., 2008). Supporting data revealed the presence of P2X7R by immunohistochemistry and electron microscopy in presynaptic terminals of neurons in the CNS (Deuchars et al., 2001). Western blot analysis and immunocytochemistry also found high concentrations of P2X7R on presynaptic terminals of mossy fiber synapses in rat hippocampus (Armstrong et al., 2002). Other evidence for constitutive localization of the P2X7R in dentate granule hippocampal neurons comes from studies by our group using EGFP-P2rx7 reporter mice expressing enhanced green fluorescent protein (EGFP) under the control of the P2X7R promoter region (Engel et al., 2012b). The GFP signal was found in scattered dentate granule neurons in the mouse (Engel et al., 2012b).

The presence of P2X7R in astrocytes under normal physiological conditions has been reported by using different techniques, from qPCR to immunofluorescence. An increase of astrocytic P2X7R expression in the nucleus accumbens of rats was
found in a stab wound model (Franke et al., 2001). P2X7R expression has also been reported in cultured astrocytes (Duan et al., 2003). However, there is little evidence of in vivo studies that support the astrocytic expression of P2X7R (Yu et al., 2008; Engel et al., 2012b).

P2X7R expression has also been observed in oligodendrocytes. Western blot and double-immunofluorescence in tissue from rat optic nerve and spinal cord sections and cultured oligodendrocytes (MBP$^+$ cells) showed a localized expression of the receptor in this cell type (Matute et al., 2007; Yu et al., 2008).

### 1.15.3 P2X7 receptor functions

As mentioned, the P2X7R is activated by high concentrations of ATP and its activation increases intracellular Ca$^{2+}$ influx. This then leads to a number of different transduction pathways involved in diverse functions (North, 2002). Probably the best understood consequence of P2X7R activation is the processing and release of the prototypical inflammatory cytokine IL-1$\beta$ (Suzuki et al., 2004). IL-1$\beta$ is translated as a 31-kDa inactive secretory protein and a proteolytical step is needed for it to be converted to the active 17-kDa mature cytokine. Many activated proteases process the pro-IL-1$\beta$ extracellular, but intracellularly the only protease responsible for IL-1$\beta$ maturation and its release is caspase-1 (Hazuda et al., 1990). Notably, caspase-1 is activated in human and experimental TLE (Henshall et al., 2000b; Vezzani et al., 2010). P2X7R has also been shown to trigger the activation of the cytoplasmic protein complex (nucleotide-binding oligomerization domain-like receptor) necessary for caspase-1 activation (Wen et al., 2010). These prototypical inflammatory cytokines have been shown to be up-regulated in activated microglia and astrocytes, triggering a cascade of downstream inflammatory events that also involves neurons in human and experimental epilepsy (Monif et al., 2009; Vezzani, 2014). As mentioned before, several studies have reported the increase of IL-1$\beta$ expression and the activation of its signalling pathway in epilepsy (Ravizza et al., 2008; Maroso et al., 2010) and anticonvulsant effects after administration of the IL-
IL-1β endogenous receptor antagonist, IL-1Ra (Vezzani et al., 2000). Additionally, NF-κB pathway can also be activated by IL-1β and TNF-α (Medzhitov and Horng, 2009). NF-κB is a protein complex that its activation leads to the transcription of genes involved in proinflammatory immune responses, cytokine production and cell survival (Ghosh and Hayden, 2008). In addition, P2X7R activation has been linked to the CREB transcription factor phosphorylation and activation. The formation of CREB/CBP complex is mediated by extracellular calcium influx and promotes expression of various genes (Wen et al., 2010). CREB activation by P2X7R has been reported to promote an anti-apoptotic survival signal (Wen et al., 2010). However, CREB also has been linked to increased hyperexcitability and its overexpression may promote epilepsy (Zhu et al., 2012b).

1.16 P2X7 receptor expression and function in diseases of the CNS

A common observation has been the upregulation of the P2X7R in numerous experimental models and human brain in chronic neurological disorders. Increased levels of P2X7R have been observed in the hippocampus and cortex of Alzheimer’s disease models (Diaz-Hernandez et al., 2012) and in the striatum in experimental models of Huntington’s disease (Diaz-Hernandez et al., 2009). Additionally, P2X7R induction has been observed in degenerating cells in Parkinson’s disease animal models (Marcellino et al., 2010; Hracsko et al., 2011). Furthermore, increased of P2X7R have been reported in experimental models of ischemia and traumatic brain injury (Arbeloa et al., 2012; Kimbler et al., 2012).

Functional evidence for the involvement of P2X7Rs in pathologies of the CNS has also been obtained. A reduction of β-amyloid-induced microglia activation was observed in P2X7R−/− mice as well as reduced β-amyloid-induced cell death using P2X7R inhibitors (Parvathenani et al., 2003; McLarnon et al., 2006; Sanz et al., 2009; Diaz-Hernandez et al., 2012). A reduction in neurodegeneration and motor coordination deficits was reported with the selective P2X7R inhibitor, Brilliant Blue G (BBG), in a Huntington’s disease model (Diaz-Hernandez et al., 2009). P2X7R
inhibition using BBG also attenuated contralateral rotations in a rat model of Parkinson’s disease (Carmo et al., 2014). Supporting an involvement in CNS diseases, time-dependent up-regulation of the P2X7R was also observed in neurons and glia within the cortex of rats in a model of focal cerebral ischemia (Franke et al., 2004). An improvement in recovery has been shown after the inhibition of the P2X7R by the P2X7R antagonist oxidized ATP, in the spinal cord injury model (Peng et al., 2009). Increased levels of the P2X7R and its altered expression have also been linked to other neurodegenerative diseases including amyotrophic lateral sclerosis (Cervetto et al., 2013), mechanical injury (Franke et al., 2001), and energy deprivation (Cavaliere et al., 2002; Cavaliere et al., 2004), among others. Moreover, enhanced expression of P2X7R was also observed in human tissue obtained from multiple sclerosis (MS) (Narcisse et al., 2005) and sensory nerve injury patients (Chessell et al., 2005). Thus, the therapeutical potential of P2X7R inhibition is broadly emerging for diseases of the CNS.

1.16.1 P2X7R in epilepsy

The first study to report P2X7R changes in epilepsy was by Vianna et al. in 2002 (Vianna et al., 2002). Using the pilocarpine model, they reported that hippocampal slices treated either with ATP or BzATP, a specific P2X7R agonist, increased intracellular Ca\(^{2+}\) due to an enhanced expression of P2X7R. Subsequently, a role for P2X7R in epilepsy was suggested by evidence of enhanced P2X7R immunoreactivity in microglia in the hippocampus and cortex after SE in rats (Rappold et al., 2006; Avignone et al., 2008). In addition, P2X7R immunoreactivity was also observed in the terminal nerves of CA3 and DG hippocampal subfields in epileptic rats (Dona et al., 2009). Western blot analysis showed increased levels of P2X7R protein in hippocampal samples 72 h after the induction of SE by intra-amygdala KA in mice (Engel et al., 2012b).

Although these studies supported up-regulation of the P2X7R in epilepsy, the main observations depended on immunostaining or Western blotting. Concerns have
been raised, however, with the specificity of P2X7R antibodies (Sim et al., 2004). To overcome the limitations of those techniques, work by our group took an alternative approach. EGFP-P2X7R reporter mice, which express EGFP under the transcriptional control of the P2rx7 promoter, were used to study basal expression of the P2X7R and responses to SE (Engel et al., 2012b). GFP induction was observed in scattered dentate granule neurons in control and in neurons after SE in the hippocampus, particularly in the dentate granule neurons and also in some pyramidal neurons of the CA1 region (Engel et al., 2012b). Surprisingly, neither microglia nor astrocytes showed a GFP induction in the hippocampus after SE (Engel et al., 2012b; Engel et al., 2012a).

Other purinergic receptors such as the P2X4R, P2X2R or the P2X1R have also been reported to be regulated in models of SE and chronic epilepsy (Dona et al., 2009; Engel et al., 2012b; Henshall et al., 2013; Ulmann et al., 2013). Additionally, purinergic signalling has been shown to be increased after SE and a positive feedback mechanism that intensified the activation process has also been proposed (Avignone et al., 2008).

Although there is significant support for a pro-inflammatory and pro-excitability role of P2X7Rs in epilepsy, there are also conflicting findings. Seizures induced by KA or picrotoxin (a GABA_A receptor antagonist) were not altered in mice lacking P2X7R, suggesting that P2X7R may not always be important in seizures (Kim et al., 2011b; Henshall et al., 2013). Seizures in the pilocarpine model are in fact enhanced in mice lacking P2rx7 (Kim and Kang, 2011). Additionally, some studies have reported P2X7R expression in astrocytes in vitro (Duan et al., 2003). P2X7R involvement in gliotransmitter release through Ca^{2+}-independent mechanisms in chronic epilepsy and in astrocytic apoptosis and leukocyte infiltration after SE in pilocarpine-injected rats has also been reported (Kim et al., 2010, 2011b; Crunelli et al., 2015).


1.17 Pharmacology of the P2X7R receptor

In recent years there have been important advances in our understanding of the pharmacological and physiological characteristics of purinergic receptors, with the development of a number of potent and selective antagonists for each P2X subtype (Gever et al., 2006). The first antagonists produced included suramin and pyridoxal-5’-phosphate-6-azo-phenyl-2,4-disulfonate (PPADS). However, both showed a low specificity also blocking other P2X receptors. Oxidized ATP (oxATP) is an ATP analogue compound used as a non-selective P2X antagonist. Subsequently, a number of suramin analogs were synthesized, including NF023 and NF279, and PPADS analogs, such as MRS2220 and PPNDS. Many of these showed superior selectivity for different purinergic receptors including P2X1R, P2X3R, P2X4R and P2X7R although they showed low affinity and noncompetitive antagonisms (Jacobson et al., 2002; Gever et al., 2006).

Other advances in the development of P2X7R ligands have resulted in the identification of several different classes of P2X7R antagonists, including dyes BBG, tyrosine derivatives (KN-62 and KN-04), cyclic imides, adamantane and benzamide derivatives. KN-62 is a noncompetitive uncharged antagonist ligand that has been reported for the use in receptor binding assays (Gunosewoyo et al., 2007). In addition, BBG is a potent noncompetitive P2X7R antagonist that strongly inhibited P2X7R-mediated currents in individual cells expressing P2X7R in rodents and human (Jiang et al., 2000). A more extensive range of potent, selective P2X7R ligands are required for a better understanding of the cascade of cellular processes associated with the P2X7R. For a list of the different antagonists available, please see Table 1.3 and Figure 1.8.

CE-224,535 is a selective antagonist of the human P2X7R that was developed as a disease-modifying antirheumatic drug (DMARD). Clinical trials anticipated that the antagonist would reduce leukocyte secretion of IL-1ß and IL-18. However, studies were discontinued due to the lack of clinical efficacy. CE-224,535 showed non
analgesic and anti-inflammatory efficacy in clinical trials in subjects with osteoarthritis pain of the knee (Stock et al., 2012). In addition, good results in tolerance responses and no sign of secondary effects were reported using other P2X7R antagonists such as AZD9056 in patients with chronic inflammation of the gastrointestinal tract (Ochoa-Cortes et al., 2014). Moreover, clinical studies in patients suffering from rheumatoid arthritis (RA) showed a significant inhibition of ex vivo ATP-induced monocyte IL-1β release after the administration of AZD9056 (Keystone et al., 2012). Another antagonist with potent specificity for the P2X7R is A438079 (3 - (5 - (2, 3 - dichlorophenyl0-1H-tetrazol – 1 -yl) methyl pyridine). An advantage of this antagonist is the reported inexistent interaction with a wide array of other cell-surface receptors and ion channels (Nelson et al., 2006; Donnelly-Roberts et al., 2009a).

### 1.17.1 P2X7R involvement during status epilepticus

Evidence of a causal P2X7R involvement during SE has been obtained using different P2X7R antagonists and genetic models. P2X7R involvement in microglia activation was shown by the administration of P2X7R antagonists such as BBG and PPADS, into hippocampal slices from SE-induced mice. Results showed an increase in microglia membrane currents during the administration of the P2X7R agonists and microglia membrane currents blockage during P2X7R antagonist infusion to the hippocampal slices (Avignone et al., 2008). BzATP, a P2X7R agonist, infusion was reported to increase microglia activation after SE whereas inhibition of P2X7R by OxATP, a P2X7R antagonist, reduced microglia activation in the rat hippocampus and the frontoparietal cortex (Choi et al., 2012). However, microglia activation in other brain areas such as the piriform cortex were not affected by either BzATP or OxATP-infusion after SE. These findings suggest that P2X7 receptor function differently modulates SE-induced microglial responses in distinct brain regions (Choi et al., 2012). Additionally, other studies have reported reduction of astroglial degeneration and microglial activation after treatment with PPADS and suramin,
### Table 1.3 P2X7 receptor antagonists

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Description</th>
<th>Mouse P2X7R C57BL/6 (pIC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Rat P2X7R (pIC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>Human P2X7R (pIC&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A438079 hydrochloride</td>
<td>Competitive P2X7R antagonist inactive at other P2XRs</td>
<td>6.26 ±0.07</td>
<td>6.51±0.03</td>
<td>6.91±0.01</td>
</tr>
<tr>
<td>Brilliant Blue G</td>
<td>Commonly used analytical biochemistry dye and P2X7R antagonist</td>
<td>&lt;4</td>
<td>5.09±0.06</td>
<td>&lt;4</td>
</tr>
<tr>
<td>KN-62</td>
<td>Potent non-competitive antagonist at P2X7R</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>4.97±0.12</td>
</tr>
<tr>
<td>A740003</td>
<td>Potent selective and competitive P2X7R antagonist</td>
<td>6.17±0.05</td>
<td>7.74±0.02</td>
<td>7.36±0.01</td>
</tr>
<tr>
<td>A8084598</td>
<td>A P2X7R antagonist</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GW 791343 hydrochloride</td>
<td>A P2X7R allosteric regulator; Positive allosteric modulation in rat P2X7R and negative in human P2X7R</td>
<td>-</td>
<td>-</td>
<td>6.9±0.07</td>
</tr>
<tr>
<td>A 839977</td>
<td>A potent P2X7R antagonist</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AZ10606120</td>
<td>A potent negative allosteric modeulator</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JNJ-47965567</td>
<td>A potent selective P2X7R antagonist</td>
<td>-</td>
<td>7.1±0.1</td>
<td>7.5±0.07</td>
</tr>
</tbody>
</table>

Adapted from (Michel et al., 2008; Donnelly-Roberts et al., 2009b; Guile et al., 2009; Gum et al., 2012) pIC<sub>50</sub> = The half maximal inhibitory concentrations (measurement of the effectiveness of a substance in inhibiting a specific biochemical function).

P2X7R antagonists, after SE in rats (Kim et al., 2009). The treatment with the P2X7R antagonist OxATP or BBG, showed both reduced astroglial loss in the molecular layer of the DG hippocampal subfield and in the frontoparietal cortex. In contrast, treatment with the P2X7R agonist BzATP, exacerbated astroglial death in the same brain areas (Kim et al., 2011b).
Figure 1.8 Structures of prototypical and novel antagonists of the P2X7R

PPADS, suramin and oATP non-selective antagonists; K62 and AZ compounds exhibit higher potency for the human than rat P2X7R, contrary to BBG that showed best effects on the rat P2X7R. A-740003 and A-804598 showed equal potency for both rat and human P2X7R. A43 and JNJ exhibited high specificity for P2X7R in rodents (Ray et al., 1989; Donnelly-Roberts et al., 2009b; Jarvis, 2010).
In other studies, BzATP, a specific P2X7R agonist, increased TNF-α immunoreactivity after SE in the DG hippocampal subfield and decreased neuronal damage in the CA3 hippocampal subfield. Moreover, the administration of several P2X7R antagonists including OxATP-, A438079 and A740003 increased CA3 hippocampal damage after pilocarpine-SE in rats (Kim et al., 2011a).

Work by our group provided the first experimental evidence that blocking the P2X7R could reduce seizures during SE. Both A438079 and BBG, P2X7R antagonists, decreased seizure time during SE in the intra-amygdala KA model in mice (Engel et al., 2012b). The hippocampus of these mice also displayed reduced neuronal loss. Levels of microglia activation and IL-1β expression were reduced by A438079 and BBG (Engel et al., 2012b). The co-treatment of A438079 with Lorazepam was also shown to have seizure-suppressive effects in a model of refractory SE (Engel et al., 2012b). Collectively, these data indicate P2X7R ligands may have potential use as anticonvulsants.

1.18 Molecular mechanisms regulating P2X7R expression

Although a number of findings indicate that altered P2X receptor expression has a causative role in neurodegenerative diseases (Tsuda et al., 2010; Burnstock et al., 2011; Diaz-Hernandez et al., 2012), not much is known about the control and regulation of the P2rx7 gene. P2X7R expression may be controlled by transcription factors, epigenetic mechanisms or at post-transcriptional or post- translational levels.

1.18.1 Transcription factors

Transcription factors are proteins that bind to DNA regulatory sequences. They function to promote or block the transcription of target genes. Moreover, transcription factors can bind multiple regulatory sites of the genome and due to
their multiple downstream consequences after their action, are considered to be instrumental regulators of gene expression.

A recent study has provided molecular and functional evidence that the Specific protein 1 (SP1) transcription factor plays a pivotal role in the transcriptional regulation of P2rx7 gene. SP1 is the founding member of a Zinc finger transcription factors family. The zinc finger is a small protein structural motif that coordinates one or more zinc ions to stabilize the fold; bind directly to DNA and enhances gene transcription (Rotheneder et al., 1999). SP1 is widely expressed in tissues, including brain and binds to GC box motifs in DNA promoter regions (Chu and Ferro, 2005).

Garcia-Huerta and colleagues analysed a demarcated region with four SP1 sites of the murine P2rx7 promoter using different techniques including site-directed mutagenesis. They showed a direct SP1 functionality on the P2rx7 promoter sites in neuroblastoma cells (N2) (Garcia-Huerta et al., 2012). In this study, Mithramycin A (Myt A) was used to inhibit SP1-mediated transcriptional activation. Results showed a reduction in endogenous P2X7R levels after Myt A administration in primary cultures of cortical neurons and astrocytes (Garcia-Huerta et al., 2012).

1.18.2 DNA methylation

Another important emerging field to study during epileptogenesis is the ‘long-lasting’ changes caused to the transcriptional state after the initial stimulus. There are epigenetic changes in gene expression that are not involved in nucleotide sequences alterations but could have a very important role in transcriptional regulation (Meaney and Ferguson-Smith, 2010). Therefore, epigenetic mechanisms have become a new study target as reversible processes subject to acute dysregulation during neurological diseases including epilepsy (Graff et al., 2011).

Methylation is an epigenetic process/mechanism controlling the transcriptional state of DNA, promoting either gene activation or gene repression (Hwang et al., 2013). DNA methylation takes place at cytosine bases, which are transformed to 5-
methylcytosine by enzymes known as DNA methyltransferase (DNMT). Guanine nucleotides are normally found immediately beside modified cytosine, therefore two methylated cytosine residues can be found in a diagonal position on opposite DNA strands. Cytosine methylation is usually restricted to CpG dinucleotides sequences in the genome, but in some particular regions, CpG islands, which are DNA regions with a high CpG linear sequence, can be found. The methylations of these CpG islands may lead to an alteration in gene silencing (Mostoslavsky and Bergman, 1997; Hwang et al., 2013). Methylation seems to be an actively maintained epigenetic state, although it has been observed that there is a dynamic developmental regulation of methylations and that methylations or demethylations of the genome frequently occur under certain conditions (Reik et al., 2001).

Although methylation of the P2X7R promoter has not been reported, the regulation of other purinergic receptors by methylation has been explored, including A2A. Studies in rat striatum showed that A2A receptor expression is controlled by S-adenosyl-L-methionine-mediated methylation (Villar-Menendez et al., 2014). Moreover, P2Y12 receptor expression has also been associated with gene promoter DNA methylation in clopidogrel resistance in coronary artery disease patients (Su et al., 2014).

A final potential mechanism controlling P2X7R expression is through microRNAs. These are small non-coding RNA molecules which act post-transcriptionally to reduce protein levels in cells (Ambros, 2004). P2X7R has been suggested to be regulated by several microRNAs such as miR-150 and miR-186 in cancer cells (Zhou et al., 2008). However, the transcriptional control of P2X7R through microRNA within the brain has not been explored.
1.19 Hypothesis and objectives of the thesis

The following hypothesis were tested:

1. Expression of the P2X7R is altered in the neocortex after SE in the intra-amygdala KA model
2. Expression and function of the P2X7R is altered in the hippocampus in epileptic mice in the intra-amygdala KA model
3. Treatment of epileptic mice with P2X7R antagonists will reduce spontaneous seizures and have anti-inflammatory, disease-modifying effects.
Chapter II - Material and Methods

2.1 Materials

Chemicals and General Reagents

See Table 5 for a list of chemicals and reagents including catalogue number and manufacturers.

Antibodies

See Table 6 for a list of the antibodies used for this study including catalogue number and manufacturers.

Primers

See Table 7 for a list of all primers with their respective sequences.
2.2 Methods

2.2.1 In-vivo model of experimentally induced status epilepticus and chronic epilepsy

2.2.1.1 Animals and husbandry

All animal experiments were performed in accordance with the principles of the European Communities Council Directive (86/609/EEC) and the ‘National Institute of Health’ Guide for the Care and Use of Laboratory Animals. Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC 205) under license from the Department of Social and Family Affairs. All efforts were maximised to reduce the number of animals used in this study.

Mice used for this study were male adult C57BL/6 mice (20-25g), 6-9 weeks old and were obtained from Harlan Laboratories (Bicester, UK).

P2X7R reporter mice [Tg(P2rx7-EGFP)FY174Gsat/Mmcd, stock 011959-UCD] expressing enhanced green fluorescent protein (EGFP) immediately downstream of the P2rx7 promoter were obtained from U.S. National Institutes of Health Mutant Mouse Regional Resource Centers and granted by Dr. M. Nedergaard (University of Rochester, Rochester, NY USA).

Animals were housed in a controlled biomedical facility on a 12 h light/dark cycle (8am-8pm) at 22±1 °C and humidity of 40-60% with food and water provided ad libitum.
2.2.1.2 Surgery

Surgical procedures were performed by the author and Dr. Tobias Engel. Animals were anesthetized using isofluorane (3 - 5 % in oxygen), and placed into a stereotaxic frame and body temperature was maintained by means of a feedback-controlled heat blanket (Harvard Apparatus Ltd) at 37 °C.

A midline incision was made and the skull was exposed. Three partial craniotomies were performed using a drill on the skull surface: one in the frontal cortex and the other two overlying the hippocampi. Then, screw electrodes where inserted to affix the skull-mounted electroencephalogram (EEG) (Bilaney Consultants Ltd). Following coordinates from Bregma, the anatomical point were the coronal suture perpendicularly crosses the sagital suture on the skull, a smaller cranial hole was made for the placement of the guide cannula at the following coordinate: AP=0.94 mm; L=2.85mm for the intra-amygdala injection (Engel et al., 2010). The guide cannula was then lowered to the surface of the mouse brain. Finally, the entire assembly was fixed using dental cement on top of the skull and allowed to dry completely. Afterwards, mice were moved into a 25 °C incubator to recover before induction of SE (Figure 2.1. A).

2.2.1.3 Status Epilepticus induction

For seizure induction, a 31-gauge internal cannula was lowered through the guide cannula and inserted 3.5 mm below the cortical surface to target the central and basolateral amygdala nucleus (Figure 2.1. B). Kainic acid 0.3 µg (KA) (Sigma-Aldrich) was then injected in a volume of 0.2 μl of phosphate-buffered saline (PBS, pH 7.4). Mice were connected to an EEG system and EEG was recorded for a duration of 40 minutes using a Grass Comet XL digital EEG (Medient Ltd). The anticonvulsant lorazepam (6 mg/kg) (Wyeth, Taplow, UK) was administrated intraperitoneally (i.p.) to curtail SE at 40 min post-KA injection. Mice were killed at different time points or
returned to clean cages and housed in a climate-controlled biomedical facility for 14 days.

### 2.2.1.4 Drug injections

A-438079 (Tocris Biosciences, Bristol, United Kingdom), a specific P2X7R, was delivered intracerebroventricularly (i.c.v.) (coordinates from Bregma: AP = 0.4 mm; L = 0.95 mm) 10 min prior to KA injection and 60 min post-KA injection. Intracerebroventricularly injections were carried out by Dr. Tobias Engel. Mithramycin A (150 μg/kg) (Santa Cruz Biotech), a specific SP1 inhibitor, and JNJ-47965567 (30 mg/kg) (Janssen R+D LLC, San Diego, CA, USA), a blood-brain barrier brain stable P2X7R inhibitor (Bhattacharya et al., 2013) were injected intraperitoneally (i.p.) with a 25-gauge needle. Both were administrated 10 days after SE induction, twice daily for 5 days. Control animals were injected with vehicle solutions. Drug concentrations injected are indicated in Table 2.1.

### Table 2.1 Drug injection dose

<table>
<thead>
<tr>
<th>Name</th>
<th>Stock</th>
<th>Vehicle</th>
<th>Quantity administrated</th>
<th>Dose given (for a ~ 25g mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-438079</td>
<td>10 mg</td>
<td>PBS</td>
<td>2 μl</td>
<td>1.75 nmol</td>
</tr>
<tr>
<td>Mithramycin A</td>
<td>1 mg</td>
<td>H₂O and Methanol</td>
<td>200 μl</td>
<td>150 μg/kg</td>
</tr>
<tr>
<td>JNJ-47965567</td>
<td>200 mg</td>
<td>BSE 30% in H₂O</td>
<td>100 μl</td>
<td>30 mg/kg</td>
</tr>
</tbody>
</table>
Figure 2.1 Surgical procedure for intra-amygdala injection of KA in mice

A) Electrode placement on the mouse skull. Two electrodes were placed approximately above the hippocampus, one on each hemisphere. A reference third electrode is placed on the frontal lobe. The cannula for KA injection site is indicated by a star. B) Schematic coronal section showing the intra-amygdala targeting of the internal cannula for KA injection.
2.2.2 Behaviour analysis of seizures

Mice were observed during KA-induced seizures and scored according to a modified Racine scale for mice (Borges et al., 2003). See Table 2.2 for different Score features. For the original Racine Score System, please see Chapter I. Mice were scored every 5 min for 40 min after KA injection. The highest score attained during each 5 min period was recorded by an observer blinded to treatment.

Table 2.2 Racine Score System

<table>
<thead>
<tr>
<th>Score number</th>
<th>Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal activity</td>
</tr>
<tr>
<td>1</td>
<td>Arrest and rigid posture or immobility</td>
</tr>
<tr>
<td>2</td>
<td>Stiffened or extended tail</td>
</tr>
<tr>
<td>3</td>
<td>Partial body clonus</td>
</tr>
<tr>
<td>4</td>
<td>Rearing</td>
</tr>
<tr>
<td>5</td>
<td>Rearing and falling</td>
</tr>
<tr>
<td>6</td>
<td>Tonic-clonic seizures with loss of posture or jumping</td>
</tr>
</tbody>
</table>

(Borges et al., 2003)

2.2.3 EEG analysis of long term telemetry recording

For JNJ-47965567 long-term studies and the analysis of the time of onset of spontaneous, recurrent seizures following SE induction, telemetry devices (Data Systems International, St. Paul, MN) were used and seizures were analysed as previously described (Mouri et al., 2008b). Telemetry devices were implanted in the back of mice in a subcutaneous pocket to record spontaneous seizures during epilepsy. After the electrode cranial surgery, the transmitter unit was implanted under the skin, between the shoulders of the mice and skin was sewn and wiped with iodine solution to avoid infection. The transmitter unit was then activated and
cortical surface EEG was recorded in freely-moving mice in cages placed on top of receivers (RPC-1). Continuous EEG data was transferred to a PC via a Data Exchange Matrix (DSI). All mice used in the telemetry study were subjected to three weeks of 24 h/day EEG recordings after KA microinjection. Continuous video recording was also used to allow confirmation and observed characteristic features of the spontaneous seizures. All telemetry unit implantation surgeries in this study were performed by Dr. Tobias Engel (Figure 2.2).

