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# The Purinergic P2Y Receptor Family as Novel Drug Target in Epilepsy

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# **The Purinergic P2Y Receptor Family as Novel Drug Target in Epilepsy**

**A dissertation submitted by**

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**Department of Physiology and Medical Physics**

**Royal College of Surgeons in Ireland**

**A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and  
Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of**

**Doctor of Philosophy**

**July 2018**

Supervisor: Dr. Tobias Engel

Co-supervisor: Professor David Henshall

Funded by Science Foundation Ireland (Starting Investigator Awarded to Dr. Tobias Engel)

## **Candidate Thesis Declaration**

I declare that this thesis, which I submitted to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme, this is dually acknowledged in the text such that it is possible to ascertain how much of my work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took responsible care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except such work has been cited and acknowledged within the text.

Signed: Mariana Alves

Student Number: 14130131

Date: 10<sup>th</sup> July 2018

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## Abstract

Epilepsy is one of the most common chronic neurological diseases and is characterized by recurrent seizures. Despite the existence of numerous anti-epileptic drugs, 30-40% of patients remain drug-refractory. Consequently, we must identify novel target genes with new mechanisms of action. Accumulating evidence suggests neuroinflammation drives seizure pathology and the epileptic phenotype. ATP, usually present at low extracellular concentrations, is released under pathological conditions in the brain where it activates purinergic P2 receptors including the ionotropic P2X receptors and metabotropic P2Y receptors, contributing to changes in glial function and modulation of inflammatory pathways thereby increasing neuronal network excitability.

A role for P2Y receptors has been suggested for several brain diseases and altered expression and function of some subtypes has been reported in animal models of epilepsy, particularly for the P2Y<sub>1</sub> receptor subtype. However, to date, no systematic expressional and functional characterization of the P2Y receptor family during seizures has been undertaken.

The aim of the present PhD thesis was to investigate the expressional changes and functional role of P2Y receptors during and following seizures using resected brain tissue from patients suffering from temporal lobe epilepsy, two different mouse models of epilepsy (kainic acid and pilocarpine), P2Y knock-out mice and specific P2Y receptor-targeting agonists and antagonists.

The main findings on expression of the P2Y receptor family include agonist- and P2Y receptor down-stream target specific changes in the levels of the P2Y receptors during status epilepticus. P2Y receptor up-regulation was the main response during chronic epilepsy in experimental epilepsy and in epilepsy patient brain.

Functional studies revealed important effects of P2Y receptors on seizures and epilepsy. Treatment of mice with ADP, a broad-spectrum agonist of P2Y receptors exacerbated seizure severity in a kainic acid model whereas UTP decreased seizure severity during status epilepticus. Antagonism of the P2Y<sub>1</sub> receptor prior to kainic acid

injection increased seizure severity and seizure-induced brain pathology. In line with these results, pre-treatment with P2Y<sub>1</sub> agonists protected the brain from seizure pathology. Surprisingly, and in contrast to results obtained from the pre-treatment regime, P2Y<sub>1</sub> antagonist treatment shortly following the induction of status epilepticus strongly reduced seizure severity and neuronal death and P2Y<sub>1</sub> agonist aggravated seizure pathology. In support of the P2Y<sub>1</sub> driving inflammatory process during status epilepticus, microglial cells were the first to show an increase in P2Y<sub>1</sub> levels and anticonvulsive effects were lost in P2Y<sub>1</sub> antagonist-treated mice with suppressed brain inflammation.

Finally, P2Y<sub>1</sub> antagonist treatment following status epilepticus delayed the emergence of spontaneous seizures and, when given to epileptic mice, reduced the occurrence of spontaneous epileptic seizures by over 50% during treatment.

In conclusion, the targeting of P2Y receptors, particularly P2Y<sub>1</sub>, may represent a new promising therapeutic strategy to treat status epilepticus and possibly drug-refractory epilepsy.

## Publications and Manuscripts

### Published:

1. **Alves M**, Beamer E, Engel T. (2018). The Metabotropic Purinergic P2Y Receptor Family as Novel Drug Target in Epilepsy. *Front Pharmacol.* 9:193.
2. **Alves M**, Gomez-Villafuertes R, Delanty N, Farrell MA, O'Brien DF, Miras-Portugal MT, Hernandez MD, Henshall DC, Engel T. (2018). Expression and function of the metabotropic purinergic P2Y receptor family in experimental seizure models and patients with drug-refractory epilepsy. *Epilepsia.* 58(9):1603-1614.
3. Reynolds JP, Jimenez-Mateos EM, Cao L, Bian F, **Alves M**, Miller-Delaney SF, Zhou A, Henshall DC. (2017). Proteomic Analysis After Status Epilepticus Identifies UCHL1 as Protective Against Hippocampal Injury. *Neurochem Res.* doi: 10.1007/s11064-017-2260-6.
4. Engel T, Martinez-Villarreal J, Henke C, Jimenez-Mateos EM, Sanz-Rodriguez A, **Alves M**, Hernandez-Santana Y, Brennan GP, Kenny A, Campbell A, Lucas JJ, Henshall DC. (2017). Spatiotemporal progression of ubiquitin-proteasome system inhibition after status epilepticus suggests protective adaptation against hippocampal injury. *Mol Neurodegener.* 24;12(1):21.
5. Ichikawa N, **Alves M**, Pfeiffer S, Langa E, Hernández-Santana YE, Suzuki H, Prehn JH, Engel T, Henshall DC. (2017). Deletion of the BH3-only protein Noxa alters electrographic seizures but does not protect against hippocampal damage after status epilepticus in mice. *Cell Death Dis.* 12;8(1):e2556.
6. Engel T, Brennan GP, Sanz-Rodriguez A, **Alves M**, Beamer E, Watters O, Henshall DC, Jimenez-Mateos EM. (2017). A calcium-sensitive feed-forward loop regulating the expression of the ATP-gated purinergic P2X7 receptor via specificity protein 1 and microRNA-22. *Biochim Biophys Acta.* 1864(2):255-266.
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Spontaneous Seizures and Gliosis in Experimental Temporal Lobe Epilepsy. J Neurosci. 36(22):5920-32.

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## Abbreviations

AC- Adenylyl cyclase

ADP – Adenosine diphosphate

AED – Anti-epileptic drug

AMPA –  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic

Apaf-1 - Apoptotic protease activating factor-1

APS - Ammonium persulfate

ATP – Adenosine 5'-triphosphate

BBB – Blood-brain barrier

Bcl-2 - B-cell lymphoma protein

BH3 - Bcl-2 homology 3

CA1 – Cornu ammonis 1

CA3 - Cornu ammonis 3

CCI - Controlled cortical impact

cDNA – complementary DNA

cGMP- complementary GMP

CNS – Central nervous system

COX-2 - Cyclooxygenase-2

DAG - Diacylglycerol

DG – Dentate gyrus

EAAT1 / EAAT2 - Glutamate transporters

EEG - Electroencephalogram

ERK – Extracellular signal-regulated kinases

FjB – Fluoro-Jade B

GABAA – Gamma-amino butyric acid A

GAD - Glutamic acid decarboxylase

GFAP – Glial fibrillary acidic protein

GLT - Glutamate transporter

HASCVD – Hypertensive atherosclerotic cardiovascular disease  
HFHA – High frequency high amplitude  
HMGB - High-mobility group box-1  
HS - Hippocampal sclerosis  
IGEs - Idiopathic Generalized Epilepsies  
IL-1 $\beta$  – Interleukin-1 $\beta$   
IL-6 – Interleukin 6  
ILAE- International league against epilepsy  
IP3 – Inositol 3-phosphate  
JNKs – c-Jun N-terminal kinases  
KA – Kainic acid  
KO – Knockout  
LPF - Later fluid percussion  
MAPK – Mitogen-activated protein kinase  
MCI-1 – Induced myeloid leukaemia cell differentiation  
MFS – Mossy fiber sprouting  
MRI - Magnetic resonance imaging  
mTOR - The mammalian target of rapamycin  
NMDA - N-methyl-d-aspartate  
NF $\kappa$ B - Nuclear factor-kappa B  
NO – Nitric oxide  
NOS – Nitric oxide synthase  
PB – Phenobarbital  
PHT - Phenytoin  
PKC – Protein kinase C  
PLA2 – Phospholipase A2  
PLC – Phospholipase C  
PLD – Phospholipase D  
PI3K – Phosphoinositide 3-kinase

PMI – Post-mortem interval  
PTZ - Pentylentetrazol  
P2R – Purinergic receptor 2  
P2X(R) – Purinergic receptor X  
P2Y(R) – Purinergic receptor Y  
qPCR – quantitative polymerase chain reaction  
SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis  
SE – Status epilepticus  
SRSs - Spontaneous recurrent seizures  
SV2A – Seminal vesicle secretory protein 2A  
TBI - Traumatic Brain injury  
TBS-T – Tris-buffered saline tween  
TLE – Temporal lobe epilepsy  
TLR4 - Toll-like receptor 4  
TNF $\alpha$  - Tumour necrosis factor  
TRAIL - TNF receptor apoptosis-including ligand  
UDP – Uridine diphosphate  
UTP – 5'-Uridine triphosphate

## Chapter 1 – General Introduction

## 1. Overview

The primary treatment for epilepsy is the use of anti-epileptic drugs (AEDs). These drugs control seizures by shifting the balance of inhibitory and excitatory drive in the brain (Bialer et al., 2013). 30% of patients, however, are pharmacoresistant to all available AEDs and between 40 and 50% of patients on AEDs suffer adverse effects (Baker et al., 1997). Therefore, a current major focus of epilepsy research is to develop treatment strategies that impact upon disease emergence and progression, show efficacy within the currently pharmacoresistant cohort and to lower the burden of adverse effects. To this end, the role of neuroinflammation in icto- and epileptogenesis has received much attention (Terrone et al., 2017). Purinergic signalling provides a mechanism by which inflammatory responses can lead to hyperexcitation and whereby inflammation can lead to hyperexcited networks. As such, the targeting of purinergic signalling is a promising strategy for developing new treatment options (Engel et al., 2016). Purinergic signalling is mediated via two families of purinergic receptor: ionotropic P2X channels and metabotropic P2Y receptors (Burnstock, 2007). Both subtypes respond to extracellular adenine or uridine nucleotides. Much of the focus of purinergic signalling in epilepsy has been on the P2X receptor family while the role of P2Y receptors in epilepsy has, to date, received much less attention (Engel et al., 2016, Rassendren and Audinat, 2016, Beamer et al., 2017). This thesis includes a complete characterization of the induction and expressional changes of the P2Y receptor family in two different mouse models of epilepsy. A functional role of the P2Y<sub>1</sub> receptor during status epilepticus, the development of epilepsy and during chronic epilepsy has also been shown. Our data demonstrates that P2Y<sub>1</sub> antagonism provides seizure-suppressive effects and suggests P2Y<sub>1</sub> targeting as a new therapeutic strategy to treat status epilepticus and possibly epilepsy.