![Telemetry recordings for epilepsy monitoring](image)

**Figure 2.2 Telemetry recordings for epilepsy monitoring**
Schematic showing mouse fitted with a transmitter unit on a receiver plate connected to a computer recording EEG 24 h. Camera recordings monitored 24 h a day behaviour.

### 2.2.4 Open field behaviour test

A large square chamber of dimensions 30 cm long x 30 cm wide x 15 cm height was used for the open field test as described before (Prut and Belzung, 2003). The chamber floor was white plastic and walls were of transparent Plexiglas. Animals were positioned in the centre of the arena at the beginning of the analysis and were allowed to explore the arena for 10 min. Mice were recorded by a camera placed on top of the chamber where the entire arena could be visualized (Figure 2.4). The chamber was always cleaned with 70 % EtOH between animals and trials. Total
distance travelled, velocity and time spent in the demarcated central zone of the arena were automatically analysed using Ethovision videotracking (Ethovision).

Figure 2.3 Open field arena
Arena used for the open field test. Dimension of the cage was 30 cm x 30 cm x 15 cm. Periphery and inner areas are indicated by dashed lines.

2.2.5 Brain microdissection

Mice were anesthetised by isofluorane and euthanized by cervical dislocation at different time points 1 h, 4 h, 8 h and 24 h and 14 days after status epilepticus. Decapitation was performed using sharp laboratory scissors and the brain was removed and placed into a standard petri dish, placed on top of a cold cutting board stored at -20 °C prior to use. Using a scalpel, the cerebellum was removed and brain hemispheres were divided. The mid brain was removed and the cortex from both hemispheres was separated from the brain structure and the hippocampi from both hemispheres were separated from the cortical structure. When subfields separation was required for analysis, the three hippocampal divisions (CA1, CA3...
and DG) were identified under a dissection microscope and separated from each other carefully using tweezers (Figure 2.4). Hippocampal subfields were quickly placed in separate reagent tubes and stored on dry ice before being transferred to a – 80 °C freezer.

Figure 2.4 Microdissection of hippocampal subfields
Hippocampus was removed from the brain and placed with the surface down. Using curved tweezers, tissue was separated into three samples as indicated by the dashed lines in CA1, CA3 and DG (Elmer et al., 1998).

2.2.6 Western blotting

2.2.6.1 Protein extraction

Tissue was homogenized using lysis buffer (150 mM NaCl, 50 mM Tris HCl pH8, 1% NP-40, 1mM EDTA pH8). Phosphatase and proteases inhibitors (1:1000 Aprotinin, 1:500 PMSF, 1:1000 Leupeptin, 1:1000 Vanadate) were freshly added to the lysis buffer directly before use. Guideline amounts of lysis buffer are: 100 μl per hippocampal subfield, 300 μl per whole hippocampus. Tissue was homogenized using a small plastic plunger in a 1.5 ml reagent tube. Once tissue was efficiently homogenized, samples were kept at 4 °C until protein quantification was performed. No final spin step was performed due to whole study was based on membrane receptors.
**2.2.6.2 Protein quantification**

Protein concentration was determined using the Micro BCA method (Micro BCA Protein Assay kit, Thermo Scientific) in a 96 well plate. For standard curve calibration 0, 1, 2, 3, 4 and 5 μl of 2 μg/μl BSA (bovine serum albumin) was added in wells in triplicate to 150 μl of 0.9 % NaCl. Then, 2 μl of sample was added in triplicate to 150 μl of NaCl. Reagents A, B and C from the kit were then mixed together in the following ratio 25:25:1 (v:v:v) and added to all wells. The plate was incubated in darkness at room temperature for approximately 15 min. Protein quantification was measured by absorbance using a Tecan plate reader at 560 nm.

**2.2.6.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins by size. Polyacrylamide gels are composed of two layers, a stacking gel, which has large pores and low concentration of binding agents and below this one, the resolving gel, with varying concentrations and pore size chosen depending on the size of the protein of interest. Guideline gel concentrations are depending on protein size: 15 % gel for 10 - 30 kDa proteins, 12% gel for 30 – 65 kDa proteins, 10 % gel for 66 – 100 kDa proteins and 8 % gel for 100 – 120 kDa proteins. The composition of the resolving gel is as follows: dH₂O, Tris-HCl 1.0 M, pH 6.8, 40 % Acrylamide (Sigma), 10 % SDS, 10 % ammonium persulfate (APS), tetramethylethlenediamine (TEMED). Once polymerized, gels were placed into the mini-protean tetra electrophoresis system container (BioRAD) and tank filled with 1 X gel electrophoresis buffer (250 mM glycine, 25 mM Tris-HCl, pH 8.3, 0.1 % SDS). Before loading, samples were heated in a heating block at 95 °C for 10 min. The first well of every gel was loaded with a protein size marker (Fermentas, PageRuler Plus.
Broad Range 10 – 250 kDa). Gels were run at 120 V until the blue dye front reached the end of the glass plates.

2.2.6.4 Protein Transfer

After electrophoresis, proteins were transferred onto nitrocellulose (ProTan) or PVDF membranes using semi-dry transfer. Filter paper (Whatman) and membranes were pre-soaked in semi-dry transfer buffer (25 mM Tris, 192 mM Glycine, 20 % methanol (v/v), pH 8.3). PVDF membranes were activated by the immersion in methanol during 2 min before use. Gels were placed in the following order from bottom to up: two soaked filter paper, one membrane, gel and two more soaked pieces of filter paper. The transfer was performed at 18 V for approximately 90 min. To assess transfer efficiency, 1 X Ponceau Red was used. After washing with TBST-T, membranes were blocked for 1 h at room temperature in 5 % w/v skimmed milk in Tris-buffered saline solution with Tween 20 (TBST: 1 mM Tris-HCl, pH 8.0, 15 mM NaCl, 0.05 % Tween 20).

2.2.6.5 Immunoblotting

Membranes were incubated in 5% non-fat milk with primary antibodies overnight at 4 °C shaking (different antibody concentrations used are indicated in Table 6). Excess primary antibody was removed from membranes by washing with TBS-T, 4 times, for 5 min each. The appropriate secondary antibody Horse-radish peroxidise-conjugated goat anti-rabbit or anti-mouse antibodies (dilution 1/5000 Millipore) was used to incubate the membranes for 2 h at RT. Membranes were washed again in TBS-T a minimum of 4 times for 5 min each. Membranes were immersed for 1 min in Imobilon western HRP substrate (Millipore) and then placed into Fujifilm LAS-4000 system under chemiluminescence to visualize protein bands.
2.2.6.6 Densitometry/quantification of protein signal

Images were analysed with AlphaEaseFC software (Stand Alone) using the SpotDenso option to determine the optical density of each band. Background correction was performed to all the bands detected in the membranes. Values of proteins were normalized to the loading control (β-actin or α-tubulin). Values for control vehicle-injected animals were set to 1 and changes were calculated in fold change as the ratio of change.

2.2.7 mRNA expression analysis

2.2.7.1 Homogenisation

Hippocampal and cortical tissue was obtained from mice sacrificed by cervical dislocation as described previously. Whole hippocampus or hippocampal subfields were immersed in 800 µl of Trizol reagent (QIAzol Lysis Reagent, Quiagen) and homogenized using a dounce homogenizer. Samples were then centrifuged at 12000 x g for 10 min at 4 °C. Supernatants were transferred to fresh reagent tubes and pellets containing membranes and cell debris were discarded.

2.2.7.2 Phase Separation

Samples were incubated for 5 min at RT and 200 µl of chloroform was added to each tube. Samples were manually shaken vigorously for 15 sec. Samples were incubated for 3 min at RT and then centrifuged at 13600 rpm for 15 min at 4 °C.

2.2.7.3 RNA precipitation

After the final centrifugation, two separate layers appeared, separated by a white dense interface. The top layer, the clear aqueous phase, was removed and transferred to a clear reagent tube. To precipitate mRNA, 450 µl of 2-isopropanol
(Sigma) was added to each sample and samples were shaken and kept at -20 °C overnight.

### 2.2.7.4 RNA purification

The following day, samples were centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant was carefully removed and discarded. The pellet was washed with 800 μl of 75 % EtOH. Samples were then mixed by vortex and centrifuged at 12000 x g for 5 min at 4 °C.

### 2.2.7.5 Re-dissolving the mRNA

EtOH was discarded and pellets were dried by opening the lid and leaving them at RT for 1 h until total EtOH evaporation. The pellet was then dissolved in 25 μl of DEPC water and incubated in a heating block at 60 °C for 10 min at 800 rpm. Quantification of mRNA was measured by Nanodrop (Eppendorf, UK) and samples were stored at -80 °C.

### 2.2.8 cDNA Synthesis and qPCR

#### 2.2.8.1 cDNA synthesis (Reverse transcriptase PCR)

#### 2.2.8.1.1 DNA degradation

To synthesize complementary DNA (cDNA), 1 μg (in 8 μl volume) of the extracted RNA was added to 1 μl of 10 X DNase I reaction buffer (Invitrogen) and 1 μl DNase I in DEPC water to a final volume of 10 μl. Samples were quickly spun down and incubated for 15 min at RT. To stop the reaction 1 μl of 25 mM EDTA (Invitrogen) was added to each sample and heated at 65 °C for 10 min to completely degrade DNA.
2.2.8.1.2 Reverse transcription to cDNA

For the reverse transcription to cDNA, 1 µl of random primers (doxi NTD polyT=dN6 primers) (Fermentas) was added to each sample and placed on the thermocycler under the following program:

1. 65 °C 5 min
2. 4 °C 1 min
3. 25 °C 10 min

During this process, the mastermix was prepared as follows: 4 µl 5x buffer RT (Invitrogen), 2 µl 0.1 M DTT (Invitrogen), 1 µl of 10 µM (Invitrogen), dNTPs (Fermentas), 0.5 µl RNaseOUT inhibitor enzyme (Invitrogen) and 0.5 µl of Reverse Transcriptase superscript II (Invitrogen). The program was then paused after step 2 was completely finished and 8 µl of the Master mix was added to each sample except to the negative control where the RT superscript II retro transcriptase was replaced by water. The PCR cycle was then continued:

4. 42 °C 50 min
5. 72 °C 15 min
6. 4 °C 5 min

Remaining cDNA was stored at -20 °C.

2.2.8.1.3 qPCR (SYBR Green method)

Real time quantitative polymerase chain reaction (RT-qPCR) was carried out on a Lightcycler 2.0 (Roche) using Quantitete SYBR Green PCR kits (Quiagen) and custom designed primers (Primer 3.0, Sigma Aldrich) for the selected target genes. The Master mix was prepared as follows: 10 µl Sybrgreen, 1 µl of primer mix (75 µl H2O, 12.5 µl forward primer and 12.5 µl reverse primer) and 7 µl RNase/DNase free H2O (Invitrogen). cDNA samples (2 µl) were placed in capillaries (Roche) and 18 µl of the
mastermix was added to each capillary. Capillaries were capped and spun down briefly at approximately 2000 rpm. Samples were run using the following program (Table 2.3):

Table 2.3 RT-qPCR cycles

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Target temp</th>
<th>Inc Time</th>
<th>Trans Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre Inc</td>
<td>95 °C</td>
<td>15 min</td>
<td>20.00</td>
</tr>
<tr>
<td>Amplification</td>
<td>95 °C</td>
<td>15 sec</td>
<td>20.00</td>
</tr>
<tr>
<td>50 times</td>
<td>55 °C</td>
<td>20 sec</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>40 sec</td>
<td>0.00</td>
</tr>
<tr>
<td>Melting Curve</td>
<td>94 °C</td>
<td>0 sec</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>65 °C</td>
<td>15 sec</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>95 °C</td>
<td>0 sec</td>
<td>0.10</td>
</tr>
<tr>
<td>Cooling</td>
<td>40 °C</td>
<td>30 sec</td>
<td>20.00</td>
</tr>
</tbody>
</table>

Relative mRNA transcript levels were assessed by normalising to actin using the standard ΔΔ CT method.

2.2.9 Histological studies

2.2.9.1 Fresh frozen tissue

2.2.9.1.1 Brain removal and tissue processing

Mice were anaesthetized and then subjected to cervical dislocation and carefully perfused by transcardial perfusion with 15 ml of PBS. Brains were flash-frozen in 2-methylbutane for 5 min (- 30 °C, on dry ice) and then stored at −80 °C. Coronal cryosections (18 µm thickness) were sliced on a CM1900 cryostat [Leica]. Sections were prepared at the rostral (1.2 mm- 1.4 mm posterior of bregma), medial (1.9 mm – 2.1 mm posterior of bregma) or ventral (2.8 mm – 3.0 mm posterior of bregma) hippocampus (Paxinos & Franklin, 2004).

Immunohistochemistry techniques were performed on sections and were analysed after using a Nikon 2000s epifluorescence microscope (Micron Optical) under
Ex/Em wavelengths of 472/520 nm (green) and imaged using a Hamamatsu Orca 285 camera (Micron Optical). Images were processed using Adobe Photoshop 6.0 (Adobe Systems Software Ireland Limited).

2.2.9.1.2 Fluoro-Jade B staining (FJB)

Neuronal death was assessed using Fluoro-Jade B (FJB) staining (Millipore), a fluorescein derivate, which specifically binds to degenerating neurons (Schmued and Hopkins, 2000). Sections were immersed in formalin for 30 min for fixation. Slides were then transferred to 100 % EtOH for 3 min followed by 1 min immersion in 70 % EtOH. Slides were washed in dH$_2$O for 1 min and then left shaking in 0.06% w/v KMnO$_4$ for 15 min. Then, sections twice were washed for 1 min in dH$_2$O and placed into 0.001 w/v % FJB solution in 0.1 % acetic acid solution for 30 min shaking in darkness. The following steps were performed in darkness. Slides were washed with dH$_2$O 9 times for 1 min to remove excess FJB. Slides were then covered and placed in a 37 °C oven for 2 h, to dry completely. Once slides were completely dry, they were immersed 3 times for 2 min in Histo-Clear solution (National Diagnostics). Slides were mounted with DPX mounting medium (Sigma-Aldrich).

2.2.9.2 Immunohistochemistry and Immunofluorescence

2.2.9.2.1 Free floating sections

Mice were anaesthetized, sacrificed by cervical dislocation and carefully perfused by transcardial perfusion with 5 ml of PBS followed by 15 ml of 4 % w/v parafomaldehyde (PFA). Brains were removed and kept in 4 % w/v PFA for 24 h. For vibratome sectioning, brains were immersed in 1 % w/v agarose until solidified. Sagital sections (30 µm) were taken on a VT1000S vibratome [Leica] at the same levels described in the previous section (2.2.5.1). Sections were placed in cryoprotective solution (30 % Ethylene glycol, 30 % glycerol in PBS) and stored at -20 °C.
2.2.9.2.2 Immunofluorescence microscopy

Free floating sections were washed in PBS for 5 min to remove cryoprotection solution. Sections were then immersed in 0.1 % Triton in PBS for 15 min and then in 1 M glycine for 30 min (Glycine binds free aldehyde groups formed from PFA solution to prevent unspecific background antibody binding). Sections were rinsed in PBS for 5 min and blocked in 1 % w/v bovine serum albumin (BSA) in PBS for 1 h at RT. Samples were incubated in primary antibody (in 1 % BSA) overnight at 4 °C with shaking. The next day, sections twice were washed in PBS for 5 min and incubated in secondary antibody AlexaFluor 488 or 568 (Molecular Probes, OR, USA) (in 1 % BSA) with gentle shaking in darkness at RT for 2 h. Slices were washed twice in PBS for 5 min and labelled with 4′, 6 diamidino-2-phenylindole (DAPI) (Vector Laboratories Ltd, Peterborough, UK) nuclear stain, to visualise nuclei. Tissue was washed in PBS and mounted with FluorSave (Calbiochem). Images were captured using Nikon 2000s epifluorescence microscope or confocal microscopy (Laser scanning Microscope 710 Meta [Zeiss]). Exposures 1 µm optical sections were taken at the midpoint of the 30 µm vibratome-sectioned samples, as determined by the limits of fluorescent immunoreactivity. Dimensions and alignments were identical for all fields of view along the laminar structures of each hippocampal subfield exposed (CA1, CA3 and DG). Laser power and exposure time conditions were consistent for all samples. Confocal images were acquired with a Leica TCR 6500 microscope equipped with four laser lines (405, 488, 561 and 653 nm) using a 40x and a ×63 immersion oil objective.

2.2.9.2.3 DAB (3, 3′-diaminobenzidine) Immunohistochemistry

Staining was performed as previously described (Engel et al., 2012c). Tissue was removed from cryoprotectant solution and placed in PBS. Tissue was washed twice for 5 min. PBS was replaced with 1 % H₂O₂ to inactivate endogenous peroxidases and left at RT for 45 min. Tissue was then washed twice with 1 x PBS for 10 min. PBS was removed and blocking solution was added to the slices (10 ml: 8.3 ml 1x PBS, 1 ml 10 % BSA, 0.5 ml Fetal Bovine Serum (FBS), and 0.2 ml 10 % Triton-X-100) for 1.5
h. Next, slides were incubated in primary antibody in blocking solution overnight at 4°C. The following day, primary antibody was removed and slides were washed 3 times in PBS for 10 min. For the next steps Vectastain kit (Vector Labs) was used. For this, 1 drop of biotinylated antibody and 3 drops of horse/donkey serum were added to 10 ml of 1% BSA/PBS and added to tissue sections for 90 min incubation with gentle shaking. Then, sections were immersed in the Avidin (ABC) peroxidase complex (2 drops of both reagents A and B in 10 ml of 1% BSA in PBS) for 1 h. Then slices were washed in PBS for 20 min and incubated in DAB solution (Sigma-Aldrich) for 5-15 min until the tissue colour turned brown. Sections were removed from the DAB and washed with H2O for 10 min. Slides were mounted with coverslips using Fluorosave reagent (Calbiochem). Staining was examined under a light microscope.

2.2.10 Synaptosome preparation for epifluorescence microscopy or immunostaining

2.2.10.1 Isolation of functional synaptosomes

Synaptosomes were prepared as previously published (Dunkley et al., 1986; Wang et al., 1989). First, coverslips were prepared with poly-lysine (Sigma-Aldrich) and left apart for 2 h for the final steps of the protocol. A pool of two hippocampi per group, from control and epileptic mice (14 days post-KA), was used. Tissue was homogenised with 300 µl of SAC-TES buffer (0.25 M sucrose; 5 mM TES, pH 7.4) on ice using a Glass-teflon douncer with 7 strokes at 700 rpm. Then SAC-TES buffer was added to reach a 5 ml final volume per sample. The homogenised sample was centrifuged at 2000 x g for 3 min at 4°C. The supernatant was recovered and then spun again at 9500 x g for 13 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 2 ml of SAC-TES buffer. Before continuing, a Percoll gradient (3%, 10% and 23%) was performed in a different falcon fraction tube for each sample. The re-suspended synaptosomes were then placed on the top of the percoll gradient column. Each column was centrifuged at 25000 x g for 11 min at 4°C.
103°C. The fraction left between the layers 10% and 23% corresponded to the isolated synaptosomes. Using a Pasteur pipette, the synaptosome layer was extracted and diluted in 15 ml of HBM-no Ca\(^{2+}\) buffer (140 mM NaCl, 5 mM KCl, 5 mM NaHCO\(_3\), 1.2 mM NaH\(_2\)PO\(_4\) (non hydrous), 1 mM MgCl\(_2\) *6H\(_2\)O, 10 mM glucose and 10 mM HEPES). Afterwards, the dilution was spun at 20000 x g for 11 min at 4°C and using a Pasteur pipette, the pellet was collected and re-suspended in HBM+Ca\(^{2+}\) buffer. Last, 200 µl of the dilution obtained was placed on coverslips pretreated with poly-L-lysine and laminin (Life Technologies, Madrid, Spain). Synaptosomes preparation slides were either placed in a calcium-sensitive fluorescent indicator (FURA – 2AM) for 40 min at 37°C for calcium microfluorimetric analysis or were fixed in 4% PFA for immunostaining (Figure 2.6).

2.2.1.1 Calcium microfluorimetric analysis

Measurements of free intracellular calcium concentrations were monitored using the calcium-sensitive fluorescent indicator FURA-2AM (5 µM) and analyses were performed as previously described (Diaz-Hernandez et al., 2001). Samples were incubated in the FURA-2AM with HBM solution for 40 min at 37°C. A superfusion chamber (Warner Instruments) attached to an epifluorescence microscope (Leica Microsystems Fluorescence Microscopy) was used for the fluorescent imaging. Microsystems Fluorescence Microscopy was excited via 100 x fluorine objective (Nikon TE-200, Tokyo, Japan) using an optical quartz guide at 510 nm.
Figure 2.5 Synaptosome preparation for microfluorimetric analysis
Hippocampi were extracted and synaptosome purified using a percoll gradient protocol. Samples were fixed in poly-acrilamide covers with 4% PFA for immunostaining analyses or immuresed in FURA-2 for fluorometric analyses.

Fluorescence signals were collected at 340 - 380 nm (Ex/Em). Images were taken at one second intervals. For eliciting $[\text{Ca}^{2+}]_i$ responses after 30 sec of perfusion, solution was switched to the solution containing the P2X7R agonist, BzATP (30 μM) in HBM for 30 sec and then switched back to the washing solution. After 2 min, washing solution was switched to HBM solution containing $K^+$ (100 mM) for 30 sec. $K^+$ was used as a positive $[\text{Ca}^{2+}]_i$ control to depolarize membranes and elicit a $\text{Ca}^{2+}$ response currents. Basal fluorescence was determined from images of each slide before the first application of the drug. Fluorescence data of each region of interest was analysed using MetaFluor® Fluorescence Ratio Imaging Software (Molecular Devices). The data is represented as the normalised fluorescence ratio F340/F380 that increases when $[\text{Ca}^{2+}]_i$ increases.
The variables used for the quantitative characterization of the $[\text{Ca}^{2+}]_i$ were defined as:

$$[\text{Ca}^{2+}]_i = K_d \left[ \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right] \beta$$

Where parameters correspond to $K_d$ as the dissociation constant for $\text{Ca}^{2+}$/Fura-2 (5 μM), $R$ is the relative fluorescence for each time illuminated at 340 and 380 nm. $R_{\text{max}}$ is the value of $R$ measured when $\text{Ca}^{2+}$ was saturated and $R_{\text{min}}$ when all was free of $\text{Ca}^{2+}$ and $\beta$ is the relative fluorescence measured in absence and saturation of $\text{Ca}^{2+}$ at 380 nm.

### 2.2.12 Synaptosome isolation for Western blot analysis

Synaptosome preparation for immunoblotting was undertaken as previously described (Nagy and Delgado-Escueta, 1984). A pool of two hippocampi per group, from control and epileptic mice (14 days post-KA), were homogenised in 3 ml of homogenising buffer (1 mM EDTA, 0.32 M sucrose, 1 mg/ml BSA and 5 mM HEPES, pH 7.4) in a Glass-teflon douncer with 10 strokes at 600-650 rpm at 4 °C. The homogenised samples were centrifuged at 3000 x g for 10 min at 4 °C and the supernatant was recovered (cytoplasm and synaptosomes). Afterwards, samples were spun at 14000 x g for 12 min at 4 °C and the supernatant was discarded. The synaptosome pellet was re-suspended in 220 µl of Krebs-Ringer buffer (NaCl 140 mM, KCl 5 mM, Glucose 5 mM, EDTA 1 mM and HEPES 10 mM, pH 7.4). Then, 180 µl of Percoll (45 % v/v) was added and mixed by gently inverting the tubes. The mix was spun at 18600 x g for 2 min at 4 °C. Then, the superficial layer containing the enriched synaptosomes was recovered with a P1000 tip and re-suspended in 1 ml of Krebs-Ringer buffer. Afterwards the synaptosomes were spun at 18600 x g for 30 sec and the supernatant was discarded. The pellet (synaptosomes) was re-suspended in 80 µl of Hepes-Krebs buffer (NaCl 147 mM, KCl 3 mM, glucose 10 nM, MgSO₄ 2 mM, CaCl₂ 2 mM, HEPES 20 mM, pH 7.4) and BCA assay was performed.
Once protein concentration was determined 16 µl of loading buffer was added per 100 µl sample.

2.2.13 Electrophysiological recordings

2.2.13.1 Tissue preparation

Brains were extracted from EGFP-P2rx7 mice 14 days after SE and quickly sectioned in the sagittal plane (300 µm) and glued onto an agarose cube placed on the stage of a vibratome cuvette (Integraslice 7550, Campden Inst., UK) containing saline solution (125 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 12 mM glucose, pH 7.4) at 4 °C. Brain slices were kept in saline solution continuously bubbled with carbogen (95 % O₂ / 5 % CO₂) at RT for about 6 h.

2.2.13.2 BzATP and A437980 administration and P2X7R current measurements

All patch-clamp procedures were performed by Prof. Luis Olivos-Oré (Universidad Complutense de Madrid, Madrid, Spain). Brain slices were transferred and fixed with a nylon grid to a submersion chamber attached to the stage of an upright microscope (Olympus BX51W1; UK) and superfused at a rate of 2 ml/min with the above-mentioned saline solution. For the water immersion observation, a 63 x microscope objective was used and for the GFP-cell visualization in the DG and CA1 regions of the hippocampus a DL-604 OEM camera (Andor Technology, EU) was used while the slices were illuminated. Electrophysiological recordings were performed with an EPC10/2 patch-clamp amplifier using PatchMaster software (HEKA Electronic, Lambrecht, Germany). Borosilicate capillaries (Kimble Chase, Mexico) were pulled with a Narishige PP830 puller (Tokyo, Japan) to fabricate patch pipettes having a final resistance of 5 - 6 MO when filled with a buffer solution (140 mM, N-Methyl-D-glucamine (NMDG+), 5mM EGTA, 3 mM MgCl₂, 10 mM HEPES, pH 7.2, adjusted with HCl; ≈ 290 mOsm). To measure the membrane currents in the
whole-cell configuration, membranes were filtered at 3 kHz and sampled at 10 kHz. A voltage \( V_h \) of -70 mV was used to hold the cells. Series resistance (5 - 10 MΩ) were compensated by 80 % and monitored throughout the experiment together with the cell membrane capacitance. After taking into consideration a series resistance of 10 MΩ, a neuron’s membrane capacitance of 7.06 ± 0.9 pF (n = 22 cells) and the molecular weight of NMDG⁺ (195.21 kDa), the time estimated for the equilibration between the NMDG⁺, the patch pipette and the cytoplasm of the recorded cell was approximately 10 min (Pusch and Neher, 1988). Due to this, drug application began 10 min after obtaining the whole-cell configuration. Experiments in which series resistance changed by more than 20 % or holding current exceeded 20 pA were not analyzed. All recordings were obtained at room temperature. The P2X7R agonist, BzATP (100 μM; Sigma-Aldrich, Spain), was applied onto the observed cell by means of a glass-pipette (3 - 5 μm tip diameter) connected to a pneumatic drug ejection system (PDES-02DX, NPI Electronic GmbH, Germany). Stock solutions of drugs were diluted fresh in extracellular saline, which were loaded into the application pipette a few minutes before starting the experiments. The P2X7R antagonist A438079 (10 μM) (Sigma-Aldrich, Spain) was applied 2 min before and during agonist administration.