## 1.1 Epilepsy

Epilepsy is one of the most common chronic neurological diseases affecting people of all ages. About 1% of the general population suffers from epilepsy. Epilepsy is associated with high health and social costs (Moshe et al., 2015), with an estimated annual cost of over €20 billion in Europe according to the World Health Organization in 2010. Patients with epilepsy may suffer from social stigma, discrimination and a loss of autonomy for daily activities which causes increased stress in their lives (Moshe et al., 2015). Apart from the occurrence of uncontrolled seizures, epilepsy is often associated with life quality-reducing comorbidities, such as anxiety and depression, increasing the burden on patients and the healthcare system. These conditions are up to eight times more common in epilepsy patients than in the general population (Keezer and Sander, 2016). Psychiatric comorbidity can worsen treatment response to AEDs and increase the risk of death (Fisher et al., 2014). A patient with epilepsy's mortality rate is two to three times higher than in a healthy person in the general population, contributing to a reduction of life expectancy by 2-10 years (Tomson et al., 2005, Hitiris et al., 2007).

## 1.2 Definition of epileptic seizures and epilepsy

The International League Against Epilepsy (ILAE) created a classification system to define seizures and epilepsy in 1981. Since then, these concepts have been changed and adapted to a better understanding and to facilitate communication between clinicians and patients (Chang et al., 2017). In 2005, an epileptic seizure was defined as *“the clinical manifestation of transient, abnormal, excessive, hypersynchronous discharge of a group of neurons”* (Fisher et al., 2005). In general, seizures do not last more than 1 – 2 minutes and are self-limiting (Jenssen et al., 2006) and can be classified according to their aetiology, semiology and anatomical focus (Chang et al., 2017). Seizures can affect sensory, motor and autonomic functions and can range in severity from a brief sensory experience to a major convulsive status epilepticus

(Chang and Lowenstein, 2003, Baca et al., 2011). Status epilepticus is characterized by a prolonged seizure period. This concept will be explained in more detail under the section 1.6 Status epilepticus. When seizures are recurrent and spontaneous, epilepsy is diagnosed. In 2014, a new practical and clinical definition of epilepsy was proposed by the ILAE. Epilepsy is defined by any of the following conditions:

“(1) At least two unprovoked (or reflex) seizures occurring >24 h apart;

(2) One unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years;

(3) Diagnosis of an epilepsy syndrome” (Fisher et al., 2014).

In this new definition, epilepsy is accepted as a disease rather than a disorder, emphasizing the serious nature of epilepsy. The term disease conveys a more lasting derangement of normal function, while disorder implies a functional disturbance, not necessarily lasting (Fisher et al., 2014).

A cure of epilepsy implies that the risk to have new seizures is the same as those in an unaffected baseline population. However, after a history of epilepsy this low risk is never achieved. The term “resolved” has been adapted to describe the situation when a person no longer has epilepsy, but there is no guarantee that it will never return. According to the ILAE, “epilepsy is resolved for individuals who had an age-dependent epilepsy syndrome but are now past the disease-specific age or those who have remained seizure-free for the last 10 years, with no need for seizure suppressive medicines for the last 5 years” (Fisher et al., 2014).

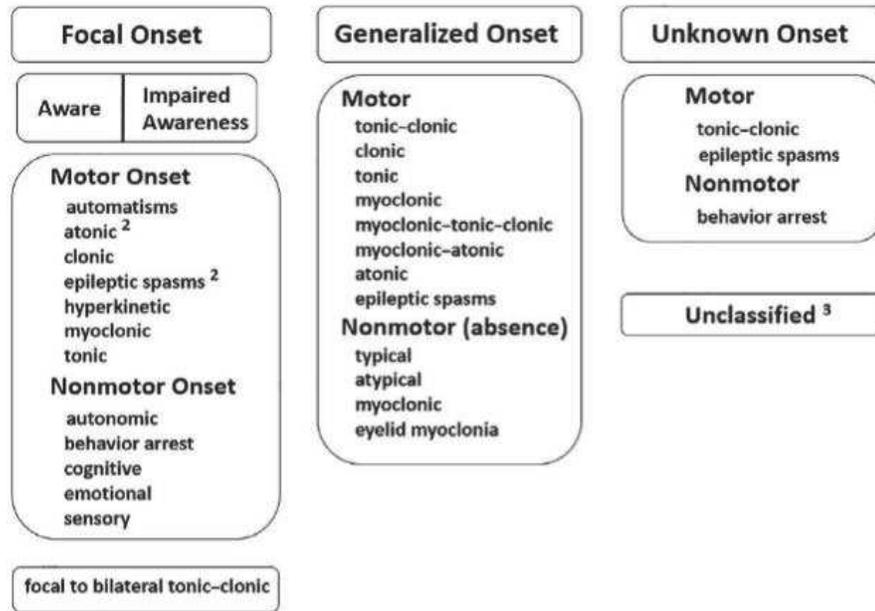
### **1.3 Classification of epileptic seizure and epilepsy**

The classification of epileptic seizures is important clinically to understand the type of seizure and personalize the treatment for each individual suffering from epilepsy.

Several attempts have been made to classify seizures and epilepsies. In 1981, ILAE proposed a classification of epileptic seizures based into two groups: partial and generalised seizures. In terms of aetiology, epilepsies were divided in two groups: idiopathic and symptomatic. However, in 1989, a third group was added to classify epilepsies: cryptogenic. Advances in the understanding of the pathogenesis of seizure and epilepsy contributed to an update in the classification system in 2010 by the ILAE. Accordingly, seizures were classified as generalised, focal or unknown. For epilepsy itself, the 2010 report proposed a new classification: genetic, structural, metabolic and unknown.

A new classification of seizure and epilepsy was announced in 2017. The ILAE proposed to classify epilepsies according to seizure type, epilepsy type and epileptic syndromes. In relation to seizure type, the new classification is based on clinical rather than pathogenic mechanisms. Here, seizures are classified in: focal, generalised or unknown onset (Figure 1.1). A generalised seizure is defined as a seizure which originates within the neuronal network. These seizures are bilaterally distributed and propagate rapidly throughout both hemispheres. These seizures can be divided into either motor or non-motor (absence) seizures. The latest classification distinguishes eight subtypes of generalized motor seizures and four subtypes of generalized non-motor (absence) seizures (Fisher et al., 2017a, Fisher et al., 2017b). On the other hand, a focal seizure originates in a specific area of the brain and is limited to a unilateral hemisphere only. A focal seizure can be classified as aware (simple partial seizures), impairment of awareness (complex partial seizure) or focal to bilateral tonic-clonic (secondary generalized seizure) (Chang et al., 2017). Both focal aware seizure and focal impaired awareness seizure types can be further categorized into motor and non-motor seizures (Chang et al., 2017).

In the case of a seizure of unknown onset, it is still unclear whether the onset is focal or generalized in an initial clinical assessment. This type of seizure can be relabelled as either a focal or generalized seizure if more clinical information is available. An example for these seizure types are epileptic spasms (Chang et al., 2017).



**Figure 1.1 – Classification of seizure types.**

The new classification of seizure types is divided into three subgroups: focal, generalised or unknown onset. In focal onset seizures, specification of the level of awareness is optional. Awareness means that the person is aware of self and environment during the seizure, even if not moving. A focal seizure can be classified in aware (simple partial seizures), impairment of awareness (complex partial seizure) or focal to bilateral tonic-clonic (secondary generalized seizure). A generalised seizure is a seizure that originates within the neuronal network which is bilaterally distributed and propagates rapidly in both hemispheres involving the subcortical or cortical structures. These seizures can be divided into either motor or non-motor (absence) seizures. For seizures of unknown onset, it is still unclear whether the onset is focal or generalized in an initial clinical assessment (Fisher et al., 2017b).

The classification of epilepsies is subdivided into four groups: focal, generalized, combined generalized and focal and unknown, and the categorization is based on the type of seizure, e.g. focal epilepsy is an epilepsy where the patient experiences focal seizures.

An epileptic syndrome is a combination of specific features, such as age of onset and seizures types. Idiopathic generalized epilepsies (IGEs) and self-limited focal epilepsies are the two major epilepsy syndromes. In IGEs, four well-established epilepsy syndromes have been identified: childhood absence epilepsy, juvenile

absence epilepsy, juvenile myoclonic epilepsy and generalized tonic–clonic seizures alone. Self-limited epilepsies have also been split into different groups: self-limited epilepsy with centrotemporal spikes, frontal, temporal and parietal lobe epilepsies (Chang et al., 2017, Scheffer et al., 2017).

### **1.3.1 Causes of epilepsy**

In recent years, our understanding of the causes of epilepsy has changed. In 1975, the causes of epilepsy were mostly unknown. Now, many cases thought to be idiopathic are believed to have genetic causes (Thomas and Berkovic, 2014). Epilepsies caused by a genetic aetiology can be related to an inherited mutation or due to a *de novo* mutation. An alteration in expression or function of genes crucial for brain development or function can lead to pathological mechanisms resulting in epilepsy. Studies performed in experimental models of epilepsy and in brain tissue obtained from patients with epilepsy suggests an important role of the mutation in genes implicated in epilepsy and epileptogenesis. Common mutations underlying epilepsy include those affecting the function of ion channels, such as the Na<sup>+</sup> channel or Sodium Voltage-Gated Channel Alpha Subunit (SCN1A) (Kasperaviciute et al., 2013). These mutations can contribute to the reduction of the action potential threshold in neurons. Syndrome of Benign Familial Neonatal Epilepsy is a genetic epilepsy resulting from a mutation of one of the potassium channel genes, KCNQ2 or KCNQ3. Molecular genetics has contributed to identifying the causative mutation in a large number of genes. However, it is important to note that genetic is not the same as inherited. For example, several *de novo* mutations have been identified in epilepsies. Patients with *de novo* mutations may now have a heritable form of epilepsy. In a case of *de novo* dominant mutation, the offspring has a risk of 50% of inheriting the mutation (Scheffer et al., 2017).

A structural abnormality of the brain is linked to an increased risk of developing epilepsy. Some examples of structural aetiologies are: stroke, brain trauma, infection.