2.2.14 DNA methylation analysis of the P2rx7 promoter region

Extraction of DNA, RNA and Protein

DNA, RNA and Protein were extracted from hippocampal subfields using the DNA/RNA/Protein purification Plus Kit (Norgen Biotekcorp; Cat.# 47800) as per protocol. All centrifugations in 2.2.7.1 section were performed at 14000 x g at RT.

2.2.14.1 Tissue homogenisation

Tissue was homogenized in 300 μl lysis solution by passing it through a 26 gauge needle until it was fully homogenized. The lysate was transferred into a RNase-free
reagent tube and spun for 2 min to pellet cell debris. The supernatant was then transferred to another RNase-free reagent tube.

2.2.14.2 Genomic DNA purification

The supernatant from step 1 was passed through a genomic DNA (gDNA) purification column. The flow-through was retained for RNA purification and was stored at -20 °C. Genomic DNA bound to the columns was washed and eluted as per protocol. Purified gDNA was stored at -20 °C.

2.2.14.3 Total RNA purification

The flow-through from step 2 was passed through an RNA purification column. For every 100 µl of flowthrough obtained in step 2, 60 µl of 96-100 % EtOH was added. The flowthrough of this step was retained for protein purification and stored at -20 °C. Total RNA bound to the column was washed and eluted as per protocol. RNA was measured by nanodrop and stored at -80 °C.

2.2.14.4 Bisulfite conversion of DNA

Bisulfite conversion of DNA is necessary to deaminate unmethylated cytosine and produce uracil in DNA. This step is considered the "gold standard" for downstream applications to assess DNA methylation status. Methylated cytosines are protected from the conversion to uracil, allowing the use of direct sequencing to determine the locations of unmethylated cytosines and 5-methylcytosines at single-nucleotide resolution. 500 ng DNA was prepared in 20 µl of dH2O. Samples that exceeded the volume were concentrated by vacuum centrifugation at 60 °C for 45 min. Bisulfite conversion of GC regions was carried out using the EZ DNA Methylation-Gold Kit (Zymo Research; Cat # D5005). CT Conversion Reagent was prepared as per protocol. 180 µl of the CT Conversion Reagent was added to the 20 µl DNA samples and placed into a thermal cycler to perform the next steps: 98 °C for 10 min, 53 °C for 5 h, and 4 °C storage for up to 20 h. 600 µl of m-Binding Buffer was added to a
column and the sample was loaded into the same column and mixed by inverting several times before centrifugation at 10000 x g for 30 sec. 100 µl of M-wash buffer was added to the column and centrifugation was repeated. 200 µl of M-Desulphonation Buffer was added per column and incubated at RT for 20 min. Samples were spun at full speed for 30 sec. After washing twice with 200 µl of M-Wash Buffer, samples were centrifuged at full speed for 30 sec and the column was placed into a 1.5 ml reagent tube. Converted DNA was eluted with 30 µl dH₂O by centrifugation at 18600 x g for 30 sec. Bisulfite converted DNA concentration was measured on a nanodrop as single stranded DNA and stored at -80 °C.

2.2.14.5 PCR amplification of the P2rx7 promoter region

10 ng bisulfite converted DNA was prepared in a total volume of 4.5 µl of dH₂O. Bisulfite converted DNA was concentrated by vacuum centrifugation for 1 to 2 min at 30 °C where necessary.

Primer design:

A region of -2kb to +0.5kb around the transcription start site (TSS) of P2rx7 promoter region was analyzed. The location of the TSS was taken from the UCSC genome browser (Mm8, TSS at chr5: 122,904,527). The sequence used for PCR primer design was Chr5: 122,902,527–122,905,027.

Primers were designed using MethPrimer software: (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Using this program, 2 CpG Islands were found in the 2.5kb region around the TSS, including 14 CpGs. Several primers were designed and tested on converted DNA before choosing the optimum primer pair (See Table 2.4).
Table 2.4 Sequences of primers used for PCR amplification of bisulfite converted DNA

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PCR amplification:

PCR amplification was carried out using the PYROMARK PCR kit (Qiagen; Cat # 978703). The protocol mix for the PCR was: 1.5 µl of each primer, 25 µl of Master Mix, 12.5 µl of DMSO and 5 µl of Coral Red Loading buffer. The total volume per well after adding the protocol mix and 4.5 µl from each sample was 50 µl. Negative controls of unconverted DNA were used throughout.

PCR conditions:

95°C 15 min  Activation
95°C 30 sec  Denaturation
52°C 30 sec  Anneal
45 Cycles
72°C 30 sec  Extend
72°C 10 min  Final extension

12 µl PCR products were run on a 2% agarose gel.

2.2.14.6 Purification of PCR products

PCR products were purified using the QIAquick PCR purification Kit (Qiagen; Cat # 28106). All centrifugations were performed at 17900 x g at RT. All steps were performed according to the manufacturer’s instructions. The volume of PCR
product purified was 38 µl, 12 µl was used previously for the gel electrophoresis. According to the protocol, 5 volumes of the buffer added per 1 volume of product, therefore, 190 µl of the buffer PB was added per sample and placed into a QIAquick column. After centrifugation for 30 - 60 sec, 750 µl of Buffer PR was added and spun for the same time. One extra centrifugation was performed for 1 min to remove residual buffer. To elute the DNA, 30 µl of dH₂O was added into the column matrix and after 1 min, samples were centrifuged for 1 min. All samples were measured by nanodrop as double stranded DNA.

2.2.14.7 Sequencing of PCR products

Sequencing was performed by a service provider (Eurofins MWG operon, London, UK). As the company required a minimum concentration of 5 ng/µl in 20 µl, samples were made up in dH₂O accordingly. Sequenced PCR products were analysed using BiQ Analyser software (Max-Planck-Institute for Informatics and Saarland University, Saarbrücken, Germany).

2.2.15 Data analysis

Data are presented as means ± standard error of the mean (SEM). Three or more group data was analyzed using analysis of variance (ANOVA) with post hoc Fisher’s protected least significant difference test or, for two-group comparison, Student’s t-test (STATVIEW software; SAS Institute, Cary, NC, U.S.A.). The distribution of independent standard normal random variables was analysed by $X^2$ test. Racine scores were compared between groups using Harrell’s C coefficient (also known as the Wilcoxon– Mann–Whitney statistic). This calculates the probability of observing a lower score when an animal selected at random from the treated group is compared with one selected at random from the control group. As such, it is a useful measure of effect size. Calculations were carried out in GraphPad, and adjusted for clustering of data due to repeated measurements on each animal. Significance was accepted at * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. 

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Chapter III – Neocortical expression of the P2X7 receptor after status epilepticus and in epilepsy

3.1 Introduction

The principle focus of this chapter was to study the expression of the P2X7R in the neocortex, identifying the location and the cell type expressing the receptor after status epilepticus and epilepsy. For this study, EGFP-P2rx7 reporter mice were used to analyse the location of P2X7R transcription after status epilepticus and epilepsy in the cortex. A second objective was to assess whether clinical behaviour (convulsions) was altered in mice treated with a P2X7R antagonist. In addition, the possibility of protection to the cortex from seizure-induce damage was assessed in the treated mice.

3.1.1 Neurophysiology of the neocortex

The cerebral cortex can be classified into two parts which are differentiated by their characteristic laminations in all mammals; the neocortex and the allocortex. The neocortex, also known as isocortex or neopallium, is the largest area of the mature cerebral cortex and it is divided into six distinct layers. The allocortex is of a much smaller area and contains less than six layers (Shipp, 2007). The neocortex and hippocampus are connected by the subiculum structure, which is the transition zone from the three to the six layered cortex. The structure of the neocortex is considered uniform and each of the six different horizontal layers (I-VI) include characteristic populations of cells based on their different densities, sizes, morphologies, inputs and outputs. However, the main cell types of neurons are excitatory pyramidal neurons (approx. 80 % of neocortical neurons), inhibitory interneurons (approx. 20 %) and glia, and the uniformity is not always consistent
(e.g. motor cortex lacks layer IV) (Lehmann and Dunbar, 2009). The circuitry of the neurons in the neocortex is distinct; the pyramidal neurons from upper layers II and III project their axons to other neocortex regions, while those in deeper layers V and VI project out of the cortex to other structures such as the thalamus and brainstem. Furthermore, neurons located in layer IV receive all the synaptic connections from outside the cortex and make local connections to other cortical layers (Lam and Sherman, 2010).

3.1.2 Neocortex and Temporal lobe epilepsy

It is known that the characteristic seizures of temporal lobe epilepsy (TLE) originate in limbic structures such as the hippocampus and parahippocampal areas (Nakasato et al., 1993). Seizures can expand and reach cortical areas in a process called “secondary generalization” (Sanabria et al., 2002). Cavalheiro and colleagues (Cavalheiro, 1995; Cavalheiro et al., 1996) suggested that the first spontaneous seizure in chronic TLE involves mainly limbic areas but these then rapidly progress over time leading to the development of more complicated behavioural seizures. Their study suggested that a kindling-like phenomenon is responsible for recruiting further structures, such as the neocortex, after several spontaneous limbic seizures. This event has been described previously in many animal models such as the pilocarpine model of epilepsy (Cavalheiro et al., 1996) and kainate model of epilepsy (Cavalheiro et al., 1982; Sloviter et al., 2007).

Although the hippocampus is invariably the main site of pathology in human TLE (Norwood et al., 2010), cell damage and long-lasting structural reorganizations are also found in local and remote neuronal networks in experimental models and humans after SE (Turski et al., 1987; Cavalheiro et al., 1991; Sloviter, 1991; DeGiorgio et al., 1992; Inoue et al., 1992). Seizure-induced neuronal death is evident in a number of extra-hippocampal brain regions such as the amygdala and entorhinal cortex in humans (DeGiorgio et al., 1992; Fujikawa et al., 2000; Sankar et al., 2008). Brain imaging studies by Bernasconi et al., showed atrophy in the entorhinal cortex ipsilateral to the seizure focus in patients with TLE and normal
hippocampal volumes. These findings emphasize the participation of the entorhinal cortex in the pathogenesis of the disease (Bernasconi et al., 2001). Cortical damage has also been reported in animal experiments using different models of SE induction, either chemically, using kainic acid (KA) or pilocarpine (Curia et al., 2008; Mouri et al., 2008a), or after electrical stimulation-induced SE (Kienzler et al., 2009). Additionally, models of cortical epileptogenesis have also reported neuronal damage in the cortex (Gutnick et al., 1992; Barkai et al., 1994). Neocortical neuronal loss is not, however, inevitable in human TLE (Bothwell et al., 2001).

3.1.3 P2X7 receptor localization and expression after SE

The focus of most research on the role of the P2X7R in epilepsy has been the hippocampus. However, P2X7R expression has also been shown in other brain regions, including the neocortex, where it has been shown to be expressed in microglia, neurons, astrocytes and oligodendrocytes (Deuchars et al., 2001; Sperlagh et al., 2002; Sperlagh et al., 2006; Diaz-Hernandez et al., 2009; Arbeloa et al., 2012; Engel et al., 2012a).

A number of studies investigated the activation of P2X7R in the neocortex in diseases of the CNS, including Alzheimer’s disease, ischemia and SE (Franke et al., 2004; Kim et al., 2010; Lee et al., 2011; Oliveira et al., 2011). Oliveira et al., reported P2X7Rs in cortical astroglia using whole-cell patch-clamp recordings (Oliveira et al., 2011). In an Alzheimer’s disease model in mice, the upregulation of P2X7R expression and the production of ROS in microglia were found parallel to the Aβ increase in the neocortex (Lee et al., 2011). After stroke, an increase in P2X7R immunoreactivity in neocortex was observed in microglia cells (Franke et al., 2004).
3.1.4 P2X7R activation after seizures

There is limited data on P2X7R expression in the neocortex after SE. Kim et al., observed a P2X7R regulation of leukocyte infiltrations in the rat frontoparietal cortex following SE (Kim et al., 2010). Previous studies have reported an enhanced P2X7R immunoreactivity in microglia in the piriform cortex of rats following induction of SE by systemic KA (Rappold et al., 2006). Other work has reported a reduction in IL-1β-immunoreactive microglia in the frontoparietal cortex of rats after inhibiting P2X7R after SE (Kim et al., 2010). Choi et al. suggested a role for the P2X7R in region-specific microglia activation and reported an increase of microglia activation in the frontoparietal cortex after SE induced by pilocarpine (Choi et al., 2012). In the intra-amygdala KA model, P2X7R expression is selectively increased in the hippocampus, but expression in the neocortex has not been explored (Engel et al., 2012b).

As an alternative resource to study the expression of the P2X7R, and to avoid reliance on currently available P2X7R antibodies, or P2X7 receptor-deficient mice, a transgenic reporter mouse has been developed with the Egfp (enhanced green fluorescence protein) gene upstream of the ATG start codon of the p2rx7 gene. This new transgenic mouse was generated by Dr. M. Nedergaard (University of Rochester, Rochester, NY, USA). In this model, cells transcribing P2rx7 can be identified by GFP fluorescence. Previous work showed increased P2rx7 transcription levels in neurons within the hippocampal brain region after SE (Engel et al., 2012b).

3.1.5 P2X7R antagonists as anti-seizure agents

Previously, studies using agonists and antagonists for P2X7R as well as genetic tools, have confirmed that its targeting during SE can have effects on seizures and seizure-induced cell death in the hippocampus (Kim et al., 2011a; Engel et al., 2012b). There has been minimal focus on the neocortex but Klaft et al. reported a minor reduction in the amplitude of the slow field potentials in slices from medial
entorhinal cortex in epileptic rats by the administration of A740003, a P2X7R antagonist (Klaft et al., 2012).

3.2 Chapter objectives

**Hypothesis:** P2X7R expression is increased in extrahippocampal regions, such as the neocortex after SE induced by intra-amygada KA. Seizures and seizure-induced neocortical damage will be reduced in animals treated with P2X7R antagonist.

**Aims:** To analyse P2X7R expression in the neocortex after SE and in chronic epilepsy and to analyse effects of P2X7R inhibition on clinical seizures and seizure damage in the neocortex after SE.
3.3 Results

3.3.1 Intra-amygdala KA-induced status epilepticus produces injury to the neocortex

The intra-amygdala KA model of focal-onset SE was used in this study to analyse the expression and functional effects of targeting the P2X7R on the neocortex (Araki et al., 2002; Mouri et al., 2008a). To characterize the extent of extra-hippocampal damage resulting from seizures in the model, coronal brain sections were stained with the neuronal cell death marker FluoroJade B (FJB). Damaged neurons were found in the neocortex, as well as the hippocampus 24 h after SE (Figure 3.1 A). Damage was prominent in cortical layers V and VI which presented FJB-positive cells 24 h after SE (Figure 3.1 B). No damage was found in any of the cortical layers in control mice.

3.3.2 P2X7R protein levels increase in the neocortex after SE and in epilepsy

Western blotting was performed to explore whether P2X7R levels are increased in the neocortex after SE and in chronic epilepsy. Tissue was obtained at different time points 1 h, 4 h, 8 h and 24 h after SE and at 14 days, when animals are displaying frequent spontaneous seizures (Mouri et al., 2008a). Results showed a significant increase of P2X7R protein levels in the neocortex 24 h after SE (Figure 3.2 A, B) and in epileptic mice compared to time-matched controls (Figure 3.2 C, D).
Figure 3.1 Cell death in neocortex after *status epilepticus*

**A)** Representative photomicrograph showing FJB staining in the mouse brain 24 h after SE. Note, cell death is evident mainly in ipsilateral CA3 and layers V and VI in neocortex. **B)** Higher magnification photomicrographs (20x lens) showing FJB-positive cells in cortex (layers V and VI) from mice subjected to SE. Scale bar A) 500 μm; B) 150 μm.
3.3.3 Increased neuronal transcription of P2X7R in the neocortex after SE

To determine the location within the cortex and the cell types expressing the P2X7R after SE, a P2X7R reporter mouse, which expresses GFP under the transcriptional control of the P2rx7 promoter, was used (Engel et al., 2012b). P2X7R reporter mice displayed a normal response to KA with similar behavioural changes during SE when compared to wildtype C57BL/6 mice (data not shown).

GFP immunoreactivity, indicative of constitutive transcription of P2rx7, was seen in cortical layers II and III in control animals. A similar amount of GFP positive cells were also found in this region in mice 8 h after SE (Figure 3.3 A, B). GFP positive cells in layers II and III co-localized with NeuN, a neuronal marker, in brain sections from control mice and from mice culled 8 h after SE (Figure 3.3 C).

Neurons were also found to be the main population expressing GFP in sections 8 h after SE in cortical layers V and VI; cortical region showing majority of damage. No GFP expression was observed in layers V and VI in control animals (Figure 3.4 A, B).

Studies have reported expression of the P2X7R in microglia after SE in the piriform cortex (Rappold et al., 2006). Accordingly, sections from control and SE reporter mice were co-stained with the microglial marker Iba-1. Iba-1 labelled cells had typical appearance of microglia with small soma and ramified branches. No co-localization was observed between the GFP positive signal and Iba-1 positive cells in either control or 8 h after SE EGFP-P2rx7 reporter mice (Figure 3.5 A).

Last, brain sections from reporter mice were co-stained for glial fibrillary acidic protein (GFAP), a marker for astrocytes, and GFP. No co-localization was observed between GFP positive cells and GFAP positive staining in any of the cortical layers analysed in either control mice or mice subjected to SE (Figure 3.5 B).
Figure 3.2 Increased P2X7R levels in the neocortex 24 h after status epilepticus induction and in chronic epilepsy

A) Representative Western blot (n = 1 per lane) and B) graph showing a significant increase of P2X7R protein levels in the neocortex 24 h after SE. α-Tubulin is shown as guide to protein loading (n = 4 per group). C) Representative Western blot (n = 1 per lane) and D) graph showing increased P2X7R protein levels in the neocortex in control and epileptic mice (Epi). α-Tubulin was used as loading control (n = 4 per group). T-test. * p < 0.05 compared to control.
Figure 3.3 Constitutive P2X7R transcription in cortical layers II and III

**A, B)** GFP-positive signal in neocortex of EGFP-P2rx7 reporter mice (see arrows) in control and 8 h after SE. **C)** Representative staining showing co-localization of GFP positive cells (green) and NeuN positive cells (red) of control EGFP-P2rx7 reporter mice in cortical layers II – III. Scale bar A, B) = 150 μm, C) = 25 μm.
Figure 3.4 Neuronal P2rx7 transcript after SE in the neocortex in mice

GFP positive cells (green) and NeuN positive cells (red) in sections from EGFP-P2rx7 reporter control and 8 h after SE mice. Arrows point to double-labelled cells. Note no GFP positive cells in layers V and VI of the neocortex in control EGFP-P2rx7 reporter mice. Scale bar =12 μm.
Figure 3.5 Lack of microglia or astrocyte induction of P2X7R after SE in the neocortex of mice

**A)** GFP fluorescence (green) and Iba-1 (red) fluorescence in neocortex from EGFP-P2rx7 reporter mice 8 h after the induction of SE. Arrows indicate GFP and Iba-1 positive cells. **B)** GFP fluorescence (green) and GFAP (red) in the neocortex from EGFP-P2rx7 reporter mice 8 h after SE. Arrows indicate GFP and GFAP positive cells. Scale bar = 12 μm.
3.3.4 Increased neocortical expression of the P2X7R in experimental epilepsy

Next, expression of GFP was investigated in reporter mice 14 days after SE, when all mice display regular spontaneous seizures (Mouri et al., 2008a; Jimenez-Mateos et al., 2012). To establish which cell population exhibit increased P2rx7 induction in epilepsy, double-staining was performed using GFP and the same set of cellular markers (NeuN, Iba-1 and GFAP), as previously used to determine cells showing P2X7R induction after SE. The neocortex layers V and VI again displayed the highest number of GFP-positive cells.

Double-label fluorescence microscopy revealed neurons as the main cell population displaying GFP induction in epileptic EGFP-P2rx7 reporter mice when compared to controls (Figure 3.6). Analysis of microglia staining showed the presence of some double staining between Iba-1 and GFP. GFP-positive microglia displayed large somata and thick primary processes characteristic of microglia in their active state (Figure 3.7 A, B). In contrast, no co-localization between GFP and GFAP (astrocytes) was observed in the neocortex of epileptic reporter mice (Figure 3.7 C).
Figure 3.6 Increased neocortical GFP induction in neurons in chronic epilepsy

Representative photomicrographs showing immunofluorescence staining for GFP (green) and NeuN (red) in the neocortex vehicle control and 14 days post KA injection SE-treated reporter mice. Arrows mark GFP-expressing and NeuN-positive cells. Scale bar = 12 μm.
Figure 3.7 Neocortical induction of GFP in microglia in EGFP-P2rx7 reporter mice with epilepsy

A) GFP fluorescence (green) and Iba-1 (red) staining in brain sections from epileptic mice. B) High magnification of a GFP-positive microglia cell (arrow). C) GFP (green) and GFAP (red) fluorescence in the neocortex of epileptic EGFP-P2rx7 reporter mice. Note absence of co-localization. Scale bar A, C = 12 μm. Scale bar B = 25 μm.
3.3.5 P2X7R inhibition decreases convulsive behaviour during status epilepticus and protects the neocortex against seizure-induced damage

We previously reported that electrographic seizures were reduced in mice treated with A438079, a specific P2X7R antagonist (Engel et al., 2012b). To establish whether P2X7R inhibition also reduces clinical seizure behaviour during SE, mice were injected intra-cerebroventricularly with A438079 (1.75 nmol), 10 min before SE induction. Behaviour was analysed every 5 min using a modified Racine scale-type scoring system which assesses motor behaviour on a 1 – 6 scale. Typical convulsive behaviour in mice after KA begins with a score 1 - 2 on the Racine scale, which includes mild (facial automatisms) and immobility. Animals typically progress to stage number 3 – 4 after 15 minutes, which involves tail extension, forelimb clonus and head bobbing. Mice then return to score 1 – 2 or continue with rearing and falling behaviours (stage 5). Short periods of tonic-clonic seizures with loss of posture and jumping can also occur during seizures (Racine score 6) as previously described in Material and Methods.

Higher scores progress over the 40 min after KA, averaging a score of 4 – 5 by 30 – 40 min. In this experiment, vehicle treated mice subject to SE showed typical convulsive behaviour beginning with a score 1 – 2 in Racine scale, with facial automatisms and immobility. After 10 – 15 min they reached score 3 – 4 on the Racine scale, developing to stage 5 towards the end of the 40 min period of SE. Clinical behaviour was reduced in A438079 treated SE mice when compared to vehicle-injected mice. Mice showed similar scores for the first 10 – 15 min, but never progressed to score 4 or beyond on the Racine scale during the 40 minutes of SE (Figure 3.8 A). Statistical analysis was performed with the Harrell’s C statistical analysis, which showed a coefficient of 0.72 (95 % CI 0.53–0.92, *p = 0.034) (see Material and Methods), meaning there was a 72 % probability that a mouse treated with A438079 showed a lower Racine score than a vehicle mouse.
To investigate the potential effects of A438079 on SE-induced neocortical injury, sections from control and SE mice were stained 24 h post-KA injection with FJB to mark irreversible neuronal degeneration. In vehicle-treated SE animals, damage was found predominantly in layers IV to VI in the neocortex. Cell counting analysis confirmed a significantly lower (approximately 50 %) number of FJB-positive cells in A438079 treated mice when compared to vehicle-injected SE mice (Figure 3.8 B, C).
Figure 3.8 P2X7R inhibition decreases seizure severity during SE and protects against neocortical damage

A) Graph showing the maximum Racine Scores reached per 5 min period during 40 min after KA injection from Vehicle and A438079 (A43) treated mice (n=7 per group). B) Graph showing the number of FJB-positive cells in vehicle and A438079 treated mice after SE. C) Representative photomicrographs (10x lens) showing FJB-positive cells in neocortex layers IV to VI 24 h after SE in mice treated with the P2X7R inhibitor A438079 and vehicle-injected animals. Scale bar = 150 μm. T-test. *p < 0.05.
3.4 Discussion

The present chapter investigated the expression and function of the ATP-gated P2X7R during SE and in experimental epilepsy in the neocortex of mice. Increased expression of the P2X7R was found after SE in this brain region and levels were also increased in neocortex of epileptic mice. Neurons were found to be the main cell population transcribing P2rx7 after SE, as revealed by studies in EGFP-reporter mice. In epilepsy, microglia also transcribed P2rx7, suggesting recruitment of further cell types with disease progression. Astrocytes, however were not found to transcribe P2rx7 in the model. Finally, injection of mice with an antagonist of the P2X7R resulted in a reduction in clinical behaviour during SE and protected the neocortex from damage after SE. These findings implicate the neocortex as an important site of P2X7R expression, further clarifying the cells involved in this process and extend the evidence that the P2X7R is a potential new target to protect against seizures and seizure-induced cell death.

3.4.1 Increased expression of P2X7R in the neocortex after SE and epilepsy

Pathological studies have revealed that prolonged or repeated seizures are associated with extrahippocampal damage in humans, including damage to regions such as the piriform and the entorhinal cortex (Sankar et al., 2008). Reduction in cortical thickness has also been reported in cross-sectional and longitudinal imaging studies of patients with pharmacoresistant TLE (Lin et al., 2007; Bernhardt et al., 2010). However, other data has failed to observe neuronal loss in the neocortex in epilepsy (Bothwell et al., 2001). If protection is needed for structures such us the neocortex after SE, then an important step is to identify the underlying cell and molecular mechanisms that provoke cell damage or excitotoxicity. Also, it is important to identify the affected cells.
ATP acts as a transmitter and co-transmitter in neurons and glia and may play a role in modulating seizure severity and duration (Dale and Frenguelli, 2009). A major finding in the present chapter was increased expression of the P2X7R in the neocortex after SE in mice when compared to the neocortex of control mice. Previous data obtained with the same model reported a similar increase of P2X7R in the hippocampus 24 h after SE (Engel et al., 2012b). These findings suggest a consistent increase of the P2X7R in affected brain regions after SE. Increased expression of the P2X7R was also found in the neocortex of epileptic mice when compared to the same area in control mice. These data are consistent with previous studies where an increase of P2X7R expression was observed in the hippocampus in chronic experimental epilepsy (Dona et al., 2009). Hence, the P2X7R upregulation found in epileptic tissue suggests that the mechanism of action and the increased levels of the receptor is a common feature of seizure activity not a feature limited to SE.