In order to identify structural lesions, an appropriate magnetic resonance imaging (MRI) study would be required. Mesial temporal lobe epilepsy with hippocampal sclerosis is an example of epilepsies with a structural aetiology (Scheffer et al., 2017). Epilepsy can also result from a known infection in which seizures are the main symptom of the disorder. Some examples of causes which can lead to infectious epilepsies are neurocysticercosis, tuberculosis, HIV, cerebral malaria, subacute sclerosing panencephalitis, cerebral toxoplasmosis and congenital infectious, caused by the Zika virus (Scheffer et al., 2017). In a metabolic aetiology, epilepsy results from a known or presumed metabolic disorder, such as a metabolic defect with manifestations or biochemical alterations throughout the body (porphyria, uraemia or aminoacidopathies). Most metabolic epilepsies have a genetic basis; however, some may be acquired (cerebral folate deficiency) (Scheffer et al., 2017). Immune aetiology results from an immune disorder. Different neurological antibodies have been described in auto immune encephalopathy and / or epilepsy. Voltage-gated potassium channel antibody, anti-NMDA (N-methyl-d-aspartate) receptor encephalitis and anti-leucine-rich glioma-inactivated 1 (LGI1) encephalitis are the most common autoimmune conditions leading to epilepsy (Scheffer et al., 2017).

Though some epilepsies have well established aetiologies, the causes are still unclear in many other epilepsies (unknown). In this situation it is not possible to make a specific diagnosis. All forms of epilepsy with normal imaging and no metabolic, immune or genetic aetiologies are included in this category (Scheffer et al., 2017).

#### **1.4 Temporal Lobe Epilepsy**

Temporal lobe epilepsy (TLE) is the most prevalent form of acquired epilepsy in adults (accounting for around 40% of all epilepsies in adults) and also the most drug-refractory. Patients suffering from TLE are typically resistant to AEDs (Pitkanen and Sutula, 2002). TLE can be caused by different factors, such as head injury, stroke, brain infections, structural lesions in the brain, or brain tumors, however the exact causes

are not always identified. Hippocampal sclerosis (HS) is the most common pathological finding in TLE. The incidence of HS in TLE varies between 43% to 73% (Tai et al., 2018) and is characterized by neuronal loss in the hippocampus, amygdala and endothelial cortex, neurogenesis, gliosis, inflammation, reorganization of neuronal microarchitecture and reorganization of the extracellular matrix (Rao et al., 2006, Pitkanen and Sutula, 2002, Tai et al., 2018). These molecular and cellular changes may act simultaneously to produce seizures and contribute to the progression of the epileptogenic process (Pitkanen and Lukasiuk, 2011).

#### **1.4.1. Neuronal cell death**

According to the ILAE classification, HS can be classified into three subtypes based on different patterns of neuronal loss. HS type 1 is characterized by neuronal loss in Cornu Ammonis (CA)1, CA3 and CA4 region, while HS type 2 only presents neuronal loss in CA2 and HS type 3 presents loss in the CA4 region. Type 1 is the most severe form (Blumcke et al., 2013, Tai et al., 2018). In situations where hippocampal specimens showed little or no neuronal loss, these are classified in non-HS (Blumcke et al., 2013, Tai et al., 2018). Neuronal damage is mainly observed in glutamatergic neurons and gamma-aminobutyric acid (GABA) interneurons (Dingledine et al., 2014). This neuronal loss may lead to epilepsy via loss of inhibitory neurons or indirectly through the formation of maladaptive new circuits in response to the loss of synapses (Kobayashi and Buckmaster, 2003, Sloviter and Bumanglag, 2013), and reactive gliosis and inflammation (Vezzani et al., 2011). Cell death induced by seizures is mainly associated with two different processes: necrosis and apoptosis (Henshall and Meldrum, 2012). Necrosis is the predominant form of neuronal death after prolonged seizures (status epilepticus) (Niquet et al., 2007). Different studies performed in mouse models of status epilepticus showed the presence of necrotic neurons at 24 hours and 72 hours after induction of status epilepticus rather than apoptotic neurons (Fujikawa et al., 2000, Fujikawa et al., 1999). Also, in the developing brain of mice (post-natal day

14) it has been shown that at 24 hours after the induction of status epilepticus, the majority of CA1 neurons had a necrotic morphology and 72 hours later all of them were necrotic (Niquet et al., 2007). Experimental models of epilepsy have also reported alterations in the expression and activity of apoptosis-associated proteins, including B-cell lymphoma protein (Bcl-2) family proteins (Henshall and Meldrum, 2012). For example, mice lacking the pro-apoptotic Puma gene showed less damage in CA3 and fewer seizures after induction of status epilepticus (Murphy et al., 2010, Engel et al., 2010). On the other hand, loss of the anti-apoptotic Mcl-1 and Bcl-w were associated with enhanced hippocampal damage and earlier onset of status epilepticus (Murphy et al., 2010, Mori et al., 2004, Henshall and Engel, 2013).

In conclusion, both necrotic and apoptotic pathways are activated following status epilepticus. However, whether single, brief seizures lead to cell death remains a matter of debate (Henshall and Meldrum, 2012).

#### **1.4.2. Gliosis**

Glial cells, including microglia, astrocytes, oligodendrocytes, ependymal cells and radial glia, have an important role in the central nervous system (CNS) supporting neuronal cytoarchitecture and providing homeostatic regulation. They also play an important role in modulating synaptic function and plasticity (Burda and Sofroniew, 2014). These cells have a specific role and a specific response to damage caused by different diseases. For example, alterations in the function of glial cells after brain injury can cause seizures and initiate epileptogenesis (Wetherington et al., 2008). Astrocytes are the most predominant cell type in the brain and they are implicated in numerous physiological functions in the brain, including: maintenance of the water, ion, pH and transmitter homeostasis of the synaptic interstitial fluid; regulation of cerebral blood flow (vasomodulator); metabolic support, providing nutrients to neurons, promotion of the myelinating activity of oligodendrocytes, glial scars formation for nervous system repair, long-term potentiating roles (learning and

memory processes in the hippocampus) and support maintenance of the blood brain barrier (Ishibashi et al., 2006, Kimelberg and Nedergaard, 2010).

Astrocytes are also implicated in the generation of  $\text{Ca}^{2+}$  waves which are the result of a constant influx of  $\text{Ca}^{2+}$  into astrocytes (Jacob et al., 2014). This process leads to an increase of extracellular ATP and release of gliotransmitters, such as glutamate, D-serine, nitric oxide (NO) and acetylcholine (Ben Achour and Pascual, 2010). Additionally, astrocytes are also involved in synaptic scaling, which is a form of homeostatic plasticity responsible for the balance between excitatory and inhibitory inputs. However, astrocytes can react to different insults in the central nervous system, changing the normal cellular, molecular and functional features to pathological conditions. Reactive astrogliosis is characterized by hypertrophy of the cell, proliferation and scar formation (Sofroniew and Vinters, 2010). Astrogliosis by itself can promote neuronal hyperexcitability and cytotoxicity (Ortinski et al., 2010, Robel et al., 2015). For example, changes in glutamate transporters (EAAT1, EAAT2) contribute to an increase in glutamate levels in synapses (Sarac et al., 2009), leading to hyperexcitability. Furthermore, a study performed in mice lacking the glutamate transporter GLT-1 showed increase levels of synaptic glutamate and seizures after an induction of seizures by pentylenetetrazol (PTZ) (Tanaka et al., 1997).

Microglia are involved in the immune response, maintenance of homeostasis, phagocytosis of foreign material, release of cytotoxic substances, proteases, cytokines and neurotransmitters (e.g. glutamate) (Gehrmann et al., 1995, Hiragi et al., 2018). In physiological conditions, microglia is found in a “resting” or ramified state. Microglia activation occurs after an injury to the central nervous system. When active, microglia undergoes morphological changes, for example adapting a rounded amoeboid shape. Microglia activation can be distinguished by two different states: M1 and M2. M1 is the classical activation state, with pro-inflammatory responses and the release of cytokines including (cyclooxygenase-2 (Cox-2), interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ), TNF- $\alpha$ , prostaglandins among others (Boche et al., 2013). The release of these cytokines may contribute to blood-brain barrier (BBB) damage (Devinsky et al., 2013,

da Fonseca et al., 2014), neuronal excitability and neurodegeneration (Hiragi et al., 2018). The M2 activation state is associated with anti-inflammatory responses and can inhibit activated microglia which are in an M1 activation state (Boche et al., 2013). Activation of microglia has been described in neuronal injury and seizure induction (Galic et al., 2012, Hiragi et al., 2018). However, the mechanism by which microglia modulate the structure and the function of neural circuits under epileptic conditions is still unknown (Hiragi et al., 2018).

### **1.4.3. Inflammation**

Inflammation is characterized by the activation of innate and adaptive immunity, producing an array of inflammatory mediators, including cytokines (Nguyen et al., 2002). In the central nervous system, the main cytokines released are interleukins, TNFs and growth factors (Bartfai and Schultzberg, 1993).

Inflammation has been described to occur in various central nervous system diseases, including autoimmune, neurodegenerative and epileptic disorders. Clinical studies and experimental work in rodents support the role of inflammation in epilepsy (Vezzani et al., 2011). The enzyme responsible for IL-1 $\beta$  production is activated in resected brain tissue from TLE patients (Henshall et al. Neurology 2000) and later studies of brain specimens from drug-refractory epilepsy patients showed an increase of IL-1 $\beta$ , chromatin binding protein high-mobility group box-1 (HMGB1), IL-1R and toll-like receptor 4 (TLR4) levels, suggesting these pathways become activated in human epilepsy (Vezzani et al., 2011). Furthermore, a rapid and reversible induction of pro-inflammatory cytokines (IL-1 $\beta$ ) was reported in glial cells of the hippocampus after the induction of status epilepticus by kainic acid in rodents (Vezzani et al., 2002). Other studies showed that prolonged seizures lead to an increase of inflammatory mediators at the site of seizure onset and also in the regions of seizure propagation (Aronica and Gorter, 2007, Ravizza et al., 2008a, Fabene et al., 2010). Additionally, IL-1 $\beta$  is a potent stimulator of astrogliosis (Giulian et al., 1986, John et al., 2004). IL-1 $\beta$  decreases

astrocytic glutamate uptake through nitric oxide (NO) release, contributing to neuronal excitotoxicity (Ye and Sontheimer, 1996). IL-1 $\beta$  can also increase glutamate release from astrocytes through TNF- $\alpha$  induction (Bezzi et al., 2001). Further, overexpression of IL-1R in astrocytes decreases seizure susceptibility in mice (Vezzani et al., 2000, Vezzani et al., 2002). Additionally, high levels of chemokines have been reported in the hippocampus of mice after the induction of seizures by pilocarpine or kainic acid (Manley et al., 2007, Foresti et al., 2009, Xu et al., 2009), suggesting that chemokines are also implicated in inflammation and epilepsy. Another inflammatory modulator is the toll-like receptor (TLR), which is a family of innate immunity receptors implicated in pathogen recognition (Kawai and Akira, 2007). Activation of TLRs and IL-1R were implicated in the changes of neuronal excitability through the activation of the transcription factor nuclear factor-kappa B (NF $\kappa$ B), which contributes to transcription of genes encoding cytokines and chemokines (Vezzani and Granata, 2005). The transcriptional events induced by inflammation may contribute to a reduction in seizure threshold by inducing the expression of genes involved in neurogenesis, cell death and molecular and synaptic plasticity (Vezzani et al., 2011). Also, cyclooxygenase-2 (COX-2), an inflammatory gene, has been implicated in epilepsy (Vezzani and Granata, 2005). COX-2 is up-regulated after seizures in the hippocampus and treatments with COX-2 inhibitors protect from seizure-induced damage and NMDA-induced excitotoxicity (Rojas et al., 2014). Finally, inflammation can also cause the disruption of the BBB. The BBB is formed by brain endothelial cells and is responsible for the selective transport of molecules that are essential to neuronal function. However, under pathological condition, such as inflammation, leakage of the BBB may occur. This increased permeability of the BBB may lead to the accumulation of serum albumin and IgG in the brain (Webb and Muir, 2000, Vezzani et al., 2013), contributing in turn to increased hyperexcitability states.

#### **1.4.4. Neuronal plasticity and altered neuronal architecture**

Mossy fiber sprouting (MFS) is a common alteration in HS/TLE and is characterized by a synaptic reorganization within the hippocampus (Represa et al., 1993, Okazaki et al., 1995). MFS has been associated with the development of epilepsy and epileptogenic processes (Dudek and Shao, 2004, O'Dell et al., 2012, Pitkanen et al., 2007, Bae et al., 2010). This process seems to affect mainly the inhibitory GABAergic interneurons rather than excitatory neurons (Acscady et al., 1998). This fact suggests that MFS contributes to reform inhibitory circuits that are lost during the initial damage to neurons (Gorter et al., 2001). Also, the mammalian target of rapamycin (mTOR) signalling pathway has been implicated in MFS during epileptogenesis and status epilepticus. Treatment with rapamycin decreased MFS after status epilepticus (Zeng et al., 2009, Crino, 2011, Galanopoulou et al., 2012) and produces a strong seizure-suppressive effect (Drion et al., 2016). However, seizures return on wash-out of the drug and the role of MFS remains unclear and controversial. Heng et al. (2013) showed the inhibition of MFS by rapamycin is neither anti-seizure nor anti-epileptogenic (Heng et al., 2013). In addition, Pitkanen and collaborators showed no relationship between MFS density in the hippocampus and the frequency of epileptic seizures (Pitkanen, 2010).

#### **1.4.5. Neurotransmission in Epilepsy**

##### **1.4.5.1 Glutamate**

Glutamate is the most abundant excitatory neurotransmitter in the CNS and is responsible for neuronal communication. Glutamate function can be mediated by ionotropic or metabotropic receptors. The metabotropic receptors family are G protein-coupled receptors involved in the activation of intracellular pathways, while ionotropic receptors open ion channels, contributing to the flow of ions such  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$  and  $Cl^-$ .

$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), NMDA and kainate receptors (KA) are ionotropic receptors, with AMPA being the most abundant receptor in the CNS. AMPA receptors are involved in fast excitation, resulting from a fast entry of  $\text{Na}^+$  (Traynelis et al., 2010). NMDA receptors are activated when glutamate and glycine bind to the receptor. Once activated, these receptors are permeable to cations, including  $\text{Ca}^{2+}$ , contributing to the activation of various downstream pathways. NMDA receptors are critical for normal brain function, including neuronal development, synaptic plasticity, synaptic transmission and dendritic remodelling (Paoletti and Neyton, 2007). These receptors are mainly expressed postsynaptically, but can also be localized in pre-synaptically, modulating neurotransmitter release, and contributing to short-term plasticity in different regions of the brain (Lonchamp et al., 2012, Kunz et al., 2013, Park et al., 2014). KA receptors are implicated in the regulation of neurotransmitter release and synapse formation (Bortolotto et al., 1999). These receptors are abundantly expressed in the pyramidal neurons of the CA3 region (Werner et al., 1991). High affinity KA receptors facilitate neurotransmission by regulating glutamate release, whereas low affinity KA receptors mediate depression of synaptic activity in GABAergic terminals (Jane et al., 2009).

Glutamate is also involved in pathological conditions. Glutamate-mediated excitotoxicity is the main pathological process taking place in many types of acute and chronic CNS diseases (including epilepsy (Carter et al., 2011)). Different types of glutamate receptors have been suggested to play an important role in epilepsy. For example, the study of brain tissue from epileptic patients reported an increase of AMPA receptors (Hosford et al., 1991). Furthermore, in amygdala slices from patients with intractable epilepsy, blocking AMPA receptors resulted in inhibition of interictal-like electrical activity (Graebenitz et al., 2011). In line with this, the AMPA blocker, peramppanel, is used in the clinic to treat epilepsy (Steinhoff, 2015). Over-activation of NMDA receptors has been shown to contribute to an excessive influx of  $\text{Ca}^{2+}$ , leading to excitotoxicity in the brain, which may have a causal role in some diseases, including epilepsy. Indeed, inhibitors of NMDA receptors protect against neuronal death induced

by seizures (Meldrum, 1993). Additionally, GluR5 has been implicated in the excitation of interneurons the CNS (Traynelis et al., 2010) and are critical to seizure generation and spread (Rogawski and Donevan, 1999). Less is known about the role of KA receptors. Experimental evidence suggests that GluK2 is implicated in the induction of seizures and status epilepticus (Mulle et al., 1998). Also, selective activation of GluK1 KA receptors causes locomotor arrest, forelimb extension and induces myoclonic behavioural seizures and electrographic seizure discharges in the hippocampus (Cossart et al., 1998, Christensen et al., 2004, Aroniadou-Anderjaska et al., 2012). In conclusion, numerous studies have demonstrated that glutamate plays an important role in epilepsy, leading to the development of several AEDs. Considering that seizures are caused by an imbalance between excitatory and inhibitory systems, it is important to understand in which way GABA contributes to seizure generation and epilepsy as well.

#### **1.4.5.2 $\gamma$ -amino butyric acid (GABA)**

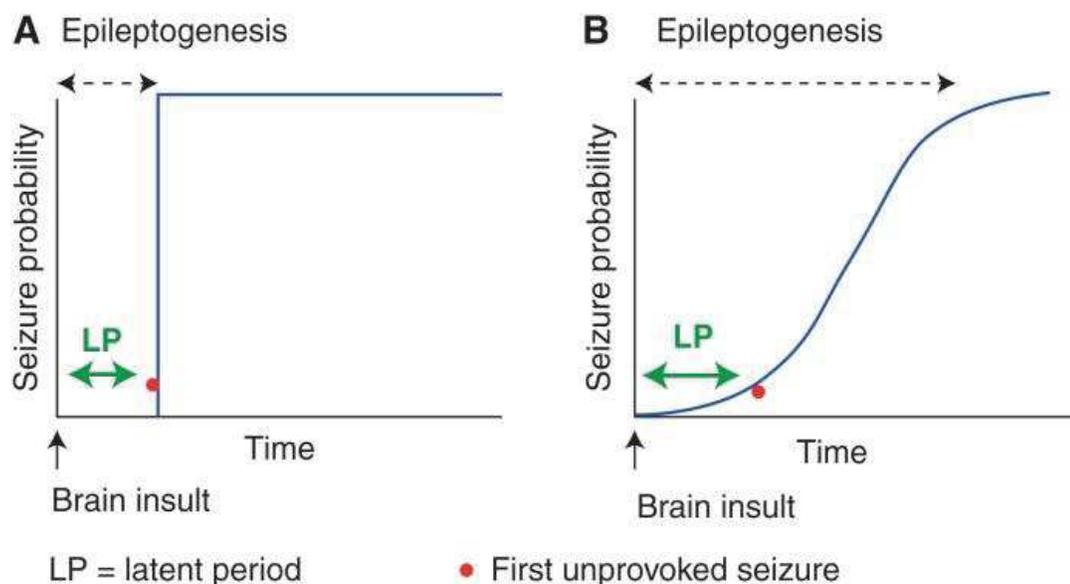
GABA, the main inhibitory neurotransmitter in the brain, regulates the balance between inhibitory and excitatory neuronal responses. GABA is produced in GABAergic axon terminals by the enzyme glutamic acid decarboxylase (GAD), and when released into the synaptic space, acts via two types of receptors: ionotropic GABA<sub>A</sub> receptors or metabotropic GABA<sub>B</sub> receptors. GABA<sub>A</sub> receptors mediate fast inhibitory neurotransmission in the brain by gating Cl<sup>-</sup> entry into the cell, while GABA<sub>B</sub> receptors mediate their effects via modulating K<sup>+</sup> conductance and Ca<sup>2+</sup> entry (also inhibiting neuronal excitation) (Wu and Sun, 2015). *In vivo* studies have shown that many drugs used to induce seizures (pentylenetetrazol, picrotoxin and bicuculline) are GABA receptor antagonists. On the other hand, GABA receptor mimetics, such as diazepam and phenobarbital, have an anticonvulsant action. These observations support the hypothesis that compromised GABAergic inhibition could lead to the development of epilepsy (Joshi et al., 2013). GABA allosteric modulators agonists, such as

benzodiazepines and barbiturates, also showed anticonvulsant effects and led to seizure suppression (Study and Barker, 1981, Macdonald and McLean, 1987). Several studies have demonstrated a critical role of GABA receptor mediated neurotransmission in the generation of seizures, and the development of epilepsy (Joshi et al., 2013). Therefore, it comes to no surprise that many of the currently anticonvulsive and anti-epileptic drugs (AEDs) used in clinical work through GABA. However, GABA<sub>A</sub> receptors are internalized by endocytosis during status epilepticus, thereby becoming inactive and reducing effects of drugs targeting GABA receptors. This process of GABA receptor internalization has been proposed as one of the main mechanisms leading to pharmacoresistance in patients with status epilepticus (Jacob et al., 2009).