3.4.2 Increased neuronal P2X7R transcription in the neocortex after SE and neuronal and microglial P2X7R transcription in epilepsy

There has been significant controversy regarding the cell type expressing P2X7R in the brain. Initially, P2X7R expression was reported to be present exclusively on glial cells (Collo et al., 1997). Immunohistochemistry analysis showed a predominantly microglia expression of the P2X7R in the rat brain 24 h after systemic KA (Rappold et al., 2006). However, other studies have supported expression of the P2X7R also in neurons (Armstrong et al., 2002; Sperlagh et al., 2006; Engel et al., 2012b). Armstrong et al., detected the P2X7R on presynaptic terminals of mossy fiber synapses in the rat hippocampus (Armstrong et al., 2002). Later, in situ hybridization studies demonstrated that neurons, oligodendrocytes, and microglia express P2rx7 mRNA in the rat brain (Yu et al., 2008).

In the present study, an EGFP-P2rx7 reporter mouse was used to identify cells transcribing P2X7R. Our group was the first to use a P2X7R reporter mice [Tg(P2rx7-
EGFP)FY174Gsat/Mmcd, stock 011959-UCD] expressing enhanced green fluorescent protein (EGFP) immediately downstream of the P2rx7 promoter. Reporter mice were obtained from U.S. National Institutes of Health Mutant Mouse Regional Resource Centres and granted by Dr. M. Nedergaard (University of Rochester, Rochester, NY, USA). P2X7R reporter mice represent a unique tool to investigate P2X7R expression in the intra-amygdala KA model (Engel et al., 2012b). The first study found P2X7R induction in neurons as the main cell type in mice transcribing P2X7R after SE, mainly in the DG subfield of the hippocampus. Our results here are consistent with previous data reported by our group and show evidence of P2X7R expression in neurons as the main cell type expressing the receptor in the neocortex after SE and in epilepsy. The increased GFP signal was mainly observed in the cortical layers V and VI in SE and epilepsy. Respectively, these layers contain large pyramidal cells and a highly variable population of excitatory neurons, which project to local and distant cortical areas (Silva et al., 1991; Ahissar and Kleinfeld, 2003). Hence, it is tempting to speculate that the increase in GFP signal observed in these layers of the neocortex during SE and in epilepsy is a result of the increased expression of the P2X7R during seizures, maintaining the hyperexcitability state in those layers and propagating it to other regions of the brain. Also, P2X7R expression in layers V and VI might contribute to further damage observed after SE.

An unexpected finding in the present study was a constitutive expression of GFP in the cortical layers II and III. This signal was found to co-stain with neurons. This is the first study to find such expression in these cells and illustrates the high value of a reporter mouse to reveal novel cell types transcribing the P2X7R. Further studies, perhaps combining in situ mRNA with GFP could help to confirm these findings.

Microglia are the best understood cell population to express P2X7R. Previous studies have shown a P2X7R upregulation in microglia in the cortex after SE in a systemic KA rat model (Rappold et al., 2006) and a pilocarpine model (Choi et al., 2012). In our reporter mice, basal expression of GFP was not found in microglia after SE, however, microglial P2X7R induction was found in the neocortex of epileptic mice. Thus, P2X7R is expressed differently according to the experimental
model used. P2X7R induction is only high in microglia during epilepsy in our model, suggesting the importance of additional mechanisms regulating P2X7R transcription in developing recurrent spontaneous seizures. Furthermore, ATP release or another signal produced in the epileptic brain could be the turn-on mechanism for P2X7R in microglia cells exclusively during the chronic epileptic phase in the intra-amygdala KA model. Therefore, two phases of induction may occur; an early change principally confined to neurons and a subsequent induction in microglia. These data conflict somewhat with studies that support the P2X7R expression as a key mechanism for microglia activation, since microglia are activated by 24 h in cortex (Rappold et al., 2006; Avignone et al., 2008; Monif et al., 2009). However, our data showing a GFP increase in microglia in epileptic mice is consistent with results demonstrating that P2X7R controls microglia activation and with those reporting an increase of P2X7R currents in activated microglia after SE (Avignone et al., 2008).

It remains unclear whether astrocytes express P2X7R. Previous work has suggested P2X7R activation induces apoptosis in astrocytes, implying they can express the receptor (Salas et al., 2013). Here, P2rx7 induction was not detected in astrocytes either after SE or in epilepsy. This is consistent with hippocampal studies in which P2X7R was not induced after SE in this model (Engel et al., 2012a). While this suggests astrocytes are not important for P2X7R signalling in this model, we cannot exclude that the reporter mouse, for technical reasons, might not allow astrocytes visualization. This could be tested, for example, by staining for the P2rx7 mRNA by in situ hybridization.

3.4.3 P2X7R inhibition decreases convulsive behaviour during status epilepticus and protects the neocortex against damage

GABA-potentiating drugs remain frontline treatments for SE (Wasterlain and Chen, 2008). However, due to the internalization of the GABA_A receptor and other mechanisms, available treatments are ineffective in approximately 30 % of patients
(Wasterlain and Chen, 2008). Therefore, it is necessary to identify new anticonvulsants with other mechanisms of action. Previous data obtained by our group demonstrated that P2X7R antagonists reduced electrographic seizures when given alone and potentiated the anticonvulsive effects of Lorazepam in combination (Engel et al., 2012b). Damage to the hippocampus was also reduced in mice treated with P2X7R antagonists during SE (Engel et al., 2012b). However, that study did not investigate clinical behaviour. The clinical behaviour in the intra-amygdala KA model has only been briefly described in previous studies and no quantification of the behaviour scores (Racine scores) was reported (Mouri et al., 2008a). The Racine scale is a well recognised useful tool to correlate severity of seizures through seizure behaviour. Furthermore, neuronal degeneration is typically dependent on the Racine stage and the SE duration (Rossi et al., 2013). Racine's scale was originally developed for the amygdala-kindling model, and is frequently used as read-out of seizure intensity in other experimental seizure models (Luttjohann et al., 2009). In the present study, seizure behaviour was analysed for 40 min until anticonvulsant injection. Behavioural features started with immobility (stage 1 - 2), followed by tail extension and forelimb clonus (stage 3), head bobbing and rearing (stage 4 - 5) and rearing and falling (stage 6). Short periods of tonic-clonic seizures with loss of posture and jumping also occurred during the seizures. Nevertheless, not all models of SE induction show the same pattern of Racine score behaviour. For example, in the pilocarpine-induced SE model mice show continuous seizures fluctuating from stage 3 to 5 with generalized seizure activity in just a few minutes after pilocarpine injection (Rossi et al., 2013). During pilocarpine SE, mice never regain normal behaviour between the seizures (Cho et al., 2014). However, Racine's scale is not always adequate for assessing seizures intensity. For example, in the Pentylenetetrazol (PTZ)-induced seizures model the EEG patterns observed from animals differ from previous models and the behaviour shown during seizures is quite different to the features defined on the Racine's scale (Luttjohann et al., 2009).
The present study found that A438079 decreases convulsive behaviour in the i.a. KA model. A438079 treated mice never scored higher than a 3 on the Racine scale (corresponding to partial/focal limbic seizures), and did not display tonic-clonic seizures. This suggests that A438079 blocks aspects of secondary generalization of seizures. The site of this effect is likely to be outside the hippocampus, and could be within the neocortex. Indeed, tonic-clonic seizures presumably require activation of the motor cortex. The earlier finding of constitutive expression of P2X7R in neocortex may be significant in this regard (Shah et al., 2004). Thus, neocortex P2X7Rs may facilitate seizure spread, which was prevented by A438079. Consistent with this, neocortical injury was also reduced in A438079-treated mice. Taken together, the data indicate a role for the P2X7R in neocortical propagation or secondary generalization of seizures in this model. Supporting behaviour seizures analysis, neuronal damage was assessed in tissue from A438079 and vehicle mice, showing a clear reduction of cortical damage in treated mice.

The strong anticonvulsant effects observed with A438079 contrast with reports of seizure effects of P2X7R activation in the pilocarpine model. Kim and Kang reported that seizures were increased in P2X7R−/− mice after pilocarpine administration (Kim and Kang, 2011). Some in vitro work supports this data, an inhibitory action of P2X7R activation was reported in the CA3 field recordings during mossy fiber pathway stimulation from hippocampal slices (Armstrong et al., 2002). These data suggest the contribution of P2X7R to seizures may be strongly model-dependent. This may also be related to how and when ATP is released; high amounts due to cell lysis or vesicle-based controlled release. Indeed, the intra-amygdala KA model produces a consistent distinct lesion to the CA3 area of the hippocampus, whereas hippocampal lesions do not occur in the systemic KA and picrotoxin models and are highly variable in the pilocarpine model. The mechanism by which the P2X7R inhibitor protected against seizure induced cell death is still unclear, although direct neuroprotective effects of P2X7R antagonists have also been reported in other models, including Huntington’s disease and ischemia (Diaz-Hernandez et al., 2009; Arbeloa et al., 2012). Direct astroglial protective effects of P2X7R antagonists have
been demonstrated in other epilepsy models (Kim et al., 2009; Kim et al., 2011b). This may be a result of blocking the cell lysis-inducing pore forming function of the P2X7R. However, it is most likely a result of reduced seizure severity (Engel et al., 2012b).

In summary, this chapter shows increased P2X7R levels in the neocortex after SE and in experimental epilepsy. Powerful effects of P2X7R inhibition were observed in reducing clinical seizures during SE and on protecting the neocortex from damage by SE. Together, these data support the potential of P2X7R ligands as new drug targets for status epilepticus and perhaps, epilepsy.
Chapter IV – Expression and transcriptional control of the P2X7 receptor in the hippocampus in epilepsy

4.1 Introduction

4.1.1 Active inflammatory process during epilepsy

The P2X7R may function as an important link between brain excitability and inflammation in epilepsy. Increasingly, immune and inflammatory mechanisms have been suggested in some forms of epilepsy. It is not yet clear, however, if inflammation might be a consequence or a cause of epilepsy (Vezzani, 2005; Gorter et al., 2015). Innate immunity activation is coordinated by a wide array of inflammatory mediators among which cytokines have a key role, including interleukins (ILs), interferons (IFNs), tumour necrosis factor (TNFs) and growth factors ([TGF]-β). The release of cytokines can be driven in the CNS by immunocompetent and endothelial cells, as well as glia and neurons (Vezzani, 2005; Vezzani et al., 2011). These inflammatory mediators have been observed in surgically resected brain tissue from patients with refractory epilepsies, including temporal lobe epilepsy (TLE). Therefore, due to the evidence of inflammatory processes in human epilepsy, experimental rodent models have been used to identify the triggers of brain inflammation during epilepsy and to unravel the link between inflammation and seizures (Marchi et al., 2014).

Evidence that seizure activity induces brain inflammation and continuing recurrent seizures promote chronic inflammation has been obtained from various experimental studies with rodents subjected to SE (Minami et al., 1991; Vezzani et al., 1999b). Results showed an early upregulation of proinflammatory cytokines, including IL-1β, TNF-α and IL-6, after the induction of SE mainly in microglia and
astrocytes. Various mechanisms are possible by which inflammatory cytokines may promote epilepsy. First, IL-1β release activates IL-1R1, which induces the transcription of genes encoding downstream mediators of inflammation via NFκB-dependent mechanisms (Aronica et al., 2012). Moreover, the activation of IL-1R1 can inhibit the reuptake of glutamate from astrocytes and increase glutamate release from glial cells by secondary pathways, including via TNF-α (Bezzi et al., 2001). Increased levels of extracellular glutamate trigger the release from glial cells of gliotransmitters (ATP, glutamate, or D-Serine) that are potent modulators of neuronal activity, synaptic transmission and plasticity (Fellin et al., 2004). Studies have reported that IL-1β also downregulates AMPA receptor expression and phosphorylation in hippocampal neurons in a Ca²⁺- and NMDA-dependent manner (Lai et al., 2006). IL-1β is also a potent stimulator of astrogliosis, which is also known to promote epilepsy (de Lanerolle et al., 2010). In vivo evidence for the importance of IL-R1 stems from studies where resistance to seizures was observed in IL-1R type I-deficient mice after bicuculline-induced seizures (Vezzani et al., 2000).

In recent years, extracellular ATP has been considered a potent danger signal (Burnstock and Verkhratsky, 2010). ATP is locally released at the inflammation site by neurons and astrocytes and triggers the activation of the ATP-gated P2X7R provoking the maturation and release of IL-1β (Ferrari et al., 2006). This chapter will explore inflammation and upregulation of proinflammatory molecules in epilepsy, using the intra-amygdala KA model of SE in mice.

### 4.1.2 P2X receptors expression in epilepsy

P2X receptors (P2Xs) are found throughout the entire brain and have been reported to be expressed by neurons and glia (Verkhratsky et al., 2012; Henshall et al., 2013). A number of P2X receptors have been shown to be altered after SE in the hippocampus. P2X1R has been found up-regulated in the hippocampus after SE (Avignone et al., 2008). P2X2 was reported down-regulated after SE and in seizure-
sensitive gerbils (Kang et al., 2003; Engel et al., 2012b). Studies on P2X4R have shown both up-regulation and down-regulation in the hippocampus after SE (Avignone et al., 2008; Dona et al., 2009). Recently, a study has reported a P2X4R up-regulation in hippocampal microglia after SE in rats (Ulmann et al., 2013). No change has been observed in P2X3R (Seguela et al., 1996) and P2X5R after SE (Engel et al., 2012b).

In the setting of epilepsy, Papp et al. showed altered expression of a number of P2XRs in the hippocampus (Papp et al., 2004b). Dona et al. studied the P2X2R, P2X4R and P2X7R immunoreactivity in hippocampal sections from rats that underwent SE induced by pilocarpine. In control animals, P2X4R immunoreactivity was present in the cell bodies and dendrites of pyramidal layers of the CA1 and CA3 hippocampal regions, whereas the P2X2R was found in the cell bodies of pyramidal layers and hilus of the DG. Additionally, western blot analyses showed a decrease in P2X4R protein levels in the CA1 and CA3 hippocampal subfields and in the hilus area in epileptic rats. Immunostaining for the P2X7R was visible in pyramidal neurons of the CA1 and CA3 hippocampal subfields and in the hilus area of the DG during the acute and latent period. Strong reactivity in the mossy fibers of the CA3 region, in the hilus and in the supragranular region of the DG was observed during chronic epilepsy. Results were supported by western blot analyses which showed an increase of P2X7R in the chronic phase of epilepsy and a decrease in P2X4R at the same time point (Dona et al., 2009). These results suggest important roles for P2XRs in disorders of excitability.

4.1.3 Control of P2X7R expression: Involvement of epigenetics?

Research on epigenetic mechanisms in epilepsy remains limited. Evidence includes rapid, spatio-temporal changes in histone modifications after seizures (Tsankova et al., 2004). Huang and colleagues showed that the acetylation of the histone H4 in neurons from the CA3 hippocampal subfield in rats was reduced at the GluR2 promoter but increased at the brain-derived neurotrophic factor (BDNF) promoter
after pilocarpine SE (Huang et al., 2002). Other studies have emphasised the role of DNA promoter methylation. The first study looked at methylation of Reelin in human TLE patients compared to controls finding increased methylations suggesting epigenetic silencing (Kobow et al., 2009). Expression of DNMT enzymes that are required for de novo and maintenance of DNA methylation have been shown to be increased in the temporal neocortex of TLE patients (Zhu et al., 2012a).

Recently, a genome-wide study of DNA methylation revealed selected changes in CpG islands and promoter regions in the mouse hippocampus after SE (Miller-Delaney et al., 2012). Lubin et al., also found that decreased Bdnf DNA methylation levels were strongly correlated with abnormally high levels of Bdnf mRNA in the epileptic hippocampus during memory consolidation. Results showed that increased levels of Bdnf DNA methylation improves memory in epilepsy (Parrish et al., 2015). Given the tight, restricted expression of P2X7R, reported to be predominantly expressed in microglia and neurons of the DG hippocampal subfield (Engel et al., 2012b), it is possible that the promoter of the P2X7R is hypermethylated in some cell types or certain hippocampal areas. Therefore, methylation of the P2rx7 promoter region was investigated here as a possible mechanism of P2X7R transcriptional control in epilepsy.

### 4.1.4 Sp1 as a novel transcription factor regulating P2rx7 transcript levels

An alternative mechanism of gene expression regulation is through transcription factors. These are proteins that bind to specific DNA sequences and control the transcription of a particular gene (Tan and Khachigian, 2009). There have been few studies to date addressing what such controllers of P2X7R expression might be. A recent study by our collaborators provided molecular and functional evidence that the specificity protein 1 (Sp1) transcription factor plays a key role in the transcriptional regulation of P2X7R. They identified a region in the murine P2rx7 promoter which contains four Sp1 binding sites and confirmed its functionality by site-directed mutagenesis and Sp1 overexpression/down-regulation in
neuroblastoma cells (Garcia-Huerta et al., 2012). Garcia-Huerta et al., showed that Mithramycin A, a DNA binding transcriptional inhibitor that binds to GC rich sequences in a competitive way with the Sp1 transcription factor, reduced P2X7R expression in neuroblastoma cells (Garcia-Huerta et al., 2012). Whether Sp1 controls P2X7R in epilepsy is unknown. However, studies have reported the activation of Sp1 transcription factor after KA-induced seizures. An increase of Sp1 transcription factor activity was reported 2 weeks after induced seizures, as determined by analyses of Enk-Sp-1 activity using electrophoresis mobility-shift assays (Feng et al., 1999). The increased Sp1 activity occurred in parallel with the emergence of mossy fiber sprouting, suggesting that elevated Sp1 activity may drive long-term changes in the plasticity of hippocampal function in epilepsy (Feng et al., 1999). Therefore, given previous studies, expression of Sp1 transcription factor may be involved in the control of the P2X7R in SE and in epilepsy.

4.2 Chapter objectives

Hypothesis: IL-1β and P2X7R are up-regulated in the hippocampus in the intra-amygdala KA model of TLE, which is controlled by Sp1 and epigenetic factors.

Aims: To characterize the inflammation process in this model analysing microglia, astrocytes and pro-inflammatory cytokine levels in epilepsy, to assess the expression of the different P2X family channels in epilepsy and last, to explore the role of Sp1 transcription factor and DNA methylation on the control of P2X7R expression.
4.3 Results

4.3.1 Intra-amygdala KA-induced SE as a model to study epilepsy

A well-characterized model of focal-onset SE by unilateral intra-amygdala KA (0.3 μg) was used to study hippocampal expression and control of the P2X7R in epilepsy (Mouri et al., 2008a). Seizures emerged 10 – 15 min after microinjection of KA into the right amygdala, as confirmed by the emergence of electrographic polyspiking and behavioural convulsions (Figure 4.1 A). Electrographic seizures continued throughout EEG recording (40 min). Vehicle-injected animals showed no behavioural changes during the 40 min. After 40 min, mice were intraperitoneally injected with lorazepam to curtail seizures and reduce morbidity and mortality. Brain sections from animals 24 h after SE showed restricted damage mainly in the ipsilateral CA3 hippocampal subfield (Figure 4.1 B, C, D).
Figure 4.1 Electrographic seizures and hippocampal injury following seizures evoked by intra-amygdala KA injection in mice

A) EEG pseudocolor heat map of frequency and amplitude data in control mice and after intra-amygdala KA injection in mice and representative EEG traces from control and during SE animals. B, C) Representative photomicrographs illustrating neuronal death (FJB) in the CA3 stratum pyramidal (arrows) 24 h after SE. Hippocampal subfields CA1 and dentate gyrus (DG) are largely spared. Scale = 500 μm. D) Higher power views FJB-positive cells in CA3 from left panel. Scale bar = 180 μm.
4.3.2 Evidence of epileptic seizures involving the ipsilateral hippocampus in the intra-amygdala KA-induced SE mouse model

As previously reported, all mice subject to intra-amygdala KA-induced SE go on to develop spontaneous recurrent seizures (epilepsy) after a short latent period. These epileptic seizures emerge 3-5 days post KA-injection and occur at a frequency of 1-5 per day (Mouri et al., 2008a) (Figure 4.2 A). However, no previous study has presented data to support the involvement of the hippocampus during these spontaneous seizures.

As supporting evidence of recent intense neuronal firing, the expression of the activity-regulated immediate early genes *C-fos* and *Arc* was measured within microdissected ipsilateral CA1, CA3 and DG hippocampal subfields in epileptic mice (14 days after SE) using RT-qPCR. All experimental samples were assessed versus time-matched non-seizure vehicle controls. *C-fos* and *Arc* expression was significantly higher in all hippocampal subfields in epileptic animals when compared to control non-epileptic mice (Figure 4.2 B, C).

To further characterise neuropathological changes in the epileptic hippocampus, we assessed protein levels of markers of neurons and glia. Western blot analysis of hippocampal lysates showed increased levels of glial fibrillary acidic protein (GFAP) in the CA3 subfield in epileptic tissue when compared to control animals (Figure 4.2 D, F). In contrast, NeuN levels, a specific neuronal marker, were lower in the CA3 hippocampal subfield of epileptic mice compared to controls (Figure 4.2 E, G). As the CA1 and DG hippocampal subfields are spared damage in this model, neuropathological analyses were not performed in these areas (Engel et al., 2012).
Figure 4.2 Recruitment of the ipsilateral hippocampus during epileptic seizures and presence of astrogliosis and hippocampal sclerosis

A) Cortical electrodes placement for EEG recordings. B) Typical electrographic spontaneous seizure recorded with a telemetry device in a mouse 14 days after i.a. KA-induced SE. C) Higher resolution EEG trace during spontaneous seizure. D, E) Real-time qPCR measurement of neuronal activity regulated early-genes c-fos and Arc (normalized to actin) for CA1, CA3 and DG hippocampal subfields of control and epileptic mice (n = 9 mice per group). E, F) Western blots showing GFAP and NeuN protein levels in CA3 hippocampal subfield of control and epileptic mice (n = 5 mice per group, n = 1 sample per lane). α-Tubulin is shown as loading control. G, H) Densitometry analysis of the CA3 levels of GFAP and NeuN protein. T-test. *p < 0.05. **p < 0.01. All data are mean ± s.e.m.
4.3.3 Increased molecular markers of inflammation in the hippocampus in epilepsy

Inflammation is a common pathophysiological response after seizures and previous studies reported that microglia and astrocytes are the primary source of cytokines (Vezzani, 2005). To explore whether inflammatory responses occur in this model of epilepsy, the levels of *Iba-1*, as a microglia marker, *GFAP*, as an astrocyte marker, and *IL-1β* and *TNF-α* as pro-inflammatory cytokines were examined. All genes analysed showed a significant increase in mRNA levels in the CA1, CA3 and DG hippocampal subfields from epileptic animals when compared to controls. (Figure 4.3 A, B, C, D).

To support these results, Western blot analysis was performed for some of these markers. Results showed an increase of the microglia marker Iba-1 in the whole hippocampus (Figure 4.3 E, G) and mature IL-1β in each hippocampal subfield of epileptic mice when compared to time-matched control mice (Figure 4.3 F, H).

In agreement with Western blot data, immunofluorescence analysis of Iba-1 showed a remarkable enhanced microglia expression in the hippocampus of epileptic mice, particularly in the CA1 and CA3 subfields when compared to control (Figure 4.4 A, B).
Figure 4.3 Increased molecular markers of inflammation in the hippocampus in epilepsy

A, B, C, D) Real-time PCR analysis of microglia activation (Iba-1, IL-1β and TNF-α) and astroglial (GFAP) messenger RNA levels in CA3, CA1 and dentate gyrus (DG) hippocampal subfields of control and epileptic mice (n=9 per group). E) Representative western blots (n=1 per lane) of microglia marker, Iba-1 in the whole hippocampus and F) IL-1β levels in CA1, CA3 and DG hippocampal subfields in control and epilepsy (n=4 per group). α-Tubulin is shown as loading control. G, H) Densitometry analysis of Iba-1 and IL-1β protein levels. T-test. *p < 0.05. **p < 0.01. ***p<0.001.
Figure 4.4 Increased microglia expression in the hippocampus in epilepsy

**A, B**) Representative Iba-1 staining of whole hippocampus from control and epileptic mice. Scale bar = 500 μm. White boxes show magnification views of Iba-1 staining in microglia cells in CA1 and CA3 of the hippocampus. Scale bar = 100 μm. **C**) Representative negative control of the CA3 area from epileptic mice. Scale bar = 100 μm.
4.3.4 Up-regulation of P2X receptors in the hippocampus in epilepsy

To examine whether P2XRs are altered in the present model of epilepsy, qPCR was used to first examine the expression of transcripts for P2rx1, P2rx2 and P2rx4 due to their alteration after SE published in previous studies (Dona et al., 2009; Engel et al., 2012b; Ulmann et al., 2013).

P2rx1 mRNA levels were increased in all hippocampal subfields in epileptic mice (Figure 4.5 A). In contrast, P2rx2 mRNA showed a decrease in CA1 only; no differences between groups were observed for CA3 and DG hippocampal regions (Figure 4.5 B). The P2rx4 transcript showed the highest increase in the hippocampus in epilepsy, with a 4 – 7 fold increase in all hippocampal subfields when compared to levels in control animals (Figure 4.5 C).

To support these mRNA findings, protein levels of these receptors were analysed by Western blot. Protein levels of P2X1R and P2X2R were not different between epilepsy and control samples for any subfields (Figure 4.6 A, B, C, D). For the P2X4R, protein levels were higher only in the CA1 and CA3 when compared to controls (Figure 4.6 E, F).
Figure 4.5 Altered P2XR transcript levels in the hippocampus in epilepsy

A, B, C) Real-time qPCR measurement of P2rx1, P2rx2 and P2rx4 mRNA levels for the CA1, CA3 and DG hippocampal subfields of control and epileptic mice (n = 9 per group). T-test. *p < 0.05. **p < 0.01. ns - not significant.
Figure 4.6 Altered hippocampal P2XR levels in epilepsy

A, C, E) Western blots (n=1/lane) showing expression of different P2XR subtypes (P2X1R, P2X2R and P2X4R) within the 3 main subfields of the hippocampus in vehicle-injected control and epileptic mice (n=9 per group). α-Tubulin is shown as guide to loading. B, D, F) Graphs showing semi-quantification analysis of each protein in all hippocampal subfields in vehicle-injected control and epileptic animals. T-test. *p< 0.05. ns-not significant.
4.3.5 Increased P2X7R expression in hippocampal subfields in epilepsy

A key function of P2X7R activation is the processing and release of IL-1β (Ferrari et al., 2006; Rappold et al., 2006). Having established the increase of IL-1β levels in the hippocampus in epilepsy, we continued to analyse hippocampal P2X7R expression in the hippocampus in epilepsy. Real-time qPCR showed increased P2X7R induction in the hippocampus in epileptic mice. All hippocampal subfields showed higher P2rx7 mRNA levels in epilepsy when compared to control (Figure 4.7 A). The CA3 hippocampal subfield showed the highest increase of P2rx7 mRNA expression among the three hippocampal regions.