#### **1.4.6. Epileptogenesis**

Epileptogenesis is the process that turns a normal brain into an epileptic brain after a transient insult to the brain (Pitkanen et al., 2015). The concept of epileptogenesis was revised recently by the ILAE. According to the new terminology, the process of epileptogenesis refers to “the development and extension of tissue capable of generating spontaneous recurrent seizures (SRSs), resulting in (1) the development of an epileptic condition, and/or (2) progression of epilepsy after it is established”. Now, epileptogenesis no longer refers only to the period between the initial insult in the brain and the diagnosis of epilepsy, but also includes the mechanisms of progression that still occur even after the diagnosis of epilepsy (Figure 1.2) (Pitkanen et al., 2015). This definition has been challenged, however. In a recent article, Sloviter argued that progression is better referred to as epileptometagenesis since the genesis is the birth and any progression occurs after (Sloviter, 2017). Epileptogenesis is characterized by the interplay of many factors including ongoing cell death, inflammation and synaptic and axonal plasticity changes (Pitkanen et al., 2015). This can be triggered by febrile seizures, status epilepticus, hypoxia-ischemic injury or

head injury (Hart et al., 1990, Annegers et al., 1998, Walker et al., 2002, Pitkanen et al., 2015). Nowadays, there are numerous experimental models to study the pathophysiology of the epileptogenic processes. Injection of chemoconvulsants, such as kainic acid or pilocarpine, or repetitive electrical stimulation of brain structures (hippocampus, amygdala) can lead to chronic epilepsy with spontaneous seizures (Pitkanen et al., 2015). All these models show seizure propagation and damage in the limbic structures, mainly in the hippocampus. As occurs in the human brain, these models also present a latent period after the initial injury (2-3 days) (White, 2002), cell death, gliosis, axonal sprouting, among others. Some limitations have been raised regarding the use of mouse models of epilepsy to study epileptogenesis. One of these limitations is related to the different time-frame between animals and humans until the occurrence of the first spontaneous seizure following a precipitating injury (Sloviter and Bumanglag, 2013). Different approaches have been proposed to delay or interrupt the process of epileptogenesis following status epilepticus (Sloviter and Bumanglag, 2013). These include the suppression of seizure spread, surgical excision of the seizure focus (Gotzsche et al., 2012), the regulation of ion channel function (Richichi et al., 2008), inflammation, among others.



### **Figure 1.2 – New concept of epileptogenesis.**

**(A)** The “previous” concept of epileptogenesis was defined by a latent period, which represents the time between the precipitating insult and the occurrence of the first unprovoked clinical seizure. **(B)** Recently, the concept of epileptogenesis changed and is now considered to be prolonged outside of the latent period, which is still defined as the time from the brain injury and the first clinical seizure. However, sub-convulsive seizures may occur before the first clinical seizure. Also, seizure frequency and severity progressively increase over time, suggesting that epileptogenesis can continue indefinitely (Pitkanen et al., 2015).

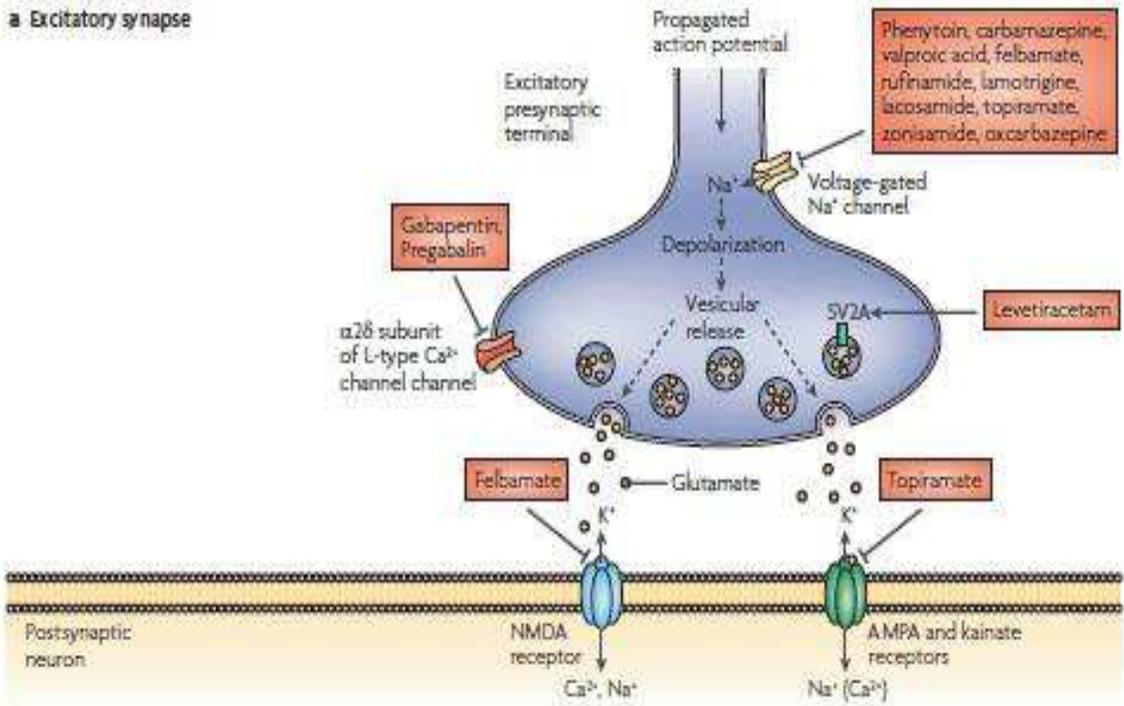
### **1.5 Current treatments for epilepsy**

The main objective of epilepsy treatment has been to control seizures with minimum side effects to the patients, resulting in a better quality of life (Duncan et al., 2006). Currently, there is no cure for epilepsy; however, in approximately 60% of patients suffering from epilepsy, seizures are controlled with pharmacological or non-pharmacological approaches. Around 30% of epileptic patients do not respond to any of the treatments available or, the side effects are unacceptable (Kwan and Brodie, 2000, Duncan et al., 2006).

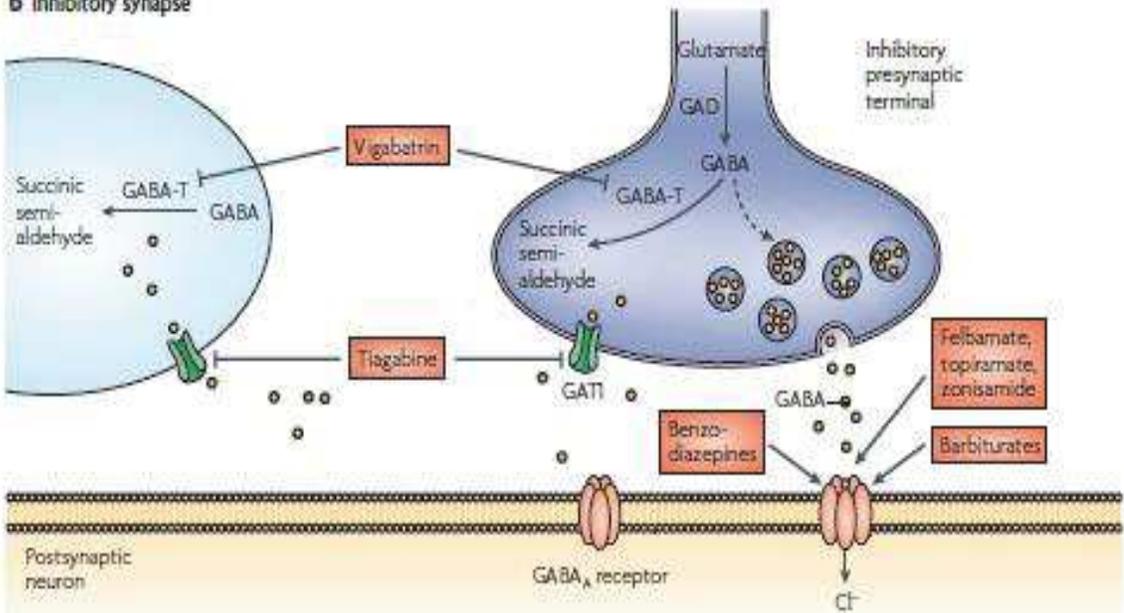
Potassium bromide was the first effective anti-seizure drug used in 1850. Since then, several AEDs have been discovered and used for the treatment of epilepsy. Phenobarbital (PB) was discovered in the early 20<sup>th</sup> century and it is widely used to this day, mainly to treat neonatal and childhood seizures (Yasiry and Shorvon, 2012). Phenytoin (PHT) was discovered in 1937 and presented a better tolerability and clinical efficacy compared to PB and since then it has become a major first line AED (Brodie, 2010, Yasiry and Shorvon, 2012). In the following years, more AEDs were introduced to the clinic: carbamazepine, benzodiazepines and valproic acid (Bialer and White, 2010). A second and third generation of AEDs have been licensed for clinical use. The second generation of AEDs include vigabatrin, lamotrigine, felbamate, gabapentin, levetiracetam, oxcarbazepines among many others, and the third generation is comprised of: eslicarbazepine acetate and lacosamide (Bialer and White, 2010).

Over 25 AEDs are currently used in the clinic (Bialer and White, 2010). Despite the relatively large range of options available, these AEDs fit into three broad categories. (i) increasing inhibitory transmission (e.g. the glutamate decarboxylase catalyst, Gabapentin), (ii) decreasing excitatory transmission (e.g. the non-competitive AMPA receptor antagonist, Perampanel) and (iii) blockade of voltage-gated ion channels (e.g. Na<sup>+</sup> channel blocker, Lamotrigine) (Bialer and White, 2010) (Figure 1.3). However, in most cases, AEDs have multiple actions and are incompletely understood. For example, Topiramate exerts an inhibitory effect on Na<sup>+</sup> conductance, enhances GABA neurotransmission via unknown mechanisms, and antagonises AMPA receptors (Shank et al., 2000). While there is a superficial diversity in mechanisms, all treatment options rely on the concept of redressing a balance between excitatory and inhibitory drive. This has proven largely successful in controlling seizures, but no treatments have been developed that act on the emergence or progression of the epileptic condition. Further, approximately 30% of patients remain pharmaco-resistant to all available AEDs; in most cases leaving surgery as their sole remaining option (Moshe et al., 2015). Choice of treatment strategy is based on seizure type, epilepsy syndrome, health problems, other medication used, lifestyle of the patients and other considerations such as pregnancy (Moshe et al., 2015, Kinney and Morrow, 2016). AEDs are the frontline treatment for epilepsy. Although strides have been made in terms of safety, tolerability and pharmacokinetics with the new generation of AEDs (such as felbamate, gabapentine, lamotrigine or oxcarbamazepine), the number of patients resistant to all treatments remains the same for the last 80 years (Bialer et al., 2013, Moshe et al., 2015). The search for mechanisms which could disrupt the emergence or progression of the disease remains elusive. Therefore, there is an urgent need to identify new drug targets which can show efficacy in patients who are currently refractory to available treatment and can demonstrate a disease-modifying effect.

**a Excitatory synapse**



**b Inhibitory synapse**



**Figure 1.3 – Mechanisms of action of current AEDs at excitatory and inhibitory synapses.**

**(A)** Targets of AEDs at excitatory synapses include: voltage-gated Na<sup>+</sup> channels, synaptic vesicle glycoprotein 2A (SV2A), the  $\alpha 2\delta$  subunit of the voltage-gated Ca<sup>2+</sup> channel, AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, and NMDA (*N*-methyl-d-aspartate) receptors. **(B)** AED targets at inhibitory synapses:  $\gamma$ -aminobutyric acid (GABA) transporter GAT1 (also known as SLC6A1), GABA transaminase (GABA-T) and GABA<sub>A</sub> receptor-mediated Cl<sup>-</sup> currents. Adapted from (Bialer and White, 2010).

**1.5.1 Alternative therapeutic approaches (non-pharmacological)**

Patients with epilepsy are considered drug resistant when they do not respond to two or more AEDs. In this case, alternative approaches are available, such as **surgical resection** of the epileptic focus, **vagal nerve stimulation (VNS)** and **ketogenic diet**.

A **surgical intervention** is an option when it is possible to identify the site of seizure focus through imaging and/or electrophysiological studies. This method is the main option to treat medically intractable epilepsy (refractory TLE) (Wiebe et al., 2001; Bell et al., 2009). **VNS therapy** is also used to treat medically intractable epilepsy. Intermittent electrical stimulation is applied to the cervical vagus nerve, causing desynchronization of the cerebral cortical activity, attenuating seizure activity (Ghanem and Early, 2006). Finally, the **ketogenic diet** is used mainly in children with refractory epilepsy. This diet focusses on an intake of high-fats, regular proteins and low carbohydrates. Fat is converted into fatty acids and ketone bodies, replacing the glucose for ketone bodies as an energy source. High levels of ketone bodies in the blood have been associated with a decrease of the frequency of epileptic seizures (Freeman et al., 2007). As a limitation, this therapy rarely stops all seizures and also requires close monitoring with a dietician and strong support from the family.

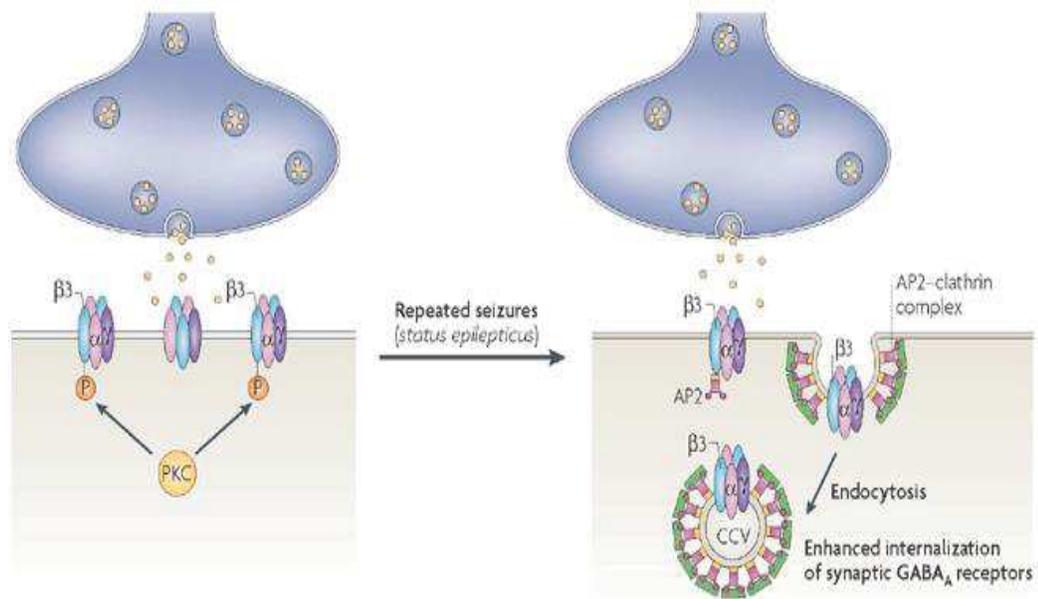
## 1.6 Status epilepticus: definition and aetiology

Status epilepticus is a prolonged seizure period (longer than 5 minutes) or more than one seizure without complete recovery of consciousness between them (Betjemann and Lowenstein, 2015). This is in contrast to most epileptic seizures which normally last no more than 1-2 minutes and are self-limiting. Seizures persisting for 30 minutes or more can cause irreversible brain injury and are a medical emergency with high mortality rates (Meldrum and Brierley, 1973, Betjemann and Lowenstein, 2015). The incidence of status epilepticus in Europe is 10-41 per 100 000 with a mortality rate of 10-20% in adults (Walker, 2018).

The aetiologies of status epilepticus in adults are frequently divided into acute (e.g.: stroke, hypoxia, systemic infection, trauma, CNS infections) and chronic (e.g.: low concentration of AEDs, alcohol misuse, tumour) (Betjemann and Lowenstein, 2015). In children, febrile status epilepticus, caused by high temperature, is the most common aetiology, representing one third of the cases (Singh et al., 2010).

Status epilepticus occurs when the mechanisms to terminate seizures fail. As described before, GABA<sub>A</sub> receptors internalized by endocytosis, become inactive, reducing the GABA inhibitory effects. Decreases in synaptic GABA<sub>A</sub>, resulting from enhanced endocytosis, have been reported in animal models of status epilepticus (Figure 1.4). Additionally, the loss of these synaptic receptor populations, which are normally benzodiazepine-sensitive, may explain the rapid development of pharmacoresistance in patients with status epilepticus and also may explain the failure to terminate seizures (Jacob et al., 2009). Repeated seizures and status epilepticus can cause profound damage to different brain structures (e.g. hippocampus), reactive gliosis and alteration of neuronal networks (Wasterlain et al., 2009, Fujikawa et al., 2000) leading to serious neurological complications. Activation of the immune system and inflammation may be involved in the pathogenesis of ongoing status epilepticus and subsequent epileptogenesis (Gorter et al., 2015). The release of anti-inflammatory mediators after status epilepticus has been suggested to prolong brain injury-induced

inflammation and its adverse effects on brain homeostasis (Devinsky et al., 2013). Status epilepticus can also induce disruption of the BBB and its consequent leaking, contributing to the development of epilepsy (Marchi et al., 2014, Vezzani et al., 2011). For example, BBB disruption may cause leakage of serum proteins that leads to glial activation and inflammation, reducing the threshold for seizures (Gorter et al., 2015).



**Figure 1.4 – Dysregulation of GABA<sub>A</sub> receptor trafficking after repeated seizures.**

Repetitive seizures lead to status epilepticus and contribute to a reduction in the phosphorylation of the GABA<sub>A</sub> receptor  $\beta$  subunits by PKC. Decreased numbers of synaptic GABA<sub>A</sub> receptors, resulting from endocytosis, lead to reduced synaptic inhibition as well as decreased benzodiazepine sensitivity. Adapted from (Jacob et al., 2009).

### 1.6.1 Current treatments in status epilepticus

When treating status epilepticus, time is a key factor and terminating the seizure is the number one priority for preventing lasting damage (Betjemann and Lowenstein, 2015). A protocol for treatment of status epilepticus has been developed whereby a first line treatment is administered within 5 – 10 minutes of seizure onset, a

second line treatment is administered within 20 – 40 minutes and a third line treatment around 60 minutes following seizure onset (Shorvon et al., 2008). The most effective first line treatment is achieved with benzodiazepines, such as lorazepam, diazepam or midazolam (Betjemann and Lowenstein, 2015). Evidence supporting the best treatment strategy for second and third line treatments is weaker, however, current practice involves the use of AEDs such as fosphenytoin, valproic acid or levetiracetam (Glauser et al., 2016) and anaesthetic drugs (Betjemann and Lowenstein, 2015). Approximately 30% of status epilepticus patients are, however, refractory to available drug treatment. Refractory status epilepticus is particularly associated with adverse clinical outcomes (Novy et al., 2010). As for epilepsy, because of the high percentage of drug resistance, status epilepticus requires new drugs with a novel mechanism of action.

### **1.7 Animal models of status epilepticus and epilepsy**

Animal models are essential to study the mechanisms underlying epileptogenesis and seizure generation. Some of these models replicate the natural history of symptomatic focal epilepsy with an initial epileptogenic insult, which is followed by an apparent latent period and by a subsequent period of chronic spontaneous seizures. There are over 100 experimental models available and these models can be classified as electrical stimulation-induced (e.g. kindling), chemiconvulsant-induced (e.g. pilocarpine, kainic acid and pentylentetrazol), brain pathology models (e.g. traumatic brain injury) and genetic models (e.g. absence seizures model) (Kandratavicius et al., 2014). Some of these models will be explained in detail below.

#### **1.7.1. Kindling model**

The kindling model of epileptogenesis consists of repetitive electrical stimulation of limbic brain structures (amygdala, hippocampus, and the olfactory

lobe), leading to a progressive and permanent state of hyperexcitability (Goddard et al., 1969, Becker, 2018). In the kindling model, the mechanism of action is mainly based on the an increase of excitatory transmission through NMDA receptors in the hippocampus, particularly in dentate granule cells (Kohr et al., 1993). This causes membrane depolarization and the activation of pathways dependent on  $Ca^{2+}$  as a second messenger (Mody and Heinemann, 1987). In fact, following the kindling process, NMDA receptors open easily and more often. However, months after kindling stimulation, the NMDA receptor conductance decreases, but seizure susceptibility is permanent, suggesting that NMDA receptors may be important for the induction of kindling, but not for the maintenance of the seizure-sensitive state (Buckmaster, 2004). Furthermore, the classic kindling model does not always lead to the development of spontaneous seizures (seizures are evoked). A prolonged kindling protocol may cause “overkindling”, which can lead to the generation of spontaneous seizures. Also, neuropathological alterations such as mossy fibre sprouting, extensive cell loss, reactive astrogliosis and BBB dysfunction are almost absent in the kindling model (Becker, 2018).

### **1.7.2. Traumatic Brain injury**

Brain trauma is one of the most common risk factors in developing seizures and epilepsy (Pitkanen and Immonen, 2014, Becker, 2018). Around 10 – 20 % of the symptomatic epilepsies are caused by traumatic brain injury (TBI) (Pitkanen and Immonen, 2014). In this model, the damage pattern is regionally more extended, and it is marked in the ipsilateral hemisphere, including cortical, limbic and thalamic structures. Several alterations at metabolic, cellular and molecular levels occur from minutes to months after injury (neurogenesis, axonal distress and impaired dendritic arborisation), leading to epilepsy (Becker, 2018).