To support qPCR results, western blot analysis was performed to establish P2X7R protein levels in epilepsy. Western blotting detected a band at the predicted molecular weight of ~70 kDa in control hippocampal subfields. Epileptic animals showed higher levels of P2X7R in CA1 and DG hippocampal subfields. P2X7R protein levels were not different in the CA3 hippocampal region in epileptic mice when compared to controls (Figure 4.7 B, C).
Figure 4.7 Increased *P2rx7* mRNA and P2X7R protein levels in hippocampal subfields in epilepsy

A) Real-time qPCR measurement of *P2rx7* mRNA levels for the CA1, CA3 and DG hippocampal subfields of control and epileptic mice (n = 9 per group). B) Representative blots showing P2X7R protein levels (n = 1/lane) within the 3 main subfields of the hippocampus in control and epileptic animals. α-Tubulin is shown as loading control. C) Semi-quantitative analysis of P2X7R protein levels for the different subfields in control and epileptic mice (n= 9 per group). T-test. *p< 0.05, **p < 0.01. ns-not significant.
4.3.6 P2rx7 transcriptional regulation by the SP1 transcription factor

A recent study showed that Specificity protein 1 (Sp1) was the mediator of the transcriptional regulation of P2X7R in N2a cells (Garcia-Huerta et al., 2012). To explore whether SP1 was involved in P2X7R regulation after SE, expression of Sp1 was analysed by PCR. This showed no major Sp1 mRNA changes over time in the hippocampus after SE (Figure 4.8 A). However, as the major increase of P2X7R was found 8 h after SE in the DG hippocampal subfield, the Sp1 mRNA levels were analysed in that particular region at the same time point. Results showed that Sp1 mRNA levels were higher in the DG hippocampal subfield 8 h after SE induction when compared to time-matched controls (Figure 4.8 B). This data was supported by Western blot analysis. Results revealed a progressive increase in SP1 protein levels in whole hippocampus over time after SE (Figure 4.8 C). Densitometry analyses showed a peak in expression at 8 h and 72 h after SE when compared to control (Figure 4.8 D).

Next, SP1 expression was examined in the hippocampus after intra-amygdala SE, when animals had developed epilepsy. Real-time qPCR results showed no differences in Sp1 mRNA levels in any of the three hippocampal subfields in epilepsy (Figure 4.9 A). However, Western blot analyses of SP1 protein levels showed higher levels in the CA3 and DG hippocampal regions in epilepsy when compared to control animals (Figure 4.9 B, C).
**Figure 4.8 Upregulation of Specific protein 1 (Sp1) transcription factor after SE**

**A)** Real-time qPCR measurement of Sp1 mRNA levels in the hippocampus after SE (n = 5 per group). **B)** PCR measurement of Sp1 mRNA levels in the DG hippocampal subfield 8 h after SE (n = 5 per group). **C)** Representative western blots (n = 1 per lane) showing increased Sp1 protein levels in the hippocampus after SE. **D)** Densitometry analysis of Sp1 abundance in the different time points compared to control (n = 6 per group). T-test. *p < 0.05. **p < 0.01. ns-not significant.
Figure 4.9 Increased Sp1 expression in hippocampal subfields in epilepsy

A) Real-time qPCR measurement of Sp1 mRNA levels for the CA1, CA3 and DG hippocampal subfields of control and epileptic mice (n = 9 per group). B) Representative blots showing Sp1 protein levels (n = 1/lane) within subfields of the hippocampus in control and epileptic animals (n = 9 per group). α-Tubulin is shown as a loading control. C) Semi-quantitative analysis showing Sp1 protein levels for the different subfields in control and epileptic mice (n= 9 per group). T-test. *p < 0.05. ns-not significant.
4.3.7 Effect of SP1 inhibitor on P2X7R expression in epilepsy

Previous studies reported that pharmacologic inhibition of SP1 by Mithramycin A reduced P2X7R expression (Garcia-Huerta et al., 2012). This is thought to be due to allosteric competition with the promoter site. To investigate whether Mithramycin A affects expression of P2X7R in epilepsy we tested this inhibitor in the model.

The experimental plan was as follows (Figure 4.10 A); mice were subjected to SE induced by intra-amygdala KA and were then injected for 5 consecutive days twice daily with Mithramycin A (150 µg/kg in 150 μl saline vehicle), from day 10 to day 14 post-KA injection. Mice were then sacrificed at day 14 post-KA for the analysis of P2X7R expression.

Treatment of mice with Mithramycin A resulted in a small, non-significant decrease in Sp1 mRNA levels in each hippocampal subfield in epileptic animals when compared to the vehicle control group (Figure 4.10 B). Analyses of P2rx7 mRNA levels showed no difference between groups in the CA1 and CA3 hippocampal subfields. However, P2rx7 mRNA expression was strongly reduced in the DG hippocampal region after five days of Mithramycin A treatment (Figure 4.10 C).

Levels of the immediate early gene C-fos expression was analysed to assess neuronal activity in each hippocampal subfield after Mythramycin A or vehicle treatment. A strong reduction of C-fos mRNA expression was observed in drug treated animals when compared to the vehicle group (Figure 4.10 D). These data suggest Sp1 may regulate expression of P2X7R within the DG and reduce seizures in this model of epilepsy.
Figure 4.10 The SP1 inhibitor, Mithramycin A, reduces P2X7R expression in vivo

A) Diagram showing the Mithramycin A (Myt) experimental study plan. Mice were injected with 150 µg/kg of Myt, twice daily during the last four days before being sacrificed.

B, C, D) Real-time qPCR measurement of Sp1, P2rx7 and early-gene C-fos mRNA levels in the CA1, CA3 and DG hippocampal subfields in vehicle and Mithramycin A treated animals (n = 4 per group). T-test.*p< 0.05. ns-not significant.
4.3.8 Analysis of the methylation status of the P2rx7 promoter in epilepsy

To extend the data on mechanisms controlling P2X7R transcription in epileptic mice, we next explored the possible role of DNA methylation, a key epigenetic mechanism that typically inhibits gene expression. Hypermethylation of CpG islands (CGIs) at gene promoters plays a key role in the silencing of numerous genes and recent work has suggested DNA methylation is altered in experimental and human epilepsy (Kobow et al., 2009; Miller-Delaney et al., 2012; Zhu et al., 2012a; Miller-Delaney et al., 2015). P2X7R hypermethylation has not yet been analysed in the brain, however, studies have shown changes in cancer cells (Zhou et al., 2009). Accordingly, the methylation status of the P2rx7 promoter was analysed using DNA samples from each hippocampal subfield focusing on a 2.5 kb region around the Transcription Start Site (TSS). Methylation status was assessed using the bisulfite sequencing technique (Figure 4.11 A). Briefly, this process involves the conversion of the cytosines in single-stranded DNA into uracil residues, which will be recognized as thymine in the PCR amplification and sequencing. However, 5-methylcytosine (5mC) are resistant to the conversion and remain as cytosines allowing 5mCs being distinguished from unmethylated cytosines. The methylation status in the loci of interest can be determined by PCR using specific methylation primers after the bisulfite treatment (Li and Tollefsbol, 2011). We identified 14 CpG islands in the promoter region of the mouse P2rx7. The numbering of the 14 CpG islands analysed were given respectively from the distance to the TSS. Number 1 was the furthest while number 14 is the closest.

No obvious P2rx7 promoter region DNA methylation pattern was found in CA1, CA3 or DG hippocampal subfields in naïve animals. There was, however, a trend among the 14 CpG islands analysed toward hypermethylation in the CA1 subfield, whereas the DG hippocampal region seemed to have fewer CpG islands methylated. The CA3 hippocampal subfield showed no consistent pattern of DNA methylations among the 14 CpG islands of the P2rx7 promoter (Figure 4.11 B, C).
Next, DNA methylation status of the 14 CpG islands was analysed in epileptic mice. Bisulfite sequencing analyses showed no clear pattern of methylation differences in the 14 CpG islands from the CA1, CA3 and DG hippocampal subfields in epilepsy when compared to control animals. The number of DNA methylation events found in the 14 CpG islands analysed was very homogeneous between groups in each hippocampal subfield, with the exception of two islands that showed variance in methylation patterns (Figure 4.12 A, B). CpG island number 3 seemed to be more consistently hypermethylated in control animals when compared to the epileptic group. In the CA1 hippocampal subfield, analyses showed 2 out of 3 hypermethylations in CpG island number 3, whereas the epileptic group showed only 1 out of 5 hypermethylated islands. In the CA3 subfield, there was no difference in the methylation status of CpG island 3 with 1 out of 4 methylated in the control group and 1 out of 5 methylated in the epilepsy group. Last, analyses of the DG showed the CpG island number 3 methylated in 2 out of 4 in the control group, while in the epilepsy group only 1 out of 5 methylations were found (Figure 4.12 C). CpG island number 8 also showed slight differences in DNA methylation patterns between hippocampal subfields and groups. In the CA1 hippocampal subfield, the CpG island number 8 showed no DNA methylation in the control group, while 1 out of 5 samples was methylated in the epileptic group. In the CA3 hippocampal subfield, there was no difference in the number of methylation found in CpG island number 8. Finally, 3 out of 4 control samples showed DNA methylation at the site 8 CpG island in the DG hippocampal region, whereas only 2 out of 5 samples were found methylated in the epileptic group in this region (Figure 4.12 D).
Figure 4.11 DNA methylation of the P2rx7 promoter region in naïve hippocampus

A) Diagram representing the bisulfite sequencing technique. B) Diagram representing the bisulfite sequencing data on number of methylations in the 14 CpG islands found 2.5 kb region upstream the P2rx7 transcription start site (TSS) in naïve animals (n = 3). Blue filled dots represent methylated islands and empty dots represent unmethylated islands. C) Graph showing the total number of methylations found in each hippocampal subfield in naïve mice (n = 3).
Figure 4.12 DNA methylation of the \textit{P2rx7} promoter in epilepsy

\textbf{A)} Diagram representing the bisulfite sequencing data on number of methylations in the 14 CpG islands found 2.5 kb region upstream the \textit{P2rx7} transcription start site (TSS) in control and epileptic animals (\(n = 3\)). Blue filled dots represent methylated islands and empty dots represent unmethylated islands. Red boxes indicating islands found with differences between groups.

\textbf{B)} Graph showing the total number of methylations found in each hippocampal subfield in control and epileptic animals (\(C = 4, \text{Epi} = 5\)). \textbf{C, D)} Graph showing the number of methylations found in CpG islands 3 and 8 in each hippocampal subfield from control and epileptic animals. T-test. ns-not significant.
4.4 Discussion

This chapter provides background and supporting evidence of the involvement of inflammation and expression of P2X7R signalling in the intra-amygdala KA model. The expression of different P2XRs was investigated in mice that developed epilepsy, revealing selected subfield-specific changes and marked upregulation of P2XRs in the hippocampus. Finally, studies here suggest a role for the Sp1 transcription factor in controlling P2X7R in epilepsy, but exclude a significant role for DNA methylation. Together, these results expand our understanding of the expression and control of P2X7R and ongoing inflammatory processes during epilepsy.

4.4.1 Recruitment of the hippocampus during spontaneous epileptic seizures in intra-amygdala KA-induced SE mouse model

The present study used a focal-onset SE model that propagates seizures from the amygdala via the entorhinal cortex to the hippocampus (Ben-Ari et al., 1980). The advantage of this model is the avoidance of direct neurotoxic effects that are a feature of other models in which KA is injected into the hippocampus, or the variable damage observed in the hippocampus when SE is evoked by systemic pilocarpine or systemic KA (Sloviter et al., 2007).

Previous work has shown that epileptic seizures emerge after 3 – 5 days after SE in this model (Mouri et al., 2008a). The hippocampus of these mice displays expected neuronal loss in the CA3 subfield and astrogliosis. However, no previous studies looked for evidence that the hippocampus was involved or recruited during spontaneous seizures. It is likely that the hippocampus is important, since behavioural seizures in the mice display classic limbic features, such as immobility, tail extension, forelimb clonus or rearing and falling. Studies here began by looking for indirect evidence of the recruitment of the hippocampus during/after spontaneous seizures in the model by assessing the up-regulation of the transcripts of the immediate early-genes (IEG) C-fos and Arc, which are activated by recent
intense neuronal activity (Dudek, 2006). The use of IEG transcription to ‘map’ epileptic seizures is a common technique. Although it cannot determine where seizures originate, it is highly sensitive to show which areas are recruited. In the present study C-fos and Arc transcript levels were found to be increased in each hippocampal subfield. These data suggest that the entire hippocampal network circuit gets ‘recruited’ after epileptic seizures, consistent with other models where the hippocampus is also involved during seizure activity (Sloviter, 1994; Toyoda et al., 2013). However, there are limitations to the approach. Single-cell resolution for example, using immunohistochemistry, could confirm the cells involved as neurons. Another technique would be to perform depth electrode recordings in the DG hippocampal region to confirm this is a site of seizure onset (Sloviter and Bumanglag, 2013). Nevertheless, these data provide useful supportive evidence for hippocampal involvement in spontaneous seizures in this model.

The standard injury in the model is a lesion mainly restricted to the ipsilateral CA3 subfield (Mouri et al., 2008a). This characteristic damage was confirmed here by immunostaining and western blot analysis of the neuronal protein NeuN. The cell death observed in the CA3 hippocampal region found here is consistent with previous data from studies where the same cell damage was found (Mouri et al., 2008a; Engel et al., 2013). Since NeuN is a gene that must be expressed, it remains possible that NeuN expression is reduced rather than an overall loss of neurons. However, FJB staining showed irreversible damage to CA3 neurons excluding this possibility. Taken together, these data expand our knowledge of this model. The injury is highly consistent, unilateral and is associated with later involvement of the hippocampus in spontaneous seizures. The injury to the hippocampus is very different to that perpetrated in the intra-hippocampal model, where all subfields are damaged and the DG develops profound dispersion (Bouilleret et al., 1999). This is not commonly observed in the resected human hippocampus (Sutula and Pitkanen, 2001). Therefore, the intra-amygdala KA model, with a restricted hippocampal lesion, is a valuable tool to study patho-mechanisms of epilepsy.
4.4.2 Inflammation in the hippocampus in the intra-amygdala KA model of epilepsy in mice

Seizure activity leads to the production of inflammatory molecules that, in turn, affect seizure severity and recurrence (Aronica et al., 2012). In addition, studies suggest that seizure-related or injury-related inflammation might contribute to the enhanced ion channel expression, cell loss and synaptic reorganization, which are important mediators of the development of hyperexcitable circuits in the brain. This may lead to epilepsy after insults such as SE (Buckmaster and Dudek, 1997). The present study provides various lines of evidence supporting inflammatory responses in the i.a. KA model of epilepsy.

At a cellular level, western blot analysis of hippocampal tissue from epileptic mice showed increased expression of the astrocytic protein GFAP and the microglia marker Iba-1. This was supported by PCR which showed an increase of both GFAP and the microglia marker Iba-1 mRNA and immunoreactivity in brain sections of epileptic mice. This is in agreement with studies suggesting an active participation of glial cells in the release of proinflammatory cytokines, including IL-1β and TNF-α, in the rodent hippocampus after SE and in epilepsy (Vezzani et al., 1999b; Rizzi et al., 2003). An important observation in the present study was that the increased glial expression levels were not restricted to the main site of damage in CA3, but also evident in the DG and CA1 hippocampal subfields, where much less neuronal loss occurs. This is consistent with other rodent models in which significant activation of glial cells and the release of cytokines occur in the absence of neuronal loss (Vezzani et al., 1999b; Vezzani et al., 2000). These observations suggest that seizure-induced brain inflammation is not necessarily related to cell death and, importantly, does not appear to drive or promote cell death since the CA1 and DG are spared despite the high increase of microgliosis and astrogliosis (Vezzani and Baram, 2007). The key question would be by what mechanisms does gliosis promote epilepsy? A number of potential mechanisms are possible for astrocytosis. Studies have reported that the upregulation of the adenosine-removing enzyme
adenosine kinase (ADK) in astrocytes, causes a deficiency in adenosine, increasing neuronal excitability and favouring seizure generation (Boison, 2008). Reactive astrocytes have shown defective reuptake of neurotransmitters including glutamate, from the synapse increasing therefore neuronal excitability (Aronica et al., 2012). Additionally, astrocytes play an important role in neuronal metabolism through their role in shuttling energy substrates and glutamine back to neurons (de Lanerolle et al., 2010). Loss of glutamine synthetase has been found in areas with neuronal loss and increased glial density such as the CA1 and CA3 hippocampal subfields in epilepsy (van der Hel et al., 2005).

A specific focus in this chapter was in the link between gliosis and inflammation mediators. The specific functions of many inflammatory mediators, in epilepsy remain incompletely understood; however, there is evidence for an active role of IL-1β, TNF-α and IL-6 in seizure generation and exacerbation in epilepsy (Vezzani et al., 2008; Rao et al., 2009). For example, inhibition of ICE/caspase 1, the main enzyme that synthesises IL-1β has anticonvulsant effects in several animal models (Vezzani et al., 2000). However, inflammation is also important for post-injury recovery. For example, neutrophil and microglia activation after brain injury are essential to repair and recovery and when the early phase of inflammation is blocked (particularly microglia responses), this is detrimental to injury outcome (Roth et al., 2014). The challenge is ideally to find the pro versus anti-seizure inflammatory responses and target these appropriately.

The present study demonstrates that several known inflammatory mediators are increased in this model of epilepsy. This data extends previous findings from our group on acute inflammatory changes such as increased levels of mature IL-1β within the ipsilateral CA3 subfield of the hippocampus (Engel et al., 2012b).
4.4.3 P2X receptors in epilepsy

ATP is released into the extracellular medium under pathological conditions such as inflammation or cell death inducing a wide variety of pathophysiological responses through the activation of P2XRs that are present on the surface of glia and neurons. Only a few studies have investigated the expression of these receptors after SE. Increased expression of P2X7R was reported in the hippocampus after SE (Engel et al., 2012b) and in epilepsy, mainly in microglia (Rappold et al., 2006) and on glutamatergic nerve terminals (Dona et al., 2009). P2X2R was found to be decreased in the hippocampus after SE (Engel et al., 2012b). P2X4 was found to be increased in hippocampal microglia after SE (Avignone et al., 2008; Ulmann et al., 2013), although decreased levels were reported in the rat hippocampus in epilepsy (Dona et al., 2009). The present study assessed expression of several P2XRs in the hippocampus in mice that developed epilepsy after SE in the i.a. KA model. Alterations in transcript levels of P2X1-4 receptors were found in several hippocampal subfields. However, there was high degree of specificity; in some cases the increase was observed in each hippocampal subfields, such as for P2rx1 and P2rx4 mRNA, whereas others were more region specific. For example, P2rx2 mRNA was only to be found increased in the CA1 subfield. These findings likely suggest individual transcriptional control mechanisms exist for P2XRs as well as reflecting different cellular origins of the expressed receptors and the differences in hippocampal injuries between subfields. The activation of P2X2R have been reported to promote inhibitory transmission within the hippocampus (Khakh and North, 2006). Previous work from our group showed a reduction of P2X2R protein levels during SE (Engel et al., 2012b). The present study showed P2rx2 mRNA levels significantly decreased in the CA1 hippocampal subfield in epileptic mice, although no important alteration of the receptor in protein levels was observed in the hippocampus during epilepsy. Agonists of this receptor may therefore have potential applications rather SE, but not in epilepsy.
A large and consistent increase was found for P2X4R in all hippocampal subfields in this epilepsy model. This suggests a general response, not restricted to cell death, which would be consistent with glial responses driving the change since gliosis was found in each subfield, whereas neuronal loss was restricted to CA3. Consistent with this hypothesis, previous work showed P2X4R to be expressed in microglia (Ulmann et al., 2013). Further, recent work showed antagonism of P2X4R blocks microglial responses to brain injury (Roth et al., 2014). While we did not investigate the affected cell types due to the difficulty of identifying the receptor with immunohistochemistry analyses, it is likely that the increased P2X4R response in our study, reflects an increase of P2X4R in microglia, which were also increased in our model of epilepsy. In contrast to our study, Dona et al. reported a reduction in P2X4R during the chronic epileptic phase in rat induced by pilocarpine (Dona et al., 2009). However, the differences observed in P2X4R expression could be due to the hallmarks of each model; the pilocarpine model triggers extensive damage to CA1 subfield and hilus, whereas the intra-amygdala KA model produces damage in the CA3 region. Moreover, the use of antibodies with uncertain specificity could result in erroneous findings on P2X4R.

4.4.4 P2X7 receptor expression in the hippocampus in epilepsy

As discussed, inflammation is an ongoing process in epilepsy, and may contribute to the enhanced excitability in epilepsy. Therefore, the regulation of the production and release of pro-inflammatory cytokines including IL-1β is an important focus of study (Smith et al., 2001). In the present study we focused mostly on P2X7R expression as it is known to control the release of IL-1β (Ferrari et al., 2006).

Previous studies from our group reported the upregulation of P2X7R protein levels in the mouse hippocampus after SE induced by intra-amygdala injection of KA (Engel et al., 2012b). Studies here showed P2rx7 transcription is also increased in each hippocampal subfield in epileptic mice in the model. Notably, protein levels of P2X7R were only increased in the CA1 and DG hippocampal subfields and no
alteration was observed in the CA3 hippocampal region. This is consistent with data obtained in a previous study, where P2X7R protein levels were only increased in the CA1 and DG hippocampal subfields after SE (Engel et al., 2012b). The lack of alteration in the expression of P2X7R protein in the CA3 hippocampal subfield could suggest post-transcriptional mechanisms prevent protein production (e.g. micro-RNA). Alternatively, these data suggest that the P2X7R is transcribed by CA3 pyramidal neurons but then is transferred to the presynaptic compartment (i.e. axon terminal), where the protein could be translated and functional (Armstrong et al., 2002). The CA3 pyramidal neurons project to the CA1 region, where P2X7R protein was increased. This data is supported by previous work where an upregulation of P2X7R protein was observed in the terminal mossy fibers in epileptic rats induced by pilocarpine (Vianna et al., 2002; Dona et al., 2009). It is also tempting to speculate that the increased expression of P2X7R is responsible for the upregulation of IL-1β and TNF-α, thereby contributing to hyperexcitability in the brain.

4.4.5 P2X7R expression control by the Sp1 transcription factor in epilepsy

The unique and select expression patterns of P2rx7 in the brain suggest complex mechanisms regulating its expression. So far, little is known about the transcriptional control of P2X7R in epilepsy. Emerging data have implicated Sp1 as a possible transcription factor for P2X7R regulation in neuroblastoma cells (Garcia-Huerta et al., 2012). An early finding here was that Sp1 protein levels were induced in the mouse hippocampus progressively over time after SE, peaking at 8 h and 72 h. These results were supported by PCR analyses where a significant increase of Sp1 mRNA was found 8 h after SE in the DG hippocampal region. This corresponds closely with the peak of P2X7R induction found in the DG hippocampal region in the same model (Engel et al., 2012b). Analysis of Sp1 expression in epilepsy in the i.a. KA model found no difference in the Sp1 mRNA levels in any of the hippocampal subfields, but Sp1 protein levels were increased in the CA3 and DG. These subfields correspond to the two regions in which P2rx7 mRNA was also most increased;
suggesting Sp1 could be a candidate transcription factor for P2X7R in epilepsy. To test this idea, Mithramycin A (Myt A), a specific Sp1 inhibitor was tested in the model. *In vitro* studies showed that Myt A binds with high affinity to GC-rich DNA (Van Dyke and Dervan, 1983). Footprinting and gel shift analyses also showed that Myt A and Sp1 competitively bind to a GC-rich motif in the Myc (Snyder et al., 1991) and c-Src (Remsing et al., 2003) promoters. Indeed, the ability of Myt A to bind to GC-rich DNA and displace the Sp1 family proteins from binding sites in numerous genes has been a much used model in research studies (Sleiman et al., 2011).

Based on these data, Myt A was administrated for five days to mice after SE. Mice received daily injections of Myt A to block Sp1 activity. This was found to reduce Sp1 transcript levels in the hippocampus in epilepsy. Results showed a reduction of *P2rx7* mRNA levels in the DG hippocampal subfield. The CA1 and CA3 hippocampal subfields did not show a significant decrease in *P2rx7* mRNA levels, but there was a strong trend. These data suggest Sp1 may drive P2X7R expression in the DG, but not other brain regions in epilepsy. Although these data support a role for Sp1, unequivocal proof requires direct analysis of Sp1 binding to the *P2rx7* promoter, such as by chromatin immunoprecipitation assays (Chip assay), which was not undertaken here.

**4.4.6 Epigenetic control of P2X7R expression in epilepsy**

The present study also explored a role for DNA methylation in P2X7R expression control. The role of DNA methylation in the pathology of epilepsy has become apparent through a number of studies. *Reelin* promoter methylation has been linked to reduced reelin protein that leads to granule cell dispersion, a characteristic feature of TLE pathology (Kobow et al., 2009). Research by our group identified genome-wide changes in DNA methylation following SE in the i.a. KA model. In particular, hypo-methylation was found prominent after SE, although *P2rx7* was not identified specifically in that study (Miller-Delaney et al., 2012). Due to the growing studies of epigenetic changes in epilepsy, the possibility of P2X7R expression control by DNA methylation was explored. Methylation is an attractive
mechanism to control $P2rx7$ promoter because its expression is highly restricted in the adult brain (Collo et al., 1997).

The methylation state of 14 different CpG islands found 2.5 kb upstream from the transcription start site (TSS) in the $P2rx7$ promoter region were studied in the hippocampal subfields from naive mice first, and then in epilepsy. The results obtained from the naive samples did not show a clear methylation pattern, but a tendency to lower levels of methylation was found in the DG whereas the CA1 had more methylated islands. This is in good agreement with data from a reporter mouse which showed that P2X7R was expressed by a subset of neurons in the DG (Engel et al., 2012b). Analysis of these CpG islands in epilepsy did not show any clear evidence of methylation alterations in the $P2rx7$ promoter region in epilepsy. Therefore, it is unlikely that DNA methylation alone influences P2X7R expression in epilepsy. It remains possible that within single cells there are important DNA methylation patterns, but the tissue sampling, containing a mix of glia and neurons missed this. This could be tested, for example, by laser capture microdissection.

In summary, the present chapter provides new insights into the expression of the P2X7R in the hippocampus in experimental epilepsy. Here we found evidence of inflammation supporting a P2X7R upregulation in areas of injury and gliosis. Moreover, the experiments here provide initial evidence for mechanisms controlling P2X7R expression in epilepsy.
Chapter V. Functional evidence of P2X7R involvement in epilepsy

5.1 Introduction

5.1.1 Aberrant expression and function of neurotransmitter receptors in epilepsy

Altered distribution of neurotransmitter receptor subtypes have been proposed as a major pathogenic mechanism for hyperexcitability in epilepsy (Dudek, 2010). Increased expression of the ionotropic GluR2/3 receptor has been reported in TLE hippocampal tissue (Das et al., 2012). Studies have reported the alteration of mGluR1 and mGluR5, two excitatory class I metabotropic glutamate receptors in the hippocampus in the electrical kindling and KA rat models. Immunostainings and transcript levels results showed an increase of these receptors in the DG hippocampal subfield. These findings could correspond to functional alterations of class I mGluRs observed in seizure models and may significantly contribute to hippocampal hyperexcitability in human epilepsy (Blumcke et al., 2000). Alteration of GABA receptor functions has been frequently observed in epilepsy which could also mediate modifications of the normal inhibitory tone in the brain, increasing network excitability (Brooks-Kayal et al., 1998; Nusser et al., 1998). A profound loss of hilar GABAergic cells was observed in the hippocampus in a hippocampal KA mouse model after SE. However, the number of GABA receptors was found increased in the granule cell somata and dendrites of the DG in epilepsy (Bouilleret et al., 2000). Much is known about neurotransmitter receptor alterations in epilepsy, however, little is known about the alterations of the P2X7R in the hippocampus in this disease.
5.1.2 P2X7R function in the hippocampus in epilepsy

In early work, the function of the P2X7R was primarily linked to microglia activation. Ferrari et al., showed that treatment of N13 microglial cells with oxidized ATP (oxATP), a selective irreversible P2X7R inhibitor, inhibits release of IL-1β (Ferrari et al., 1997). Supporting this study Monif et al., showed that overexpression of the P2X7R drove the activation and proliferation of microglia in rat primary hippocampal cultures and the treatment of these cells with oxATP resulted in a significant decrease in the number of activated microglia (Monif et al., 2009).

Experiments have also proposed important P2X7R functions in neurons. Whole-cell patch-clamp recordings in sympathetic preganglionic neurons from spinal cord slices showed that BzATP caused a depolarization of the neurons, resulting in action potential firing (Deuchars et al., 2001). P2X7R activation effects were also studied by Armstrong et al. in the mossy fiber-CA3 pathway. Dentate granule cells were stimulated in hippocampal slices from immature rats and field recordings were made in the CA3 hippocampal subfield. BzATP application resulted in a decrease in field potentials, suggesting that P2X7R activation could provoke synaptic depression in the hippocampus (Armstrong et al., 2002). Supporting data from electron microscopy examination revealed P2X7R immunoreactivity in presynaptic terminals in all hippocampal subfields, specifically in large synaptic terminals of the DG and CA3 and in smaller and asymmetric-like type of synapse in the CA1 (Sperlagh et al., 2002). However, antibody specificity is critical for these data to be accepted (Sim et al., 2004). Hippocampal rat slices perfused with ATP and BzATP showed an increase of GABA and glutamate release by the activation of P2X7R in the hippocampus (Sperlagh et al., 2002). Together, these studies suggest P2X7R localization on nerve terminals in the hippocampus in addition to microglia and perhaps other cell types in the brain (Sperlagh et al., 2002).
5.2 Chapter objectives

**Hypothesis:** The hypothesis to be tested is that P2X7R expression and function is increased in neuronal terminals/synapses in the intra-amygdala KA model of epilepsy and contributes to hyperexcitability.

**Aims:** First, to obtain individual cell resolution evidence that neurons transcribe and express the P2rx7 in the intra-amygdala KA model of epilepsy. Second, to confirm the presence of the P2X7R protein in the synapse and third, to obtain evidence of altered P2X7R function in epilepsy.
5.3 Results

5.3.1 Increased P2rx7 induction in the hippocampus in epilepsy

To obtain single-cell resolution evidence of the induction of the P2X7R within neurons in epilepsy, the P2X7R reporter mouse that expresses GFP under the transcriptional control of the P2rx7 promoter was used. This allows to visualize, at a single-cell resolution, the transcriptional induction of the P2X7R under physiological condition and in epilepsy. For these experiments, mice were killed 14 days after SE, when mice experience frequent daily spontaneous seizures (Mouri et al., 2008a). Brain sections from reporter control and epileptic mice were analysed for GFP immunofluorescence by confocal microscopy. Constitutive GFP fluorescence was readily observed without the need for an anti-GFP antibody, in both control and epileptic brains. However, anti-GFP antibody was used throughout the study to enhance GFP signal, thereby avoiding to miss cells with low GFP induction.

Analysis of non-hippocampal brain regions in these mice revealed both constitutive and epilepsy-associated patterns of GFP expression. A strong GFP signal was observed in control mice in the molecular layer of the cerebellum, which contains the axons of the granule cells and the dendrites from Purkinje and neuroglia cells located in the nuclear layer. Intense GFP fluorescence in the dendritic field in the molecular layer could be due to the diffusion of the GFP protein. In the thalamus of control mice there was no GFP expression. In contrast, there was GFP fluorescence in cell somas in the ventral nucleus region of the thalamus of EGFP-P2rx7 epileptic mice. The striatum showed GFP fluorescence in EGFP-P2rx7 mice in both control and epileptic mice. Last, as reported in the first chapter, the neocortex showed constitutive GFP signal as well as an increase of GFP induction in EGFP-P2rx7 epileptic mice when compared to controls (Figure 5.1 A, B)(Figure 5.2 A, B).

Analyses of the hippocampus in P2X7R reporter mice revealed major differences in GFP fluorescence between control and epileptic animals. The strongest induction of GFP was observed in the CA1 hippocampal subfield of EGFP-P2rx7 epileptic mice.
Intense GFP signal was observed along the entire length of the stratum pyramidale which contains the cell bodies of the neurons. This pattern of induction was observed in all animals. The dendritic field of the CA1 neurons was also intensely positive for GFP, again perhaps due to the diffusion of the GFP translated protein. Constitutive GFP staining was observed in the granule cell layer of the DG in control EGFP-\textit{P}2\textit{rx7} mice and this was strongly increased in epileptic mice. In contrast, there was only limited GFP staining of the CA3 subfield in epileptic mice (Figure 5.3 A,B)(Figure 5.4 A, B).
Figure 5.1 P2X7R induction within the brain in normal brain

**A)** Photomicrographs showing GFP immunofluorescence in a sagittal section from a control EGFP-P2rx7 mouse. Scale bar = 10 mm. **B)** Higher-power images of the different brain areas expressing GFP in a control EGFP-P2rx7 mouse. Scale bar = 500 μm. (n = 3)
Figure 5.2 Increased P2X7R induction within the brain in epilepsy

A) Photomicrographs showing GFP immunofluorescence in a sagital section from an epileptic EGFP-P2rx7 mouse. Scale bar = 10 mm. B) Higher-power images of the different brain areas expressing GFP in an epileptic EGFP-P2rx7 mouse. Scale bar = 500 μm. Note dramatic induction of GFP in the hippocampal subfield CA1 (arrow). (n = 5)
Figure 5.3 Expression of P2X7R in the DG hippocampal subfield in control animals

A) Photomicrograph showing GFP immunofluorescence in the hippocampus of EGFP-P2rx7 control animals. Note constitutive expression appears mainly in cells of the dentate granule layer. Scale bar = 125 μm. B) Higher-power images of CA1 (i) and DG (ii) hippocampal subfields from EGFP-P2rx7 control mice showing the constitutive expression of P2X7R. Scale bar = 50 μm.
Figure 5.4 Increased expression of P2X7R in CA1 and DG hippocampal subfields in epilepsy

A) Photomicrograph showing GFP immunofluorescence in the hippocampus of EGFP-P2rx7 epileptic animals. Scale bar = 125 μm. B) Higher-power images of the CA1 (i) and DG (ii) hippocampal subfields from EGFP-P2rx7 epileptic mice. Scale bar = 50 μm.
5.3.2 Increased neuronal transcription of the P2X7R in the hippocampus in epilepsy

To establish which cell types display P2X7R transcriptional induction in the hippocampus of control and epileptic mice in this model, tissue from EGFP-P2rx7 control and epileptic mice was counter-stained for different cell type markers. Counter-staining sections with NeuN determined that the main GFP signal in hippocampal sections from control EGFP-2rx7 mice was from granule neurons of the DG whereas GFP expression was largely undetectable in the CA1 and CA3 hippocampal subfields (Figure 5.5).

Epileptic EGFP-P2rx7 mice showed a large increase in GFP in NeuN-positive cells in the granule cell layer of the DG. However, the largest increase of GFP was observed in the pyramidal neurons of the CA1 hippocampal subfield of EGFP-P2rx7 epileptic mice. Moreover, a minimal GFP signal in NeuN positive cells was detected in the CA3 hippocampal subfield (Figure 5.6).

To quantify the increase of P2X7R transcriptional induction in EGFP-P2rx7 epileptic mice, co-localization of NeuN and GFP positive cells was assessed. Two random fields of view from each hippocampal subfield were taken from the hippocampus of different EGFP-P2rx7 epileptic animals and EGFP-P2rx7 control mice. Results confirmed a highly significant increase in the number of NeuN and GFP double-positive cells in EGFP-P2rx7 epileptic mice when compared to control mice (Figure 5.7).
Figure 5.5 Neuronal expression of P2X7R in the hippocampus of control mice

Immunofluorescent staining showing GFP fluorescence (green) and neuronal marker, NeuN (red), in CA1, CA3 and DG hippocampal subfields from EGFP-P2rx7 control mice. White arrows show co-localization in the DG area of NeuN-positive and GFP-positive signal. Scale bar = 50 μm.
Immunofluorescence staining showing GFP fluorescence (green) and neuronal marker, NeuN (red), in CA1, CA3 and DG hippocampal subfields from EGFP-P2rx7 epileptic mice. White arrows show co-localization in the respective hippocampal subfields of NeuN-positive and GFP-positive signal. Scale bar = 50 μm.
**Figure 5.7 Increased neuronal induction of P2X7R in the hippocampus in epilepsy**

Graph showing the quantification of the number of positive GFP cells co-localized with NeuN positive cells in the whole hippocampus of control and epileptic EGFP-\textit{P2rx7} mice (n=5 EGFP-\textit{P2rx7} epileptic, n=3 EGFP-\textit{P2rx7} control). Analysis was performed by counting cells of two representative 20 µm$^2$ fields of view from each hippocampal subfield under epifluorescence microscopy. T-test. **p < 0.01.
5.3.3 Increased microglia transcription of the P2X7R in the hippocampus in epilepsy

Continuing with the analysis, the next cell type analysed for P2X7R transcriptional induction in the model was microglia. As for NeuN, immunofluorescence analysis was performed using the microglia marker Iba-1 and an anti-GFP antibody, focusing on each hippocampal subfield and comparing EGFP-P2rx7 control and EGFP-P2rx7 epileptic mice.

As expected, low GFP expression was observed in granule cells of the DG hippocampal subfield in EGFP-P2rx7 control animals. No co-localization of GFP and Iba-1 signal was detected in the hippocampal subfields from control non-seizure EGFP-P2rx7 mice (Figure 5.8). In sections from EGFP-P2rx7 epileptic mice, some microglia signal was found co-localizing with GFP in each hippocampal subfield. In particular, within the CA3 subfield co-localization of microglia staining with the GFP signal was observed around the lesion site (Figure 5.9).

5.3.4 Increased activated microglia in the hippocampus in epilepsy

Microglia cells can be found in at least two different states; active or resting. In the resting state, microglia display a characteristic morphology with a wide nucleus and small cell body with thick long branches extending away from the soma (Nimmerjahn et al., 2005). In this state, microglia are thought to be quiescent but sensing their environment. Microglia become activated after injury in the CNS and quickly display changes in cell morphology, with a more concentrated and clear nucleus with thin and fewer ramified branches. It is in the activated state that the release of pro-inflammatory cytokines occurs (Monif et al., 2010). Next, the different microglia states expressing GFP (or not) were counted in the hippocampus from EGFP-P2rx7 epileptic mice. The goal was to determine which microglia state was dominant in cells expressing GFP in epilepsy (Figure 5.10 A).
For the analysis, all sections were stained with Iba-1 as the microglia marker and anti-GFP to visualize GFP expression. Firstly, each Iba-1 positive cell was classified into one of the two states. Secondly, the positive or negative co-localization with GFP was determined for each microglia cell, already classified as resting or activated. Results showed a significantly higher number of activated microglia cells expressing GFP in the hippocampus of EGFP-\textit{P2rx7} epileptic mice. Data was analysed by $X^2$ test with $^*p < 0.05$ (Figure 5.10 B).

5.3.5 Absence of \textit{P2rx7} transcription in astrocytes in the hippocampus in epilepsy

To determine if transcription of \textit{P2rx7} occurred in astrocytes in the hippocampus of epileptic mice, immunohistochemistry analysis was performed on EGFP-\textit{P2rx7} epileptic tissue using a GFAP antibody as an astrocyte marker and anti-GFP antibody. In control mice, no GFP signal was detected co-localizing with GFAP signal in any of the hippocampal subfields. In epileptic mice, the GFAP signal was strongly increased, particularly in areas of injury such as the CA3, suggesting an increase in activation and proliferation of astrocytes. However, no co-localization was found between GFP and the GFAP signal in any of the hippocampal subfields from epileptic mice (Figure 5.11).
Figure 5.8 Microglia expression of P2X7R in the hippocampus of control mice

Immunofluorescence staining showing GFP fluorescence (green) and microglia marker, Iba-1 (red), in the CA1, CA3 and DG hippocampal subfields of EGFP-P2rx7 control mice. Note absence of co-localization in each hippocampal subfields between Iba-1 positive and GFP positive signal. Scale bar = 100 μm.
Figure 5.9 Microglia expression of P2X7R in the hippocampus in epilepsy

Immunofluorescence staining showing GFP fluorescence (green) and microglia marker, Iba-1 (red), in the CA1, CA3 and DG hippocampal subfields of EGFP-P2rx7 epileptic mice. White arrows indicate co-localization in the respective hippocampal subfields between Iba-1 positive and GFP positive signal. Scale bar = 50 μm.
**Figure 5.10 Increased active microglia expressing GFP**

**A)** Immunofluorescent staining showing microglia-positive cells in resting state (top panel) and active state (bottom panels). Co-localization (or absence) of GFP fluorescence (green) and Iba-1 (red) microglia marker for both states (activated and resting) is shown in both panels. Scale bar = 20 μm. **B)** Graph showing the quantification of the percentage of Iba-1-positive microglia co-localizing and not co-localizing with GFP signal in the activated and resting state in the hippocampus of epileptic mice. (n=5 EGFP-P2rx7 epileptic). $X^2$ test with $p < 0.05$. 

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194
Figure 5.11 Absence of P2rx7 transcription in astrocytes in the hippocampus in epilepsy

Immunofluorescence staining showing GFP fluorescence (green) and astrocyte marker, GFAP (red), in the CA1, CA3 and DG hippocampal subfields from EGFP-P2rx7 epileptic mice. White arrows show lack of co-localization in any hippocampal subfield between GFAP positive and GFP positive signal. Scale bar = 50 μm.
5.3.6 Enhanced functional responses of the P2X7 receptor in EGFP-positive hippocampal cells in EGFP-P2rx7 reporter mice

Next, we sought evidence of enhanced P2X7R function in epilepsy. Activation of the P2X7R triggers a depolarizing entry of sodium and calcium into cells (Buell et al., 1996). To test if P2X7R-induced currents were enhanced in epilepsy, patch-clamp recordings were performed on acute brain slices from EGFP-P2rx7 epileptic mice, comparing currents in GFP-positive cells to GFP-negative cells. The prediction would be that a GFP positive cell would display increased currents due to the ongoing induction of the P2X7R in that cell. Cells were identified under a 63 x water immersion objective and fluorescence illuminated. After successful patch configuration was obtained, a stable baseline was recorded for 2 min. Then, 10 minutes after the electrical access to the cytoplasm was established, the P2X7R agonist BzATP (100 μM for 10 s) was applied to the cell under investigation.

Selective activation of P2X7R by application of BzATP induced a non-desensitizing current that was consistently associated with an increase in membrane capacitance. These currents averaged ~ 10 pA in control (GFP-negative) cells. In contrast, BzATP-evoked inwardly directed non-desensitizing currents, averaged at –40 pA. These were higher in GFP-positive cells in both the DG and CA1 cells from the same hippocampus of EGFP-P2rx7 epileptic mice (Figure 5.12 A, B).

To confirm that BzATP-induced currents in GFP-positive cells were specific and can be attributed directly to the activation of P2X7R, A438079 (1 μM), a selective P2X7 receptor antagonist, was administered during 2 minutes simultaneously with agonist application (BzATP 100 μM for 10 sec). A438079 significantly reduced BzATP-induced currents in GFP-positive cells confirming currents were P2X7R-mediated (Figure 5.12 C).
5.3.7 Increased P2X7R localization in synaptosomes in the hippocampus in epilepsy

The P2X7R has been reported to be located on pre-synaptic terminals where it may modulate neurotransmitter release (Armstrong et al., 2002; Sperlagh et al., 2002; Alloisio et al., 2008). To test if functional P2X7R are present in synaptic sites in the epileptic hippocampus in the intra-amygdala KA model, a synaptosomes isolation protocol was performed (Chapter II, Material and Methods). Two ipsilateral hippocampi, from control and epileptic animals, were used per group for the synaptosomes extraction. Isolated synaptosomes from both groups were fixed and immunostained for P2X7R and synaptophysin, which was used to confirm the presence of synaptosomes. Co-localization between P2X7R immunoreactivity signal and synaptophysin signal was assessed in different synaptosome preparations from the hippocampus of control and epileptic mice using confocal microscopy. Results showed a higher percentage of P2X7R co-localization with the synaptosome marker in the hippocampus of epileptic mice compared to the synaptosomes prepared from control mice (Figure 5.13 A, B). To support these results, Western blot analysis was performed to compare the P2X7R protein levels in the isolated synaptosomes from the hippocampus of epileptic and control animals. Results showed that P2X7R protein levels were significantly higher in synaptosomes from epileptic mice when compared to control mice (Figure 5.13 C, D).
Figure 5.12 Enhanced functional responses to agonist stimulation in GFP-positive cells

A) Whole-cell current responses evoked by 100 μM BzATP in GFP-positive (green lines) and non-GFP-positive (black lines) cells from DG and CA1 hippocampal subfields from acute hippocampal slices from EGFP-P2rx7 epileptic mice. B) Patch clamp recordings show increased response of GFP-positive cells vs non GFP-positive cells in DG and CA1 subfields from hippocampal slices in response to application of BzATP (100μM) (GFP-positive DG n = 10, GFP-positive CA1 n = 4; non-GFP-positive DG n = 4, non-GFP-positive CA1 n = 5). C) Effect of 1 μM A438079 on whole-cell current responses evoked by 100 μM BzATP in GFP-positive cells in the absence and presence of A438079 in DG and CA1 hippocampal subfields from epileptic mice (n=3 per group). T-test, *p < 0.05. **p < 0.01. *** p < 0.005.
Figure 5.13 Increased levels of P2X7R in synaptosomes in the hippocampus in epilepsy

A) Immunostaining showing co-localization between P2X7R (green dots) and synaptophysin (syn)(red dots) from synaptosome extracted from control and epileptic mice. White arrows mark the synaptosomes in the preparations (n= 3 synaptosomes preparation slides per group). Scale bar = 12 μm. B) Graph showing the percentage of synaptosomes that were P2X7R positive in the previous immunostaining preparations (n = 3 per group). C) Representative western blot showing P2X7R protein levels in isolated synaptosomes from epilepsy and control tissue (n= 2 per lane, 5 lanes per group). Synaptophysin was used as loading control. D) Densitometry of P2X7R protein levels in isolated synaptosomes from epileptic and control animals. T-test, *p < 0.05.
5.3.8 Increased functional responses of P2X7R in synaptosomes in epilepsy

To support the immunostaining data we employed a calcium microfluorimetric analysis of the synaptosome preparations from the hippocampus of epileptic and control mice. The presence of a functional P2X7R in the synaptosome preparations was demonstrated as a change in the ratio F340/F380 that increases when $[\text{Ca}^{2+}]_i$ increases. Synaptosome preparations were loaded with FURA-2 (5 μM), an aminopolycarboxylic acidic dye that binds to free intracellular calcium $[\text{Ca}^{2+}]_i$ (Gryniewicz et al., 1985). Samples were placed in a superfusion chamber on a microscope (Figure 5.14 A), and synaptosomes were stimulated for 30 sec with the P2X7R agonist BzATP (100 μM). To verify the functionality of individual synaptosome preparations, a test response to $\text{K}^+$ (100 mM) was also performed. Therefore, after administration of BzATP, preparations were washed and then perfused with $\text{K}^+$ afterwards. Only synaptosomes that showed a $[\text{Ca}^{2+}]_i$ increase during $\text{K}^+$ administration were considered for the study. Overall, cytosolic $\text{Ca}^{2+}$ increases in synaptosome preparations were higher from the hippocampi of epileptic mice when compared to the responses obtained from the synaptosome preparations from control mice (Figure 5.14 B, C).

During calcium recordings other differences between control and epileptic responses to BzATP were noted. To investigate this, synaptosome epileptic calcium responses elicited by BzATP were studied in detail. Two distinctive types of calcium response traces could be identified. The typical trace in control synaptosomes was a single increase of $[\text{Ca}^{2+}]_i$ after the administration of BzATP followed by a rapid decrease in $[\text{Ca}^{2+}]_i$ that finished after 30 seconds. In contrast, the traces recorded in the majority of the synaptosome from epileptic mice displayed several additional $[\text{Ca}^{2+}]_i$ spikes after BzATP, termed ‘multi-response’ (Figure 5.14 D, E).
Figure 5.14 P2X7R function in synaptosome preparations

A) Pictures of the superfusion chamber (Warner Instruments) and the microscope (Nikon) used in the study. B) Representative photomicrographs from isolated synaptosomes incubated with Fura-2 during recordings. C) Graph showing the percentage of isolated synaptosomes that displayed positive responses to BzATP (30 μM) administration in control and epileptic synaptosomes (synaptosome preparations: control n=7, epilepsy=8). D) Graph showing the percentage of the synaptosomes that showed a ‘multi-response’ after BzATP administration in control and epileptic synaptosomes preparations. E) Intracellular calcium increments elicited by 30 μM BzATP and 100 mM K⁺ in synaptosomes preparations. Diagram shows the representative traces found from control synaptosomes preparations and epileptic synaptosomes preparations (individual synaptosomes analysed (epi= 990, con=708). T-test *p < 0.05. ** p < 0.01.*** p < 0.005.
5.4 Discussion

The main findings in the present chapter were an increased induction and function of the P2X7R in the hippocampus in epilepsy. Neurons and microglia were the main cell types expressing P2X7R in the hippocampus in epilepsy. Microglia found expressing GFP were, overall, in an activated state in the hippocampus in epileptic animals. Synaptosome preparations from the hippocampus of control and epileptic animals also showed increased P2X7R expression in epilepsy. Functional studies here supported these results. Patch-clamp recordings detected larger inward currents in GFP-positive cells in the hippocampus. P2X7R activation responses were also recorded from synaptosome preparations during and after BzATP superfusion and analyzed by calcium microfluorimetry. Results showed an increased number of responses from the synaptosomes from epileptic mice compared to the control preparations. In summary, the results indicate a remarkable increase in P2X7R induction in the hippocampus in epileptic mice that is associated with increased functional responses.

5.4.1 Increased P2X7R expression in the hippocampus in epilepsy

In this study the expression of P2X7R in the hippocampus was investigated using a transgenic mouse, in which GFP expression is driven by the P2rx7 promoter. The first main finding was a different GFP expression between control and epileptic EGFP-P2rx7 mice in many parts of the brain. The area of the brain that showed the most dramatic increase in both groups was actually the cerebellum. This brain region has previously reported to express functional P2X7R in the cerebellar Bergmann cells (Habbas et al., 2011). Whole-cell patch-clamp recordings from the cerebellum detected functional P2X7R at both axodendritic and somatic sites of mouse cerebellar granule neurons (Sanchez-Nogueiro et al., 2014). Our findings are consistent with previous studies where P2X7R was observed to be expressed especially in the axodendritic molecular layer of the cerebellum and in granule neurons in both control and epileptic mice. However, this is the first time that an
increase of P2X7R expression has been shown in the cerebellum in epilepsy. As the cerebellum is the region of the brain that plays an important role in motor control, we can speculate that the high increase observed in epileptic brains could be an effect of seizures propagation through this brain region. However, no studies have yet explored whether the cerebellum is important for spontaneous seizures in this epilepsy model. P2X7R upregulation was also observed in other parts of the epileptic brain, including the thalamus and the striatum. P2X7R expression has been found to be altered in these regions in other neurological diseases, including Huntington's disease (Diaz-Hernandez et al., 2009). While the thalamus is important in epilepsy a role for the striatum is less certain. Hence, the increased P2X7R induction observed in these areas could be a sign of a general inflammation response within the brain in epilepsy unrelated to seizure genesis.

Another major finding in this study was the dramatic increase of GFP in the CA1 and DG hippocampal subfields from epileptic animals compared to control mice. Basal expression of P2X7R was observed only in the DG hippocampal subfield in control animals, which is consistent with previous work that supports a constitutive P2X7R expression in normal physiological conditions (Sperlagh et al., 2006; Engel et al., 2012b). This finding is important because it leads to the idea that P2X7R is expressed in neurons and supports previous in vivo studies demonstrating a distinguished P2X7R expression in the different hippocampal subfields from mice in epilepsy (Rappold et al., 2006). By using EGFP-P2rx7 reporter mice and different cells type markers, cells expressing P2X7R could be identified. Neurons were the main cell population expressing GFP in the hippocampus in epilepsy in our model, particularly in the DG and CA1 hippocampal subfields. This data fits with previous findings in the same model where neurons were the main cell type expressing GFP after SE, particularly in the DG (Engel et al., 2012b). Our findings are in agreement with other work demonstrating neuronal expression of P2X7R in the CNS (Deuchars et al., 2001; Sperlagh et al., 2006), including the hippocampus in other TLE models (Vianna et al., 2002; Dona et al., 2009). However, this study shows for the first time the increased P2X7R expression in neurons of the pyramidal layer of the CA1
hippocampal subfield and in the granular layer of the DG in experimental epilepsy. The dramatic increase in CA1 was unexpected. This is not a site of major pathology in the model but may be recruited during epileptic seizures. In fact, a dramatic increase of the synaptic excitatory responses of CA1 networks was found by Ang et al. (Ang et al., 2006). They proposed an alternative network to the classic trisynaptic pathway (entorhinal cortex → DG via the perforant path, DG → CA3 via mossy fibres, CA3 → CA1 via Schaffer collaterals), the temporoammonic pathway, where the inputs that arrive to the CA1 subfield come from the layer III of the entorhinal cortex, where they originate. This new pathway supports the idea that the dramatic increase in synaptic excitatory responses of CA1 is independent of CA3 (Avoli et al., 2002). Interestingly, this pathway originates in layer III of the entorhinal cortex, which is known to initiate limbic seizures both in epilepsy patients and in different animal models (Avoli et al., 2002; Bartolomei et al., 2005). Supporting this idea, results from previous chapters showed evidence of P2X7R induction in that particular cortical layer. Hence, temporoammonic inputs travel directly to the CA1 area, transforming the responses of CA1 pyramidal neurons from predominantly inhibitory to powerfully excitatory supplementing an efficacious reverberating loop that is well suited for sustaining seizure activity (Avoli, 2007). The functional consequences of such a high increase in P2X7R transcription in CA1 are uncertain, and it is unclear why CA1 showed such a large transformation in its responses. An alternative explanation for the large increase of P2X7R induction observed in CA1 may be related to alterations in the innervations from CA3, which are considered as the main input to CA1. The effect could be contributing to increased excitability in CA1. However, protein changes in CA1 for P2X7Rs are modest in the epileptic mice (see earlier Chapter IV), so it is possible that something is blocking the protein translation.

P2X7R upregulation was also found in microglial cells in each hippocampal subfield in epileptic mice. These data are the first evidence of co-localization of P2X7R in microglia in the hippocampus in the intra-amygdala KA model. This extends previous evidence that microglia over-express P2X7R in the hippocampus after SE
and in epilepsy (Rappold et al., 2006; Dona et al., 2009). Detailed analysis of GFP expression revealed EGFP-P2rx7 induction in resting and activated microglia in the hippocampus of epileptic mice. The majority of the microglia cells expressing GFP in the hippocampus of epileptic mice were classified as being in an activated state, based on morphology. This is consistent with work by Monif showing P2X7R expression in activated microglia (Monif et al., 2009). Studies have proposed that activation of microglia induces a rapid increase of spontaneous excitatory postsynaptic currents in the hippocampus by the alteration of neurotransmitter release (Pascual et al., 2012). In agreement with this idea, our data showed that the main state of hippocampal microglia was activated in epilepsy. Regardless, these data support previous studies that the P2X7R is expressed by resting and activated microglia and may contribute to the pathogenesis of epilepsy.