Several animal models have been used to study the mechanism behind TBI and results showed that a significant number of animals developed spontaneous seizures

following TBI. However, the development of epilepsy is different, depending on the type of TBI model used. Later fluid percussion (LFP) consists in both focal or diffuse brain injury (Pitkanen and Immonen, 2014). Here, several features of human TBI are present, including focal contusion, petechial intraparenchymal, subarachnoid hemorrhages and traumatic axonal injury. In addition, BBB disruption, white matter damage, neuronal loss and gliosis are observed in this model (Pitkanen and Immonen, 2014). LFP induces severe damage in the ipsilateral cortex, hippocampus and thalamus, however in the contralateral side mild lesions can also be observed. A study performed by Kharatishvili et al. (2006) revealed that rats with LFP injury developed post-traumatic epilepsy in 50 % of cases (Kharatishvili et al., 2006). As reviewed by Pitkanen et al. (2014), different studies have shown that LFP injury increase seizure susceptibility, which can be detected after pentylenetetrazol (PTZ) or kainic acid administration (Pitkanen and Immonen, 2014).

Another common TBI model to study epilepsy is the controlled cortical impact (CCI) model, which is characterized by a focal brain injury, associated with a spectrum of contusion injuries (intraparenchymal petechial haemorrhages) (Pitkanen and Immonen, 2014). CCI can induce widespread cortical grey matter damage, axonal injury in the adjacent white matter, corpus callosum and capsula interna, and can also induce damage in the hippocampus and thalamus (Pitkanen and Immonen, 2014). In the CCI model, some studies showed that mice with mild and severe injury developed epilepsy after several weeks (Hunt et al., 2009, Hunt et al., 2010, Pitkanen and Immonen, 2014) and a few months later developed epilepsy (Guo et al., 2013, Pitkanen and Immonen, 2014).

TBI models are a useful and aetiologically relevant tool to study temporal lobe epilepsy (mainly because of the relevance as a risk factor). However, a key limitation is that only a low percentage of the animals develop epilepsy (Buckmaster, 2004).

### **1.7.3. Models of status epilepticus**

#### **1.7.3.1 Electrical stimulation**

Electrical stimulation model is characterized by the application of a pulse of currents at different frequencies until the animals develop status epilepticus. These currents can be applied to different regions of the brain, including the hippocampus and the amygdala (Sloviter, 1987) (Buckmaster, 2004). Around 90% of animals develop status epilepticus and a high percentage develops spontaneous seizures (around 80%) after a latency period of 6 - 18 days (Nissinen et al., 2000, Buckmaster, 2004). Furthermore, the stimulation is non-chemical and performed locally, avoiding effects in other areas. Also, animals show a pattern of hippocampal damage similar with the damage reported in human TLE (Buckmaster, 2004). Electrical stimulation has also the advantage of reproducing epileptogenic features in the intact brain with low mortality and high reproducibility. Additionally, this type of procedure can be expensive if used for long term studies (Buckmaster, 2004).

#### **1.7.3.2 Chemical stimulation**

The use of chemoconvulsant is frequently used to generate spontaneous seizures in rodents and mimics closely TLE. Animals present similar “clinical features” as humans, including, an initial injury afflicting the hippocampus and/or the temporal lobe and histopathological changes characteristic of TLE (Kandratavicius et al., 2014).

##### **1.7.3.2.1 Pilocarpine model**

The pilocarpine model is one of the main chemoconvulsant models used to study TLE (Turski et al., 1983, Buckmaster and Haney, 2012). Pilocarpine is a non-selective cholinergic muscarinic receptor agonist and is administered by a systemic or intracerebral injection in rats or mice. Acetylcholine receptors are metabotropic receptors, coupled to G-proteins, which can have different effects in the brain, such as

blocking of potassium channels, thereby increasing neuronal excitability, eventually leading to seizures (Turski et al., 1989, Becker, 2018, Buckmaster, 2004). In this model, animals need to receive atropine or scopolamine before pilocarpine administration to control peripheral cholinergic side effects (e.g. dehydration). In general, status epilepticus induced by pilocarpine develops 20 - 30 minutes after pilocarpine administration and can last for several hours. After status epilepticus, animals present a latent period (from 2 – 3 days to 2 weeks) before developing spontaneous seizures (Buckmaster, 2004). Around 40% of the animals however, do not develop status epilepticus. Additionally, a high mortality rate has been associated with the pilocarpine model (Buckmaster, 2004).

Systemic application of pilocarpine causes neuronal damage mainly in the CA1, but also in the hilar region of the hippocampus (Kandratavicius et al., 2014). Other areas in the brain, such as the amygdala and the cortex also present damage (Becker, 2018). There are several network and neurochemical similarities between human TLE and the pilocarpine model, such as neurotrophin upregulation in the hippocampus. Also, cognitive and memory deficits commonly found in TLE patients are also present in pilocarpine-injected rats (Kandratavicius et al., 2014).

#### **1.7.3.2.2 Pentylentetrazol model**

PTZ is an antagonist of GABA<sub>A</sub> used to induce seizures, particularly non-convulsive absence or myoclonic seizures. This model is used as an acute seizure model, rather than an animal model of epilepsy and may be useful for rapid screening of novel AEDs, targeting different seizure types (Kandratavicius et al., 2014).

#### **1.7.3.2.3 Kainic acid model**

The kainic acid model is the most common model to study TLE. Kainic acid is a L-glutamate analogue, causing depolarization of neurons and seizures (Becker, 2018),

preferentially targeting the hippocampus (Kandratavicius et al., 2014). Furthermore, the kainic acid model is a versatile model of focal seizures and epilepsy, because it is possible to administrate kainic acid systemically or directly into different regions of the brain, such as the ventricle, cortex, hippocampus or amygdala (Nadler et al., 1980, Bragin et al., 1999, Furtado Mde et al., 2002).

#### **1.7.3.2.3.1 Systemic kainic acid**

The intraperitoneal method is the simplest way to induce status epilepticus compared with other ways to administer kainic acid. Here, the dose is dependent on the animal species (rats were the first species used to try this method) and age (Scerrati et al., 1986). Initially, high doses of kainic acid (i.p. 12 – 18 mg / Kg) were used to induce status epilepticus. However, this was accompanied by high mortality rates, requiring an adjustment of kainic acid doses (Olney et al., 1979, Schwob et al., 1980, Nadler, 1981, Cronin and Dudek, 1988). A different approach to reduce mortality was developed later, which included multiple low doses of kainic acid until status epilepticus was elicited. This method, however, causes high variability in the frequency of epileptic seizures (Hellier et al., 1998).

In relation to the neuropathological features, systemic injection of kainic acid induces HS, including cell loss and synaptic reorganization of granule cells in rats (Becker, 2018). This includes pyramidal neuronal loss in the CA3 and CA1 regions of the hippocampus (Balchen et al., 1993). However, extrahippocampal damage is also observed, which is not common in TLE (Schwob et al., 1980, Sloviter, 1991).

This systemic method induces EEG patterns with high amplitude and low frequency spikes, associated with persistent seizures (Giorgi et al., 2005). Limitations of this method include that not all the strains present seizure-induced neuronal death, one of the major problems associated with using systemic kainic acid (Schauwecker and Steward, 1997).

#### **1.7.3.2.3.2 Intra-cerebral kainic acid**

As mentioned previously, the systemic injection of kainic acid has some limitations. To avoid these issues, an intra-cerebral kainic acid injection was developed. This consists of a local injection of kainic acid into the hippocampus (intra-hippocampal) or into the amygdala (intra-amygdala) (Schwarcz et al., 1978). An extensive pyramidal cell loss and severe granule cell dispersion in the hippocampus are observed in the intra-cerebral kainic acid model, characteristic of human TLE. Both methods (intra-hippocampal and intra-amygdala) will be explained below.

##### **1.7.3.2.3.2.1 Intra-hippocampal kainic acid**

In the intra-hippocampal kainic acid model, the dose of kainic acid can vary from 0.1 to 3 µg. Schwarcz et al. (1978) showed a unilateral degeneration of the neurons in the hippocampus followed by gliosis and atrophy of the hippocampus with 2 µg of kainic acid administered in rats. In this study the authors also showed paroxysmal discharges in the EEG and typical behaviour including immobility, staring and clonic components (Schwarcz et al., 1978). A different study (performed in mice) showed a fast onset of seizures after kainic acid injection and a loss of pyramidal and hilar neurons and MFS in the granular molecular layer of the CA3 subfield of the hippocampus (Bouilleret et al., 1999).

The intra-hippocampal model has also been used in other species, such as cats (Griffith et al., 1991), macaques (Chen et al., 2013) and guinea pig (Carriero et al., 2012).

##### **1.7.3.2.3.2.2 Intra-amygdala kainic acid**

The intra-amygdala kainic acid model was first performed by Ben-Ari et al. in rats (Ben-Ari et al., 1978, Ben-Ari et al., 1980, Ben-Ari, 1985). Some years later, the group of Professor Henshall adapted the intra-amygdala kainic acid model to mice