GFP was not observed in astrocytes in the hippocampus of EGFP-P2rx7 epileptic mice. This finding is consistent with the majority of work that shows astrocytes do not usually express this receptor (Facci et al., 2014). However, there have been some studies that reported P2X7R expression in astrocytes (Collo et al., 1997) and its involvement in the control of neurotransmitter release (Ballerini et al., 1996; Wang et al., 2002; Duan et al., 2003). It is possible, of course, that something is silencing GFP transcription in the EGFP-P2rx7 transgenic mouse model in astrocytes. Indeed, GFP expression in astrocytes has never been reported in this transgenic model (Engel et al., 2012b). It is however also possible that the intra-amygdala induction of SE does not induce P2X7R activation in astrocytes (Engel et al., 2012b). This could be further explored, for example, by co-staining sections with P2X7R antibodies and the astrocytes marker GFAP.

5.4.2 Enhanced functional responses of P2X7R in GFP-positive hippocampal cells in EGFP-P2rx7 reporter mice

There have been several reports on P2X7R expression in epilepsy, but there is a lack of studies to determine if the P2X7R is really functional. Results of patch clamp studies here showed that short pulses of BzATP in slices from epileptic mice trigger
inward currents confirming the P2X7R is functional on DG and CA1 cells. Additionally, these currents were found to be larger in GFP-positive cells when compared to GFP-negative cells from the same brain region. The most obvious interpretation of these data is that there was simply more P2X7R in the GFP-positive cells, thus currents were stronger. This is the first study showing enhanced functional P2X7R currents in the hippocampus of epileptic mice. The type of cell expressing GFP was not determined in these studies, but it is likely they are neurons according to their morphology. These results are in agreement with previous studies where P2X7R currents were fully inhibited by A438079 (Yan et al., 2011). In conclusion, these findings suggest that the P2X7R is functional in the epileptic brain.

5.4.3 Increased P2X7R levels in synaptosomes in epilepsy

Further proof of enhanced P2X7R function was obtained in synaptosome studies. The P2X7R has been reported in several studies to be located in both presynaptic and postsynaptic sites in neurons in the CNS (Buell et al., 1996; Atkinson et al., 2000; Armstrong et al., 2002). In epilepsy, it may be involved in the presynaptic release of neurotransmitters (Sperlagh et al., 2006) or post-synaptic responses (Nick and Ribera, 2000). However, not much is known about the role of P2X7R expression in synaptic functions under pathological conditions. In this study, immunostaining and western blot analysis showed increased P2X7R expression in synaptosomes in the hippocampus of epileptic mice. These findings agree with previous studies that indicate that the P2X7R is present in excitatory presynaptic terminals in forebrain regions such as the hippocampus (Deuchars et al., 2001; Armstrong et al., 2002).

Experiments here also analysed the functional response of P2X7R in the synaptosomes from epileptic mice. An important finding was that synaptosomes from epileptic mice displayed increased Ca\(^{2+}\) responses to BzATP, a P2X7R agonist. These results suggest potentially enhanced Ca\(^{2+}\) entry due to P2X7R activation in epilepsy. This increase of intracellular Ca\(^{2+}\) may enhance excitability. These results are in line with other studies where intracellular Ca\(^{2+}\) release and Ca\(^{2+}\) oscillations have been observed in NG2-like cells in epileptic tissue (Lee et al., 2007), along with
an evidence of up-regulation of the P2X7R in synaptosomes (Alloisio et al., 2008). These findings suggest the possibility that, in the epileptic hippocampus, there are cells that may be capable of \( \text{Ca}^{2+} \)-dependent exocytotic release of glutamate triggering the excitation of other neurons, and thus spreading seizure activity throughout the hippocampus (de Lanerolle et al., 2010). Also, these results are in agreement with previous studies that suggest a contribution of P2X7R in synaptic transmission, which is enhanced in the hippocampus in epilepsy (Sperlagh et al., 2002; Dona et al., 2009). This extends the evidence that the P2X7R is functionally activated and up-regulated in the synaptic region of the hippocampus in epilepsy. Synaptosome preparations from control and epileptic mice also showed an altered response, with multiple increases of internal \( \text{Ca}^{2+} \) concentration after BzATP administration. This difference in the P2X7R responses may be the result of an increase in channel number; however, our results suggest that altered channel kinetics may be another possibility.

In summary, the present chapter supports the up-regulation and expression of P2X7R in neurons and microglia in the hippocampus in epilepsy. It further demonstrates increased synaptic P2X7R levels and functionality of the receptor in epilepsy.
Chapter VI - Inhibition of P2X7R reduces spontaneous seizures and has pathology-modifying effects in epilepsy

6.1 Introduction

6.1.1 What are the functional implications of increased P2X7R in the hippocampus in epilepsy?

Results in the previous chapters revealed increased expression of the P2X7R in the hippocampus in the i.a. KA model of epilepsy. Expression of the receptor was increased in multiple hippocampal subfields at both mRNA and protein levels. Examination of synaptosomes revealed enhanced P2X7R functional responses, including driving increased Ca\(^{2+}\) currents. Immunostaining and work with the EGFP-reporter mouse also demonstrated induction of P2X7R mainly in neurons, although GFP was also present in microglia in the hippocampus in epilepsy. This latter finding is consistent with other reports showing increased P2X7R immunoreactivity in microglia and neurons in the epileptic hippocampus (Dona et al., 2009). However, whether up-regulation of the P2X7R expression has any influence on the occurrence of spontaneous seizures or epilepsy pathology is unknown.

There is an ongoing need for novel AEDs with new mechanisms of action and potential disease-modifying effects. Modulating P2X7R function might affect spontaneous seizures and influence the pathophysiology of epilepsy. In particular, as P2X7R drives gliosis, targeting it may suppress the proliferation. Moreover, microglia proliferation and activation have been reported to be driven by the P2X7R (Monif et al., 2010). Activated microglia release the inflammatory and pro-convulsive cytokine IL-1β, which has been shown to increase AMPA receptor activation (Ferrari et al., 2006; Vezzani et al., 2008). In epilepsy, astrocytes respond
to changes in the extracellular environment via modulation of neurotransmitter release and uptake (Aronica et al., 2012). Activated astrocytes also deplete the brain’s endogenous anticonvulsant, adenosine (de Lanerolle et al., 2010). Thus, P2X7R overexpression in epilepsy may modulate many important functions, including neurotransmitter release and glutamate reuptake from the synaptic junction, therefore increasing excitability in the tissues (Aronica et al., 2012). Hence, P2X7R inhibition might decrease hyperexcitability and reducing the occurrence of spontaneous seizures.

6.1.2 JNJ-47965567; a potent brain-available P2X7R inhibitor

A number of P2X7R ligands are available to test for effects on epilepsy. The choice requires careful considerations regarding potency, selectivity and ability to reach the brain after a systemic injection. A438079 has been the main antagonist used in our group for P2X7R inhibition experiments (Engel et al., 2012b; Jimenez-Pacheco et al., 2013). In mice, this drug had seizure-suppresion effects when given intracerebroventricularly before SE as shown in previous chapter III and in (Engel et al., 2012b). Tests in rats recently showed that although A438079 reaches the brain after systemic injection, with peak levels at 15 to 30 min, brain levels drop rapidly and return to baseline by 2 h (Mesuret et al., 2014). This profile makes it potentially unsuitable for tests in chronic epilepsy where sustained brain levels are needed. Therefore, alternative P2X7R antagonists must be considered for use in long-term epilepsy studies.

Recently, a new centrally permeable P2X7R antagonist, JNJ-47965567, has been described by Janssen R&D (Bhattacharya et al., 2013). The effectiveness of the compound has been tested by in vitro assays in recombinant and native systems expressing the P2X7R. Effects on P2X7R function were analysed by measurements of calcium influx, radioligand binding, electrophysiology and IL-1β release. Target engagement of the compound was proven by ex vivo receptor binding autoradiography and its effectiveness by in vivo microdialysis assays, with
measurements of IL-1β levels in the brain in freely moving rats administered with the P2X7R specific agonist BzATP and JNJ-47965567. Results showed JNJ-decreased IL-1β levels in the brain in models of depression, mania and neuropathic pain (Bhattacharya et al., 2013). The P2X7R inhibitor showed an excellent selective affinity for P2X7R, appearing higher than other compounds currently available. JNJ-47965567 exhibited target engagement in the rat brain, with a brain EC$_{50}$ of 78 ± 19 ng·mL$^{-1}$ (P2X7 receptor autoradiography). Critically, measurements of brain levels after administration suggested the drug remains in the brain longer (up to 3 h after systemic injection) than any other tested P2X7R ligand. These facts make JNJ-47965567 a more suitable candidate for long-term epilepsy studies. Therefore, JNJ-47965567 was tested for effects on spontaneous seizures in the intra-amygdala KA model.

### 6.1.3 Impact of P2X7R inhibition on the underlying pathology in epilepsy

Of the approximately 20 AEDs currently used in clinic, none have ever been demonstrated to modify the underlying patho-physiology of epilepsy (Vajda and Eadie, 2014). Recent interest in IL-1β/caspase 1 inhibitors has emerged. Clinical trials have shown potential therapeutic roles in autoimmune and inflammatory pathologies, and may also have therapeutic potential in epilepsies associated with pro-inflammatory processes in the brain (Vezzani et al., 2010). P2X7R activation lies upstream of IL-1β, thereby possibly affecting excitation and inflammation (Ferrari et al., 2006).

Microglia activation is a common early feature in affected brain structures in epilepsy and may be involved in inflammation and synaptic alterations (Pascual et al., 2012). Indeed, microglial cells are highly dynamic and express membrane receptors for neurotransmitters, including the P2X7R. When activated, microglia perform a number of roles, which may either promote tissue homeostasis or exacerbate inflammation (Lister et al., 2007). The P2X7R is constitutively expressed on microglia and its activation provokes microglia activation and the release of IL-1β.
(Ferrari et al., 2006). It is tempting to speculate that blocking the P2X7R would counteract microglia activation and in consequence, reduce IL-1β and TNF-α release, contributing to a lessening of the neuroinflammatory and hyperexcitable state in the epileptic brain.

The link between P2X7R and astrocytes is less direct than in the case of microglia, but is nevertheless an important molecular target for epilepsy. Astrocytes play an active role in synaptic transmission due to their expression of transporters and glutamate receptors (Perea and Araque, 2007). Astrocytes respond to glutamate release from neurons and trigger the release of gliotransmitters that can modulate neuronal activity, synaptic transmission and plasticity (Angulo et al., 2004). Abberant astrocyte function has long been implicated in epilepsy via a number of mechanisms. Astrocytes possess key enzymes that are not normally found in neurons, such as glutamine synthetase enzyme (GS). As glutamine synthetase catalyzes the conversion of glutamate to glutamine in astrocytes, its reduction provokes an increase in the extracellular glutamate. This, in consequence, potentially increases tissue hyperexcitability in epilepsy (de Lanerolle et al., 2010). Loss of glutamine synthetase has been reported in epileptic tissue, particularly in regions of major neuronal loss and increased glial density, such as the CA1 and CA3 hippocampal subfields (Eid et al., 2004; van der Hel et al., 2005). Other studies show that astrocyte proliferation leads to increased levels and activity of the enzyme ADK, which is responsible for the elimination of adenosine. Consequently, the overactivation of astrocytes during epilepsy contributes to the overexpression of ADK, decreasing the adenosine tone and promoting an immediate and continuous hyperexcitability state in the epileptic brain (Boison, 2009). P2X7R does not directly activate astrocytes but IL-1β is a potent trigger of astrogliosis (Rothwell and Luheshi, 2000). Thus, blocking P2X7R could potentially downregulate or block the activation of astrocytes.
6.2 Chapter objectives

**Hypothesis:** Inhibition of P2X7R in epileptic mice will reduce the occurrence of seizures and suppress gliosis.

**Aims:** First, to assess the effects of the P2X7R antagonist, JNJ-47965567, on recurrent spontaneous seizures in the intra-amygdala KA model. Second, to assess the possible behaviour changes due to P2X7R inhibition in epileptic mice. Third, to characterize any effects of treatment on the underlying pathophysiology of epilepsy, such as gliosis in epileptic mice.
6.3 Results

6.3.1 Suitability of the antagonist P2X7R A438079 for long-term epilepsy studies

Previous studies using the specific P2X7R inhibitor A438079 showed a reduction in seizure time during SE as well as neuroprotective effects in the intra-amygdala KA model (Engel et al., 2012b). However, A438079 was administered directly into the brain. In more recent work, A438079 was also found to suppress seizures in an intra-amygdala KA model in immature rats (Mesuret et al., 2014). However, this study noted a rapid elimination of the drug (Mesuret et al., 2014). Nevertheless, pharmacokinetics and pharmacodynamics may differ in mice. Therefore, A438079 was tested by systemic route in mice. Accordingly, initial tests were conducted using A438079 intraperitoneally 10 min before SE. All animals received A438079 intraperitoneally at 10 mg/kg, 30 mg/kg or 50 mg/kg. EEG was recorded during 40 min after KA and total power (µV²) was analysed for each animal. Results showed a significant decrease in total EEG power during seizures in the group of animals that received the highest dose (50 mg/kg) of A438079 systemically before SE (Figure 6.1 A). These data indicate potential suitability of A438079 for the use in chronic dosing studies in mice.

The next goal was to determine how long the compound persists within the brain after systemic injection. Naïve mice received A438079 intraperitoneally at either 30 mg/kg or 50 mg/kg, and animals were sacrificed 30 min and 4 h after A438079 administration. Liquid chromatography-mass spectrometry (LC-MS) assessment was used to measure the A438079 concentration within the brain at each time point. LC-MS measurements were done by Cyprotech Ltd. Results showed the highest concentration within the brain was reached 30 min after 50 mg/kg of A438079 injection (Figure 6.1 B).
However, at either dose, brain levels were almost undetectable by 4 h. This rapid elimination confirms A438079 unsuitable and impractical for the use in epilepsy studies where it is important to maintain high brain concentration levels. Thus, the longer-acting and more brain stable antagonist JNJ-47965567 was used for our long-term epilepsy studies.
Figure 6.1 Effects of A438079 on SE and brain levels of A438079 after systemic injection

A) Total power recorded by EEG during SE in the i.a. KA model and effect of i.p. administration of A438079 at different doses. B) Table displaying the A438079 concentration after LC-MS assessment within the brain at 30 min and 4 h time points in naïve animals after A438079 i.p. administration at different doses (30 and 50 mg/kg). Results showed the highest concentration within the brain was reached 30 min after 50 mg/kg of A438079 injection. T-test. *p< 0.05.
6.3.2 JNJ-47965567 reduces spontaneous recurrent seizures in mice

The experimental design for assessment of the effects of JNJ-47965567 on epilepsy is shown in (Figure 6.2 A). To explore potential antiepileptic effects of P2X7R inhibition, all mice were equipped with EEG telemetry units. Telemetry EEG was continuously recorded for 3 weeks after SE induction. All animals experienced the first spontaneous seizures on the third day, in agreement with the normal course of epilepsy in this model (Mouri et al., 2008a). The average epileptic seizure duration for all mice was $15 \pm 50$ s (Figure 6.2 B). All telemetry-recorded spontaneous seizures showed high amplitude / high frequency duration spikes (HAHFDs) during the 21 days of recording. Additionally, mice were also video monitored during the entire duration of the study.

Following the experimental plan, 12 animals (control n= 6 and JNJ-47965567 n= 6) were subjected to i.a. KA SE. One mouse assigned to JNJ-47965567 group did not display electrographic signs of SE after intra-amygdala KA injection. Also, no seizure behaviour classifiable by Racine score was observed during the 40 min of SE. Therefore, this animal was removed from the analysis. On reaching day 10, study animals received intraperitoneal (200 μl) injection of either vehicle or 30 mg/kg of JNJ-47965567, twice a day for 5 days. After 5 days of treatment, injections were stopped and mice were left for a further 5 days of continuous EEG recording before being sacrificed on day 21. The number of seizures per day was counted for each animal during the first period of days, from day 5 to day 10 and an average number of seizures from that period of days was obtained. Next, averaged number of seizures from treatment days were counted for each individual animal (vehicle or drug treated animals). Last, we also counted average seizure number for each mouse during the ‘wash-out’ period (Figure 6.3 A, B).

All animals displayed an average of 3 – 6 seizures per day for the first 10 days of analysis. After day 10, comparison of the averaged number of seizures between vehicle (Figure 6.4 A, B) and drug treated (Figure 6.5 A, B) groups during the 5 treatment days showed a reduction in drug treated group, without reaching
significance ($p = 0.1304$). During the ‘wash out’ period, where animals were off
drug, significantly fewer seizures were recorded in the JNJ-47965567 group
compared to vehicle animals. The analysis of groups separately showed a significant
reduction in average number of seizures in the JNJ-47965567 group during
treatment and after treatment days compared to the baseline period (Day 5 – 10).
No significant differences were observed in the vehicle group between periods of
days, ($p = 0.376$ during treatment days, $p = 0.377$ during ‘wash out’ days (Figure 6.6
A).

Significant differences in seizure duration were observed between groups only in
‘wash out’ period (Figure 6.6 B). The average of electrographic seizure duration was
$30 \pm 15$ s (range $15 – 90$ s during each period of time per animal). The vehicle group
showed a slight increase in seizure duration and during the 5 ‘wash out’ days in
compare with drug treated group.
Figure 6.2 Experimental paradigm for test of JNJ-47965567 on epileptic seizures

A) Diagram showing the experimental design. Mice were injected twice daily with JNJ-47965567 (30 mg/kg) during days 11 – 15 after SE. Mice were sacrificed 5 days after treatment administration ceased. B) Examples of telemetry-recorded spontaneous seizures in the study before treatment days. Note typical duration of seizures for 15 – 30 sec and classical features such as high amplitude / high frequency duration spikes (HAHFDs).
Figure 6.3 Reduction in spontaneous seizure number after JNJ-47965567 administration

A, B) Average of number of seizures displayed from vehicle and JNJ-47965567 treated animals in each period of time before, during and after treatment (n= 9 vehicle, n= 9 drug).
Figure 6.4 Spontaneous seizure profiles in vehicle-group mice during epilepsy recordings

**A)** Diagram showing the number of seizures per day during the baseline period (Day 5 – 10), treatment period (Day 11 - 15) and ‘wash out’ period (Day 16 – 21) in vehicle mice. **B)** Percentage of reduction or increase of seizures displayed from vehicle animals in each period of time during and after treatment compared to baseline (Day 5 - 10) (vehicle n=9).
Figure 6.5 Seizure profile in JNJ-47965567 treated mice during epilepsy recordings

A) Diagram showing the number of seizures per day during the baseline period (Day 5 – 10), treatment period (Day 11 - 15) and ‘wash out’ period (Day 16 – 21) in JNJ-47965567 mice. B) Percentage of reduction or increase of seizures displayed in JNJ-47965567 treated animals in each period of time during and after treatment compared to baseline (Day 5 – 10) (drug n= 9).
Figure 6.6 Reduction in number of seizures after JNJ-47965567 treatment

**A**) Number of seizures per day in vehicle and drug group in days 5 – 10 before treatment, 11 – 15 during JNJ-47965567 and vehicle treatment and days 16 – 21 during wash out (n= 6 vehicle, n= 5 drug). **B**) Graph represents cumulative average duration of epileptic seizures in each period of time per group. No differences were observed in average duration of epileptic seizures. *p < 0.05. ns, not significant. Data are mean ± SEM. (vehicle n= 9, JNJ n= 9).
6.3.3 JNJ-47965567 effects on animal behaviour in epilepsy

We next explored whether mice treated with JNJ-47965567 displayed any differences in behavioural test. The open field test offers an opportunity to systematically assess novel environment exploration, general locomotor activity, and provide an initial screen for anxiety-related behaviour in rodents (Prut and Belzung, 2003). Behavioural tests were performed on day 11, before the beginning of vehicle and JNJ-47965567 injections, and on day 16, when treatment administration was complete. Tracking of the animals during the open field test were analysed and different parameters were compared between groups (Figure 6.7 A). No significant differences were found between vehicle and JNJ-47965567 treated mice at either test point. Groups performed similarly in distance moved, duration side area and velocity either before or after treatment days (Figure 6.7 B, C, D). However, the variability between animals was high and it is worth mentioning that one animal from the drug treated group suffered a seizure during the open field evaluation, staying immobilized in the periphery area for 3 minutes, which could skew the results. The number of crossings through the centre of the arena reflects the exploration behaviour of an animal. Higher number of crossings may be interpreted as reduced anxiety levels in an animal. The number of crossing showed no differences between groups either before or after the 5 treatment days (Figure 6.7 E).
Figure 6.7 Behaviour analyses before and after JNJ treatment

A) Tracking plots from vehicle and JNJ-47965567 animals before and after treatment days (n= 4 per group). B, C, D) Representative graphs showing the different parameters analysed in the open field behaviour test. E) Graph showing the number of center crossings by vehicle and JNJ-47965567 animals before and after treatment (veh n= 6, JNJ n= 5 per group). ns, not significant.
6.3.4 P2X7R inhibition reduces microglia responses in the hippocampus in epilepsy

Possible effects of JNJ-47965567 treatment on microglia expression in epileptic mice were next investigated using immunohistochemical analysis of brains at the end of recordings.

Iba-1 was used as microglia marker for immunohistochemistry. Results showed a strong decrease in the number of microglia in the hippocampus of JNJ-47965567 treated mice compared to vehicle-epileptic mice (Figure 6.8). Examination of the hippocampal subfields revealed significant reductions in microglia numbers in the JNJ-47965567 treated animals in all hippocampal subfields (CA1, CA3 and DG) when compared to vehicle animals (Figure 6.9 and 6.10).
Figure 6.8 Reducton in microglial cells in JNJ-47965567 treated animals

Representative Iba-1 staining of whole hippocampus slices from 3 randomly chosen vehicle animals and 3 randomly chosen JNJ-47965567 epileptic mice. Note the clear decrease in the number of Iba-1 positive cells and immunoreactivity in mice treated with JNJ-47965567 drug. Scale bar = 200 μm.
Figure 6.9 Reduction in microglia numbers in JNJ-47965567 treated animals

A) Setting of borders for individual areas of the hippocampus for immunostaining analysis. B) Representative Iba-1 staining of each hippocampal subfield from vehicle and JNJ-47965567 epileptic mice. Lower numbers of Iba-1 positive cells and low immunoreactivity were detected in JNJ-47965567 epileptic mice slices when compared to vehicle injected epileptic mice. Scale bar = 100 μm.
Figure 6.10 Reduction in microglia counts in JNJ-47965567 treated animals

Quantification of the number of Iba-1 positive microglia found in each hippocampal subfield from vehicle and JNJ-47965567 epileptic brain slices assessed on completion of the experiment, after ‘wash out’ (n= 9 vehicle, n= 9 JNJ-47965567). *p < 0.05. ** p < 0.01.
6.3.5 Effect of JNJ-47965567 on astrogliosis in epilepsy

Next, tissue sections were stained for the astrocyte marker GFAP. Analysis of brain sections showed a strong decrease in the number of astrocytes in hippocampal sections from JNJ-47965567 treated animals (Figure 6.11). Analysis of each hippocampal subfield showed a significant decrease in astrocyte numbers in the CA1 and CA3 hippocampal subfields. The DG region did not show significant changes, although a trend to lower counts was observed in sections from animals treated with JNJ-47965567 compared to the vehicle group (Figure 6.12 and 6.13).
Figure 6.11 Reduction in astrocytes in JNJ-47965567 treated animals

Representative GFAP staining of whole hippocampus slices from 3 randomly chosen vehicle and 3 randomly chosen JNJ-47965567 epileptic mice. Note the clear decrease in GFAP positive cells and immunoreactivity in mice treated with JNJ-47965567 drug when compared to vehicle injected epileptic mice. Scale bar = 200 μm.
Figure 6.12 Reduction in astrocyte numbers in JNJ-47965567 treated animals

Representative magnification images (40X lens) of GFAP staining of each hippocampal subfield from vehicle and JNJ-47965567 epileptic mice. Notice the dramatic increase in the number of GFAP positive cells in the vehicle mice when compared to JNJ-47965567 treated mice. Clear star-shaped astrocytes can be observed in each hippocampal subfields of vehicle epileptic animals. Scale bar = 100 μm.
Figure 6.13 Reduction in astrocytes counts in JNJ-47965567 treated animals

Quantification of the number of GFAP positive cells found in each hippocampal subfield from vehicle and JNJ-47965567 epileptic brain slices assessed on completion of the experiment, after 21 days (n= 9 vehicle, n= 9 drug). *p < 0.05, ** p < 0.01; ns, not significant.
6.4 Discussion

This chapter provides supporting evidence for the P2X7R as a novel target for the prevention of seizures in epilepsy. The daily administration of JNJ-47965567 to epileptic mice produced a reduction in the number of seizures during the drug administration period. Remarkably, when the drug was discontinued, seizure numbers remained lower for the subsequent 5 days, implying a disease-modifying effect. Analysis of tissue showed a profound reduction in gliosis in the JNJ-47965567-treated mice. Together, these findings support P2X7R antagonists as a novel disease-modifying treatment for epilepsy.

To date, the management of seizures in patients with epilepsy relies heavily on antiepileptic drug (AED) therapy. Anticonvulsant activity can be obtained by modifying the bursting properties of neurons and by reducing synchronisation in neuronal networks. The sites of action of current AEDs exhibiting anticonvulsant activity comprise one or more target molecules in the brain, including ion channels, neurotransmitter receptors and neurotransmitter metabolising enzymes (Vajda and Eadie, 2014). However, although these drugs show excellent safety and tolerability improving the lives of many epilepsy patients, most still have adverse side effects and a limited effect on the underlying pathology of epilepsy (Loscher and Schmidt, 2002). There remains a need to develop new AEDs with other mechanisms of action and disease-modifying effects.

6.4.1 Limitations of A438079 for the treatment of epilepsy

Experiments in the present chapter began by assessing the potential of A438079 for use in tests on epileptic mice. A438079 has been the drug of choice in experiments testing effects of P2X7R antagonists on SE (Engel et al., 2012b; Jimenez-Pacheco et al., 2013; Mesuret et al., 2014). In much of this work, A438079 was given intracerebroventricularly to mice. While practical in preclinical work, a systemic route is preferable for long-term dosing and for clinical use. Experiments here show
systemic A438079 given shortly before intra-amygdala KA reduced seizures in the model. This complements and extends earlier findings in the same model (Engel et al., 2012b; Jimenez-Pacheco et al., 2013). However, tests of brain concentration levels of A438079 found only short-lived levels within the brain, with the drug being virtually undetectable by 4 hours. This makes it unsuitable for long-term epilepsy studies, where there would be a need to dose mice every 2 – 3 hours; this was considered to be complex and largely impractical. Thus, although A438079 has seizure suppressive effects when given systemically, its brain concentration levels drop very fast, therefore it was excluded for long-term testing against spontaneous seizures in the model.

Instead, we switched to testing JNJ-47965567, which was recently reported to display much more favourable pharmaco-kinetics and dynamics than A438079, including prolonged brain levels after intraperitoneal administration in rats (Bhattacharya et al., 2013). The drug also has a high potency and specificity against the P2X7R, with a brain EC_{50} of 78 ± 19 ng·mL (P2X7 receptor autoradiography). Together, these features led to its selection for long-term studies. Studies here are the first to test for anti-epileptic effects of a P2X7R antagonist in chronic epilepsy. The study was designed with a ‘drug - on’ and ‘washout’ period, following common guidelines (Loscher, 2011). This has the advantage that data were obtained on both acute anticonvulsant effects and, any prevailing disease-modifying effects. The treatment of mice with JNJ-47965567 (30 mg/kg/twice daily) for 5 days resulted in a ~ 50 % reduction in the number of seizures occurring in the mice compared to the pre-drug baseline recordings. However, vehicle control animals also showed a reduction in seizures during the same days, although this was less obvious. This could be due to the natural variability of seizure occurrence in the model. Indeed, typically the number of seizures tends to reduce slightly by day 10 to 13 (Mouri et al., 2008a). A second and perhaps more important factor was the need to lightly anaesthetise mice during dosing. Studies have shown that Isoflurane anaesthesia may limit the spread of epileptic activity via the enhancement of GABAergic inhibition in vivo (Detsch et al., 2002). This may have an anti-seizure effect.
Regardless, when treatment was finished, seizure rates in the vehicle group showed the expected increase and return to baseline rates in number of seizures per day. In contrast, seizure rates in JNJ-treated mice remained very low long after treatment had ceased. Together, results suggest JNJ-47965567 has both a modest anticonvulsive and a disease modifying effect.

The effect of JNJ-47965567 in reducing seizures seems to be stronger than what has been observed in studies testing other AEDs in epilepsy. A reduction in spontaneous recurrent seizures has been observed after the administration of levetiracetam, an AED, in rats using the pilocarpine model of epilepsy. However, marked differences in response to treatment were observed with some rats showing complete control of seizures and others not responding at all (Glien et al., 2002). Thus, although JNJ-47965567 trials in mice with spontaneous recurrent seizures are laborious and time-consuming, such trials should be added to the preclinical characterization of novel AEDs.

**6.4.2 Lack of effect of JNJ treatment on behaviour in epilepsy**

Anxiety is a common co-morbidity in patients with epilepsy and has been found in many models of epilepsy (Engel et al., 2013; Lenck-Santini, 2013). Open field tests are commonly used for the analysis of anxiety-related behavioural changes (Prut and Belzung, 2003). In this study, animals receiving the P2X7R inhibitor and vehicle were assessed using the open field test. Since epilepsy is associated with anxiety, animals experiencing more seizures are expected to display greater anxiety. An important question remains as to whether it is the underlying damage and pathology on the seizures causing the anxiety. Results showed no behavioural differences between groups either before treatment or after treatment. This is perhaps surprising, since it was reasonable to assume that a drug that decreases seizure would also decrease anxiety. This supports, in fact, an emerging view that it is the pathology that causes both epilepsy and anxiety (Brandt et al., 2010).
These data contrast somewhat with findings on a number of AEDs which have been reported to have anxiolytic effects. This includes diazepam (DZP), chlordiazepoxide (CDX) and pentabarbitol (PB) (Britton and Britton, 1981). Other AEDs impair behaviour, including Phenobarbital, which leads to impairments in midair righting reflex, basic associative learning, sensorimotor gating, and anxiety-like behaviour (Keith et al., 2003; Forcelli et al., 2012). More studies have reported that Phenytoin, Phenobarbital and Valproate treatment reduced locomotor hyperactivity in the open-field test in a repeated electroconvulsive seizure rat model (Hidaka et al., 2008). Thus, JNJ-47965567 appears not to impair performance in the open field test, but equally, lacks the anxiolytic benefits of certain other AEDs.

6.4.3 P2X7R inhibition leads to a diminution of microglia and astrocyte proliferation in the hippocampus in epilepsy

A second major finding in this chapter was that gliosis was strongly reduced in epileptic mice treated with JNJ-47965567. This is the first study to investigate the effects of P2X7R inhibition on gliosis in epilepsy and this finding offers an explanation for the disease-modifying effects. Gliosis has been implicated in epilepsy via a number of mechanisms including enhanced inflammation signalling and altered homeostatic functions. Importantly, previous data showed increased P2X7R expression in microglia in epilepsy (Dona et al., 2009). Data here show for the first time a significant decrease in microgliosis in all three hippocampal subfields of epileptic mice after treatment with JNJ-47965567. The simplest explanation for these findings is that blocking the P2X7R for a prolonged time returned microglia to a resting state and reduced its proliferation. Therefore, these findings support a main role of P2X7R activation in microglia proliferation during seizures in epilepsy.

It is not possible to know from the current study whether this is a permanent effect or whether microgliosis might eventually return. Indeed, the animals remained epileptic, albeit having only few seizures compared to vehicle control. Indeed, microglia responses are thought to be time-locked to the occurrence of seizures
and the extent of activation closely follows their incidence, while astrocytes appear to be involved in perpetuating inflammation even in the long-term after the initial injury (Ravizza et al., 2008). If correct, one prediction would be that microgliosis may, over extended time-periods, eventually return after JNJ-47965567 treatments stopped. However, in the clinical setting such a drug would not be stopped or interrupted. This would mean the gliosis-suppressing effects should continue.

Results here also showed a significant decrease in astrocytes in the CA1 and CA3 hippocampal subfields in the JNJ-47965567 treated animals when compared to vehicle controls. This downregulation of astrocytes in P2X7R inhibitor-treated animals is most likely an indirect effect of blocking P2X7R on other cells (e.g. microglia). Consistent with this, is the fact that reduction in microglia in JNJ-47965567 mice was slightly more obvious than astrocytes reduction in the hippocampus. In contrast, astrocytes reduction are probably secondary to reduced release of IL-1β from microglia (Rothwell and Luheshi, 2000). No difference in astrocyte numbers were observed in the DG hippocampal subfield between JNJ-47965567 treated mice and the vehicle group. This result could be due to the relatively high constitutive expression of the P2X7R in this brain region (Engel et al., 2012b). Thus, the amount of P2X7R inhibitor delivered may not be sufficient to block the native P2X7R expression in that area in epilepsy. However, as microglia number was reduced in the DG therefore, this may simply be due to the relatively small numbers of animals in the study and inter-animal variability.

Although a direct effect of P2X7R inhibitor is possibly responsible for reduced gliosis, the decrease in microglia and astrocytes could also simply be an effect of a reduction in the number of spontaneous seizures; if seizures drive gliosis then reducing seizures should decrease gliosis. Experiments here cannot exclude this as a possibility and it is not possible to know whether glial changes are direct or indirect consequences of JNJ-47965567 administration. That said, seizure rates were lower in the vehicle group during ‘drug-on’ but returned to baseline afterwards. This is supportive of the change in gliosis being a direct effect of the drug.
These are not the first data to show effects of a seizure-modulating drug on gliosis. The administration of diazepam, ketamine, carbamazepine, or phenytoin decreased GFAP immunoreactivity in slices from chronic epileptic rats in the pilocarpine model (Cunha et al., 2009). A decrease in gliosis was also observed in the pilocarpine model of epilepsy after treatment with Carbamazepine (CBZ) (Capella and Lemos, 2002). However, the scale of gliosis reduction here was far superior than in these studies. This indicates again that the P2X7R specifically and directly blocks gliosis. This property is novel and strongly supports the use of P2X7R antagonists as a new disease-modifying treatment in epilepsy.

Finally, the reductions in gliosis and seizures in JNJ-47965567 mice could also be due to blocking the P2X7R on neurons. The present thesis includes multiple lines of evidence that the P2X7R is over-expressed on neurons. Thus, anti-seizure effects might be also due to direct effects of altered neurotransmitter release. In summary, this chapter provides evidence of the involvement of the P2X7R in the development of spontaneous seizures and in the underlying pathology of epilepsy, particularly the proliferation and activation of glial cells in the hippocampus. The present study supports the use of P2X7R inhibitors as novel therapeutics with disease-modifying effects to treat and prevent epilepsy.
Chapter VII – General discussion

7.1 General discussion

The main goal of the present thesis was to explore the role of the P2X7R in the generation and maintenance of spontaneous seizures and in their pathological sequelae in epilepsy. There is a clinical need to find new therapeutic treatments that could reduce the occurrence of spontaneous seizures and modify the underlying pathology of epilepsy. Experiments here provide evidence of the expression, control and function of the P2X7R in the hippocampus in epilepsy and its contribution to the development of seizures in the disease. To that end, studies used a focal-onset SE mouse model that propagates seizures from the amygdala via the entorhinal cortex to the hippocampus (Ben-Ari et al., 1980). This model avoids direct neurotoxic effects on the hippocampus and the sometimes limited damage to that region when SE is evoked by systemic pilocarpine or KA (Sloviter et al., 2007). Previous groups have studies the P2X7R in epilepsy but a major advance in here was the use of an EGFP-P2rx7 reporter mouse as an alternative tool for the exploration of cell-specific induction of the P2X7R in different brain regions affected in epilepsy. This approach avoids the reliance on insufficient selective P2X7R antibodies (Anderson and Nedergaard, 2006). Furthermore, a new selective brain stable P2X7R inhibitor was tested to determine the effects of blocking P2X7R on spontaneous seizures and on its possible contributions to the underlying pathophysiology of epilepsy. An exciting finding was that the administration of a P2X7R inhibitor reduced spontaneous seizures and that the inhibition persisted after the drug ‘wash out’ period implying disease-modifying effects in epilepsy. In addition, gliosis was also reduced in animals treated with the P2X7R inhibitor. This property is novel and strongly supports the use of P2X7R antagonists as a new disease-modifying treatment in epilepsy.
7.1.1 Neocortical expression of P2X7R in epilepsy

Pathological studies have revealed that prolonged or repeated seizures are associated with extrahippocampal damage in humans (Sankar et al., 2008). However, extrahippocampal neuron loss within cortical regions and amygdala have been reported in some (Du et al., 1993; Hudson et al., 1993; Pitkanen et al., 1998), but not all (Bothwell et al., 2001; Dawodu and Thom, 2005) clinical studies. If protection is needed for structures such as the neocortex after SE, then an important step is to identify the underlying cell and molecular mechanisms that provoke cell damage or excitotoxicity. The P2X7R has gained recent attention in epilepsy due to its enhanced expression after an initial insult and its possible involvement in the excitatory process (Rappold et al., 2006; Choi et al., 2012), along with its role in cell death (Le Feuvre et al., 2003).

Experiments in Chapter III provide evidence of increased expression of the P2X7R in the neocortex after SE and in epilepsy in the intra-amygdala KA mouse model. Neurons were found to be the main cell population transcribing P2rx7 after SE, as revealed by studies in EGFP-reporter mice. In the epileptic mice, along with neurons, microglia also transcribed P2rx7, suggesting recruitment of further cell types with disease progression. This new approach was a major advance compared to previous studies. A constitutive expression of P2X7R was also observed in neurons from the cortical layers II – III in both control and KA animals. This was a novel finding suggesting constitutive functions of the receptor beyond the hippocampus. Interestingly, studies in Chapter V found constitutive expression in the cerebellum too. Studies have reported that neurons of the layer III receive a disproportional dense excitatory input from the limbic seizure circuit, and that excessive release of glutamate may preferentially be on the layer III neurons (Du et al., 1993). The presence of constitutive P2X7R expression in that cortical region may therefore regulate neuronal excitability in the normal brain. In addition, the massive induction of the P2X7R in the cortical layers V – VI in SE and epilepsy was an exciting finding, as cortical damage after SE was mainly observed in these layers.
The cells from cortical layers V – VI project principally into the thalamus, the claustrum and other regions of the cortex. Thus, since the cortex and the hippocampus have reciprocal connections, destruction of cortical neurons might affect neuronal communication between layers in the cortex and influence the loop activity in the hippocampal-entorhinal circuit affecting direct postsynaptic hippocampal targets (Ahissar and Kleinfeld, 2003; Thomson and Lamy, 2007). These alterations could contribute to an exacerbation of the hyperexcitability state and a functional organization of hippocampal epileptic discharges that may propagate the input to other regions of the brain contributing to the development of seizures (Pare et al., 1992; Bragin et al., 1997).

In addition, the effects of the inhibition of P2X7R in the cortex after SE were explored in this study. A major finding was the reduction in cortical damage after SE in the layers V – VI in mice treated with P2X7R antagonist. Moreover, mice treated with the P2X7R antagonist also presented a strong reduction in seizures during SE. It is therefore tempting to speculate that overexcitation of and/or damage to neocortex, particularly to the neurons in the layers V and VI, may play a significant role in the development of seizures and during epileptogenesis process. These findings show the neocortex as an important site of P2X7R expression, further clarifying the cells involved in this process and extend the evidence that the P2X7R is a potential new target to protect against seizures and seizure-induce cell death.

### 7.1.2 The role of P2X7R in epilepsy

In recent years, increasing studies were conducted on the P2X7R in the brain, particularly on its role during inflammation and excitotoxicity (Alves et al., 2013). P2X7R activity has been associated with the release of the pro-inflammatory cytokine IL-1β, which plays an essential role in both the development and maintenance of the inflammatory process (Lister et al., 2007). The release of increased amounts of intracellular ATP during inflammation increases paracrine
purinergic signalling, thereby activating P2X7R. Its activation promotes the formation of large conductance channels associated with subsequent apoptosis (Elliott et al., 2009).

Experiments in Chapter IV provided supporting evidence of the involvement of inflammation linked to P2X7R signalling in the intra-amygdala KA model of epilepsy. Up-regulated mRNA and protein expression of different pro-inflammatory cytokines were found in the hippocampus in epilepsy. Particularly, an enhanced microglia staining was detected in the CA3 hippocampal subfield which coincides with the area where injury occurs in the model (Mouri et al., 2008a). P2X7R has been proposed to regulate the release of IL-1β, mediating therefore inflammatory responses in the brain (Vezzani et al., 2011). Moreover, the release of IL-1β has been shown to have pro-convulsive effects promoting neuronal injury and gliosis (Allan et al., 2005). Therefore, this study shows evidence of the P2X7R activation in microglia and neurons in the hippocampus, which could contribute to the increase of a hyperexcitability state in the brain in epilepsy (Vezzani et al., 2011).

Experiments here also performed the first comprehensive analysis of P2X receptor expression, finding the altered expression of various P2XRs in mice that developed epilepsy. These findings reveal a selective subfield-specific expression and upregulation of the P2XRs in the hippocampus. Particularly, an increased expression of the mRNA and protein of the P2X7R in the hippocampus during epilepsy are in agreement with previous studies where the receptor was seen upregulated in epilepsy (Dona et al., 2009). Moreover, P2rx7 mRNA was found upregulated particularly in neurons and some microglia in the hippocampus, as revealed using a P2rx7 mRNA reporter mouse in which GFP is expressed under the control of the P2rx7 promoter region.

An unexpected finding was the dramatic increase of constitutive GFP fluorescence in the pyramidal neurons of the CA1 hippocampal subfield in epilepsy. This is the first study that shows such a large transformation in that area in epilepsy. The functional consequences of such an increase in P2X7R transcription in CA1 are
uncertain, and it is unclear where the input is coming from. Two different hypotheses were discussed in this study, the large transformation of the CA1 subfield could be due to the alterations during epileptogenesis of the innervations from CA3, which are considered as the main input to CA1 and as effect could be contributing to increase excitability in CA1. On the other hand, the second hypothesis suggests an alternative network to the classic trisynaptic pathway, the temporoammonic pathway, where the inputs that arrive to the CA1 subfield come from the layer III of the entorhinal cortex, where they are originated. Therefore, the increase of the excitatory responses of CA1 is independent of CA3 (Avoli et al., 2002). These two theories suggest that inputs to the CA1 area may transform the responses of CA1 pyramidal neurons from predominantly inhibitory to powerfully excitatory supplementing an efficacious reverberating loop that is well suited for sustaining seizure activity (Avoli, 2007). In addition, the GFP fluorescence in granule neurons of the DG was particularly intense in epilepsy, leading to believe that the granular staining seen in this study is likely coming from mossy fibers terminal where the receptor had been trafficked to the pre-synaptic membrane. Indeed, P2X7R has been reported to be expressed there, mediating glutamate release (Deuchars et al., 2001; Sperlagh et al., 2002).

To support previous findings and to extend evidence of the involvement of the P2X7R in the disease, functional studies in Chapter V showed an increased activation of the receptor in the hippocampus in epilepsy. Patch clamp recordings revealed an enhanced P2X7R expression in GFP positive cells from the CA1 and DG regions. Moreover, microfluorometric calcium measurement of several synaptosome preparations from epileptic mice showed evidence of increased functional responses of the P2X7R in epilepsy. These are the first data to support functional changes in epilepsy. As mentioned before, these findings are in agreement with previous studies were P2X7R has been reported to be found pre- and post-synaptically (Pankratov et al., 1998; Papp et al., 2004a; Cho et al., 2010).

Although P2X7R expression has been described in detail, the mechanisms controlling its transcription in epilepsy are unknown. The unique and selected
patterns of P2X7R in the brain suggest complex mechanisms regulating its expression. Studies in Chapter IV found a role for the Sp1 transcription factor in controlling P2X7R. Increased expression of Sp1 was observed in the hippocampus in SE and in epilepsy. To confirm these findings, Mithramycin A, a specific Sp1 inhibitor was used to explore P2X7R expression in the hippocampus in epilepsy. Treatment of mice with Mithramycin A decreased $P2rx7$ transcript levels; evidence of region-specific regulation of P2X7R by Sp1 transcription factor in epilepsy. In addition, the transcript levels of the early-gene $C-fos$ were also downregulated, which indicates a decrease in neuronal activity. However, it is not clear if the reduction of the neuronal activity occurred due to the decrease of the P2X7R expression or because Mithramycin A might also have an anticonvulsant effect.

Additionally, alternative mechanisms for the control and regulation of the P2X7R expression were considered in this study, including DNA methylation. However, results were not conclusive. Therefore, other mechanisms such as microRNAs or proteosome inhibition might be implicated in the regulation and control of the P2X7R expression in other regions of the hippocampus. Thus, new analysis of alternative control mechanisms should be considered for further studies of the receptor.

Although these results expand our understanding of the expression and control of the P2X7R in the hippocampus in epilepsy and its involvement in the ongoing inflammatory processes, further studies of alternative regulation mechanisms of the P2X7R are required that would help create a more complete understanding of the role of P2X7R in epilepsy.

### 7.1.3 Need for novel, more effective AED for epilepsy treatment

Antiepileptic drug (AED) therapy is the main treatment for the management of seizures in patients with epilepsy. Anticonvulsant activity can be obtained by modifying the bursting properties of neurons and by reducing synchronisation in
neuronal networks. The mechanisms of current anticonvulsant drug action include modulating voltage-dependent ion channels, enhancing synaptic inhibition by modulation of GABAergic receptors, and the inhibition of synaptic excitation blocking glutamate (Lingamaneni and Hemmings, 1999; Stefan and Feuerstein, 2007). However, although current AEDs have shown promising effects in many epilepsy patients, most still have adverse side effects and a limited effect on the underlying pathology of epilepsy (Loscher and Schmidt, 2002). There remains a need to develop new AED with other mechanisms of action and disease-modifying effects.

Experiments in Chapter VI provide evidence for the P2X7R as a novel target for the prevention of seizures in epilepsy. A new selective P2X7R inhibitor, JNJ-47965567, was tested in epileptic mice. A major advantage of this new compound was its high BBB permeability and its stability within the brain. This is the first time the anti-epileptic effects of a P2X7R antagonist have been tested in experimental epilepsy. A major finding was that the administration of the P2X7R inhibitor produced not only a reduction in the number of seizures during the drug administration period, but also once drug treatment was completed seizures did not return, implying disease-modifying effects. These results are encouraging and support the specific inhibition of P2X7R as an anti-epileptic and a disease modifying treatment in epilepsy. Thus, P2X7R might be a promising new therapeutic target.

Gliosis has been implicated in epilepsy and P2X7R expression has been shown to be increased in microglia in epilepsy (Dona et al., 2009). The release of IL-1β from activated microglia and astrocytes has been shown to have pro-convulsive effects by reducing seizure thresholds and inflammatory effects promoting neuronal injury as well as gliosis (Allan et al., 2005). Therefore, since IL-1β is implicated in ictogenesis and epileptogenesis, there is a strong rationale for targeting the P2X7R to reduce seizures and neuroinflammation (Vezzani et al., 2011). This is the first study showing evidence of a decrease in microgliosis in the hippocampus of epileptic mice after the treatment with the P2X7R inhibitor, JNJ-47965567. Microglia activation has been reported to modulate neuronal activity and its
activation may induces a rapid increase of spontaneous excitatory postsynaptic currents (Pascual et al., 2012). Therefore, the modulation of microglia by P2X7R inhibition might affect the release of pro-inflammatory cytokines, decreasing therefore, its proliferation and the hyperexcitability state (Vezzani et al., 2008). In addition, the inhibition of the P2X7R expression in activated microglia for several days may provoke the return of microglia to a resting state therefore reducing its proliferation. Hence, these findings support a main role of P2X7R activation in microglia proliferation during seizures in epilepsy.

Astrocytes proliferation was also found to be decreased in the hippocampus of the P2X7R inhibitor-treated epileptic animals. This could be due to an indirect effect of P2X7R inhibition on other cells such as microglia. For example, blocking microglia function reduces IL-1β release, which is a potent stimulant of astrogliosis. Indeed, the decrease in the number of astrocytes was less obvious than in microglia; in particular, no significant differences were observed in the DG hippocampal subfield. This result could be due to the relatively high constitutive expression of the P2X7R in this brain region (Engel et al., 2012b). These findings suggest that the constitutive P2X7R expression might need a longer administration or a higher amount of the P2X7R inhibitor to be blocked in the DG region in epilepsy.

Nevertheless, the reduction in the number of spontaneous seizures due to a direct effect of the P2X7R inhibition might be responsible for the decrease in microglia and astrocytes proliferation. If gliosis is caused by the recurrence of seizures, then reducing seizures should decrease gliosis. However, results here show evidence of the change in gliosis as a direct effect of the drug. These results are in agreement with previous studies showing an effect of a seizure-modulating drug in gliosis. Studies using a pilocarpine model of epilepsy have shown a reduction in gliosis after the administration of different AEDs, such as phenytoin (Cunha et al., 2009) and CBZ (Capella and Lemos, 2002). However, the scale of reduction in gliosis observed in the direct inhibition of P2X7R was far superior than in previous studies with other AEDs. Therefore, it is tempting to assume that the P2X7R specifically and directly blocks gliosis. This property is novel and strongly supports the use of P2X7R
antagonists as a new disease-modifying treatment in epilepsy. Moreover, evidence of the overexpression of the P2X7R in neurons in the hippocampus in epilepsy has been shown in this thesis. Hence, an alteration in the neurotransmitter release due to an inhibition of the neuronal P2X7R might provoke an anti-seizure effect.

In summary, this thesis provides evidence for the involvement of the P2X7R in the development of spontaneous seizures and in the underlying pathology of epilepsy, particularly the proliferation and activation of glial cells in the hippocampus. The present study supports the use of P2X7R inhibitors as novel therapeutics with disease-modifying effects to treat and prevent epilepsy.

7.2 Future work

To advance the current work and direct P2X7R inhibition towards clinical uses, the following future studies are proposed:

- **The validation of the present work in another model of epilepsy.** Considering the differences existing between animal models, translation of the present findings to another model would add confidence to this study. Our lab is currently testing the effects of a P2X7R inhibitor in other epilepsy models such as Pentylentetrazol kindling model, which provokes brief seizures, and recently it is developing a new traumatic brain injury epilepsy model. The pilocarpine model could be also used as a model of epilepsy to confirm these findings.

- **Further studies with EGFP-P2X7R protein mice.** The use of EGFP reporter animals is a sophisticated and useful tool to explore the localization and expression of the receptor. Our lab is currently working with a new transgenic mouse which expresses GFP attached to the P2X7R protein,
obtained from a collaboration with Annette Nicke, Munich, Germany. This could help with the controversy over P2X7R receptors in neurons and glia.

- **Correlation of P2X7R induction/expression with epilepsy development and seizure severity.** Two photon microscopy technique brings the opportunity to correlate the increase of the GFP expressing cells over time with the development of spontaneous seizures during the latent period of epilepsy in the same animal. This way, an increase in the number of GFP positive cells could be associated to the increased number of spontaneous seizures.

- **Further studies on long-term effects of the JNJ-47965567.** Does prolonged/sustained administration of JNJ-47965567 prevent spontaneous seizures? A longer period of dosing could be tested to investigate if long-term spontaneous seizures could be completely reduced and if neuronal damage could be abolished. EEG telemetry could be used, allowing the continuous study for several months of recurrent spontaneous seizures.

- **Epileptogenesis.** The administration of the JNJ-47965567 from the onset of SE and during the subsequent epileptogenic period during could be a possible target to prevent the development of spontaneous seizures and epilepsy. This could be used in clinical trials to prevent the development of epilepsy in patients after the first seizure onset.

- **The use of other antagonists.** The recent development of specific antagonists offers a wide range of compounds capable of blocking this receptor. For example, A8084598 has been described as a potent P2X7R inhibitor. Additionally, the role of other P2X receptors and its involvement in epilepsy could be investigated in experimental models.

- **Identifying the P2X7R effectors and downstream events.** We observed a direct involvement of P2X7R in the increase of IL-1β in the hippocampus in epilepsy. However, other mechanisms are likely involved. Fluorescence-activated cell sorting (FACS) would be a useful technique for this matter, which would work by separating the GFP positive cells from non-positive cells. PCR, proteomics and array analyses on GFP-positive cells which express P2X7R induction could then be used for more specific analyses.
• **Study the regulation of P2X7R.** Although evidence for the transcriptional control of SP1 on P2X7R expression has been shown in this study, the identification of other mechanisms that control P2X7R regulation is still needed. Our lab is already working on targeting microRNA-22, a direct regulator of P2X7R expression, with the aim of altering P2X7R expression to treat/prevent seizures. Proteosome inhibition could also be an alternative mechanism for future studies.

7.3 Conclusions

This body of work represents the first characterisation of the expression of P2X7R by using a transgenic mouse model to identify cell-specific P2X7R induction and regulation of the P2X7R in the hippocampus in epilepsy and its involvement in the development of spontaneous seizures. Moreover, in this study we showed evidence of P2X7R mediating microglia activation and the release of the proepileptic inflammatory cytokines such as IL-1β, thus showing the contribution of this receptor in the inflammatory process. In agreement with this, the treatment with the P2X7R antagonist showed a reduction in microglia activation and in the number of seizures in epilepsy, suggesting therefore a contribution of the receptor in both, seizures occurrence and in the inflammation process. Spontaneous seizures are a serious common pathologic condition. Current AEDs available are partially ineffective for many TLE patients. Therefore, P2X7R antagonists provide seizure protection through ways that other AEDs do not. This work strengthens previous findings in TLE models about the contribution of the P2X7R in the generation and the maintenance of seizures. Therefore, the reduction of spontaneous seizures due to the inhibition of P2X7R strongly supports the use of P2X7R antagonists as a new target for developing novel therapeutical drugs for disease-modifying treatment in epilepsy.


and neuroprotection by targeting the purinergic P2X7 receptor during status epilepticus in mice. FASEB J 26:1616-1628.


Henshall DC, Meldrum BS (2012) Cell death and survival mechanisms after single and repeated brief seizures.


Rogawski MA, Donevan SD (1999) AMPA receptors in epilepsy and as targets for antiepileptic drugs. Adv Neurol 79:947-963.


Sloviter RS, Bumanglag AV (2013) Defining "epileptogenesis" and identifying "antiepileptogenic targets" in animal models of acquired temporal lobe epilepsy is not as simple as it might seem. Neuropharmacology 69:3-15.