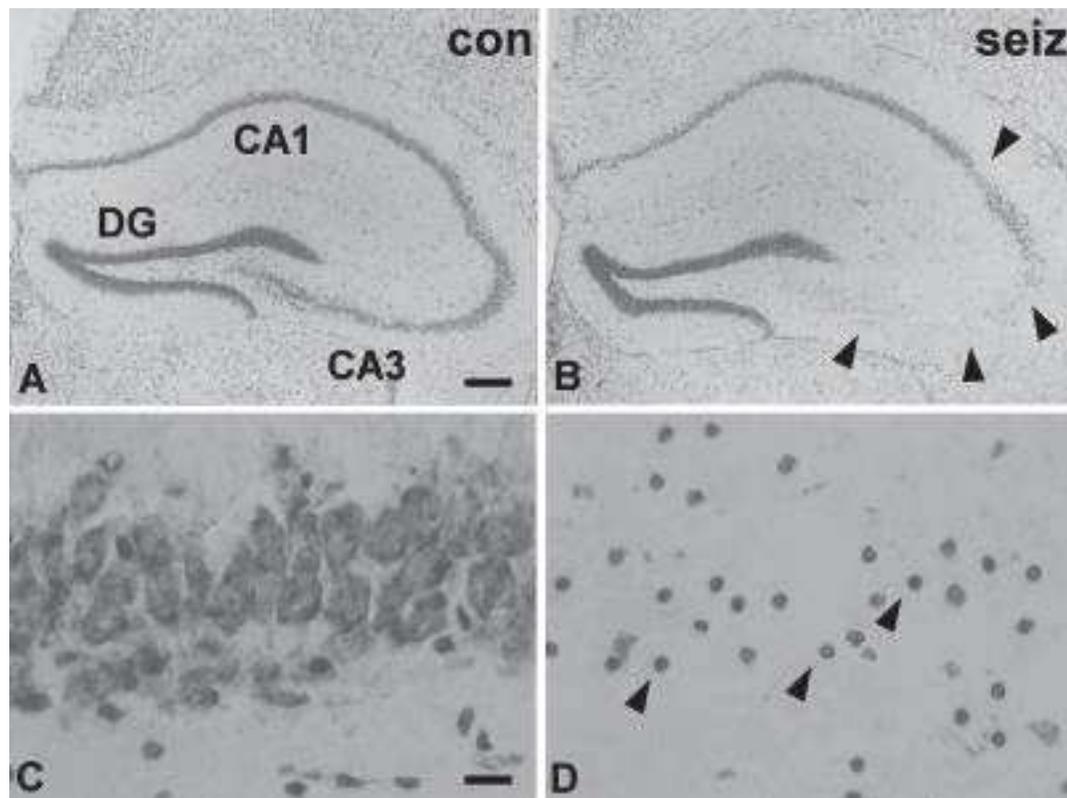
(Araki et al., 2002). After kainic acid injection into the amygdala, seizures propagate from the amygdala to the endothelial cortex and from there into the hippocampus via the perforant pathway (Araki et al., 2002, Shinoda et al., 2004). With a dose of 0.1 – 0.3 µg of kainic acid, mice showed a restricted and consistent neuronal death in the ipsilateral side of the hippocampus, mainly in CA3 (Figure 1.5). This suggests that this particular mouse model better recreates the hippocampal neuropathology of human TLE patients. These data sets establish a mouse model of focally evoked seizures in the C57BL/6 strain linked with a restricted pattern of neurodegeneration within the hippocampal subfields (Araki et al., 2002). Mice subject to intra-amygdala kainic acid showed typical behaviour changes during status epilepticus. The Racine scale includes five stages: 1) mouth and facial movements; 2) head nodding; 3) forelimb clonus; 4) rearing and 5) rearing and falling. This scale is used in research to classify and describe changes in behaviour during seizures in rodent epilepsy models (Racine, 1972). In our group, however, we use a modified Racine score to analyse the seizures behaviour (Jimenez-Mateos et al., 2012). An anticonvulsive, such as lorazepam or midazolam, is used to attenuate status epilepticus 40 minutes following kainic acid injection to minimize mortality and morbidity. Following status epilepticus mice go into a latent period of 3-5 days before experiencing their first spontaneous seizure. All mice subjected to intra-amygdala kainic acid develop epilepsy experiencing an average of 1 - 4 seizures per day (Mouri et al., 2008).

The intra-amygdala kainic acid model is characterized by unilateral damage of the CA3 region of the hippocampus and astrogliosis. The dorsal hippocampus displays similar damage to the ventral hippocampus (Li et al., 2008, Mouri et al., 2008, Tanaka et al., 2010). However, neuronal death can also be observed in the hilar region of the dentate gyrus and cortex (Mouri et al., 2008).

This model has been performed in different mouse strains including BALB/c (Shinoda et al., 2004), SJL (Tanaka et al., 2010) and FVB (Jimenez-Pacheco et al., 2016), showing similar seizure phenotype, suggesting the intra-amygdala kainic acid model as a versatile tool. Additionally, this model has been used to characterize the

electrographic seizure profile and pathology of different genetically modified mice ( $Bclw^{-/-}$ ,  $Bim^{-/-}$ ,  $Puma^{-/-}$ ,  $Chop^{-/-}$ ,  $14-3-3z^{-/-}$ ,  $bmf^{-/-}$ , P2X7-EGFP (Murphy et al., 2007, Engel et al., 2010, Murphy et al., 2010, Engel et al., 2013, Jimenez-Pacheco et al., 2016, Moran et al., 2013). Other research groups adopted the intra-amygdala kainic acid mouse model successfully (Li et al., 2008, Liu et al., 2010).

The intra-amygdala kainic acid model will be the main mouse model used in this study and a representative scheme of the model will be presented in the chapter “Material and Methods\_2.2.1.3 Status epilepticus induction”.



**Figure 1. 5 - Neuronal death in the ipsilateral side of the hippocampus.**

(A) Representative photomicrograph of the ipsilateral hippocampus from a control animal, showing the principal hippocampal fields. (B) Ipsilateral hippocampus from a mouse 24 hours after status epilepticus, showing degeneration of the CA3 pyramidal neurons. (C) High-power (40x lens) images from control brain CA3 field and (D) seizure-induced neuronal damage showing condensed and shrunken neurons 24 hours after status epilepticus. Adapted from (Araki et al., 2002).

## **1.8 Purinergic signalling in the brain**

It was not until 1972 that the role of adenosine 5'-triphosphate (ATP) as an intercellular molecule, was first described by Geoffrey Burnstock (Burnstock, 1972). Today, it is well recognized that a wide variety of nucleotides, including ATP, function as either sole or co-transmitter in both the peripheral and CNS. ATP can act as a fast, excitatory neurotransmitter or as a neuromodulator and is involved in a vast array of short- and long-term physiological and pathological processes including inflammation, cellular survival, proliferation, cellular differentiation and synaptic plasticity (Burnstock et al., 2011, Khakh and North, 2012, Idzko et al., 2014). ATP has been implicated in numerous different diseases of the CNS, including epilepsy (Burnstock, 2017).

### **1.8.1 Purine release in the brain**

Purines and pyrimidines are a well-established source of energy in all living cells. These molecules, however, also play an important role in intercellular communications within the CNS (Lecca and Ceruti, 2008, Idzko et al., 2014). Adenine and uridine nucleotides are present in almost every synaptic and secretory vesicle where they are either present alone, functioning as a fast neurotransmitter or co-stored with classical neurotransmitters (e.g.  $\gamma$ -aminobutyric acid (GABA) or glutamate) (Abbracchio et al., 2009). Under physiological conditions, adenine and uridine nucleotides are usually present at micromolar concentrations in the extracellular space; however, under pathological conditions (e.g. inflammation, hyperexcitability and cell death) extracellular nucleotide levels can reach the millimolar range (Dale and Frenguelli, 2009, Idzko et al., 2014, Rodrigues et al., 2015, Alves et al., 2018). ATP (and most likely uridine-5'-triphosphate (UTP)) can enter the extracellular space by crossing the compromised membranes of damaged and dying cells (Rodrigues et al., 2015). In addition, purines are actively released from different cell types including neurons, astrocytes, microglia and endothelial cells to act as neuro- and gliotransmitters (Lecca

and Ceruti, 2008, Rodrigues et al., 2015). Several mechanisms have been proposed to contribute to the release of nucleotides into the extracellular medium including cell damage, exocytosis of secretory granules, vesicular transport involving the vesicular nucleotide transporter (VNUT) and membrane channels such as ABC transporters, pannexins, connexins and via purinergic receptors themselves (Rodrigues et al., 2015, Lecca and Ceruti, 2008). Once released into the extracellular space, adenine and uridine nucleotides are rapidly metabolized by ectonucleotidases (e.g. ecto-nucleoside triphosphate diphosphohydrolases, ectonucleotide pyrophosphatase, alkaline phosphatases, ecto-5'-nucleotidase and ecto-nucleoside diphosphokinase) into different breakdown products including adenosine-5'-diphosphate (ADP), adenosine, uridine-5'-diphosphate (UDP) and uridine. These metabolites, in turn, are important neurotransmitters / neuromodulators in their own right, with specific receptors for each expressed throughout the CNS (Zimmermann, 2006, Burnstock, 2007).

Direct evidence for ATP release during seizures is mixed. Large elevations in ATP on electrical stimulation of the cortex (Wu and Phillis, 1978) provided the first direct evidence that high levels of neuronal activity could induce the release of ATP. Subsequently, stimulation of the Schaffer collateral in hippocampal slices was demonstrated to induce ATP in a  $Ca^{2+}$ -dependent manner, but glutamate receptor activation-independent manner (Wieraszko et al., 1989), suggesting the release of ATP was pre-synaptic. While ATP release was not detected following high frequency stimulation or electrically-induced epileptiform seizure like events in hippocampal slices (Lopatar et al., 2015), the induction of epileptiform activity in rat hippocampal slices with the use of the mGluR5-agonist, (S)-3,5-Dihydroxyphenylglycine, induced the release of ATP through pannexin hemichannels (Lopatar et al., 2015). ATP release was also elevated in hippocampal slices in a high  $K^+$  model of seizures (Heinrich et al., 2012). Dona et al, (2016) used microdialysis and high-performance liquid chromatography in order to attempt to measure extracellular concentrations of ATP and its metabolites *in vivo* after pilocarpine-induced status epilepticus and following the onset of chronic epilepsy (Dona et al., 2016). They found no change in ATP

concentrations for 4 hours following status epilepticus, but a marked increase in ATP metabolites, including AMP and ADP. Concentrations of ATP and all metabolites were reduced during chronic epilepsy, but ATP was elevated by 300% during spontaneous seizures. Because ectonucleotidases rapidly hydrolyse ATP in the extracellular space and the concentration and activity of these enzymes are increased following seizures (Nicolaidis et al., 2005), it is difficult to measure changes in ATP release directly. Less interest has been shown in investigating UTP release following seizures, however, Koizumi et al., (2007) demonstrated that following KA induced-seizure-like events in hippocampal slices, extracellular concentrations of UTP were elevated approximately three-fold (Koizumi et al., 2007).

Whereas the anticonvulsive properties of the nucleoside, adenosine, are well documented (Boison, 2016), the possible contribution of extracellular nucleotides to seizure pathology is a relatively new research area (Engel et al., 2016, Alves et al., 2018). The discovery of increased extracellular levels of ATP in seizure-prone rats was one of the first studies to suggest a functional contribution of extracellular nucleotides to seizures (Wieraszko and Seyfried, 1989). Demonstrating a direct impact on seizures, another early study showed that the microinjection of ATP analogues into the prepiriform cortex led to the generation of motor seizures (Knutsen, 1997). More recent evidence implicating extracellular nucleotides in seizure generation stems from studies showing that the injection of ATP into the brain of mice led to the development of high spiking on the electroencephalogram and exacerbated seizure severity during status epilepticus (Engel et al., 2012, Sebastian-Serrano et al., 2016). In contrast, treatment with UTP decreases the rate of neuronal firing in epileptic rats (Kovacs et al., 2013). Further, UTP metabolites such as uridine reduce epileptic seizures in patients with epileptic encephalopathy (Koch et al., 2017).

## 1.9 Purinergic receptor family

The purinergic receptors (P receptors) are sensitive to ATP, UTP and their metabolites, such as ADP, UDP and adenosine. Two classes of purinergic receptors have been identified: P1 receptors and P2 receptors (Burnstock, 2007).

### 1.9.1 P1 receptors

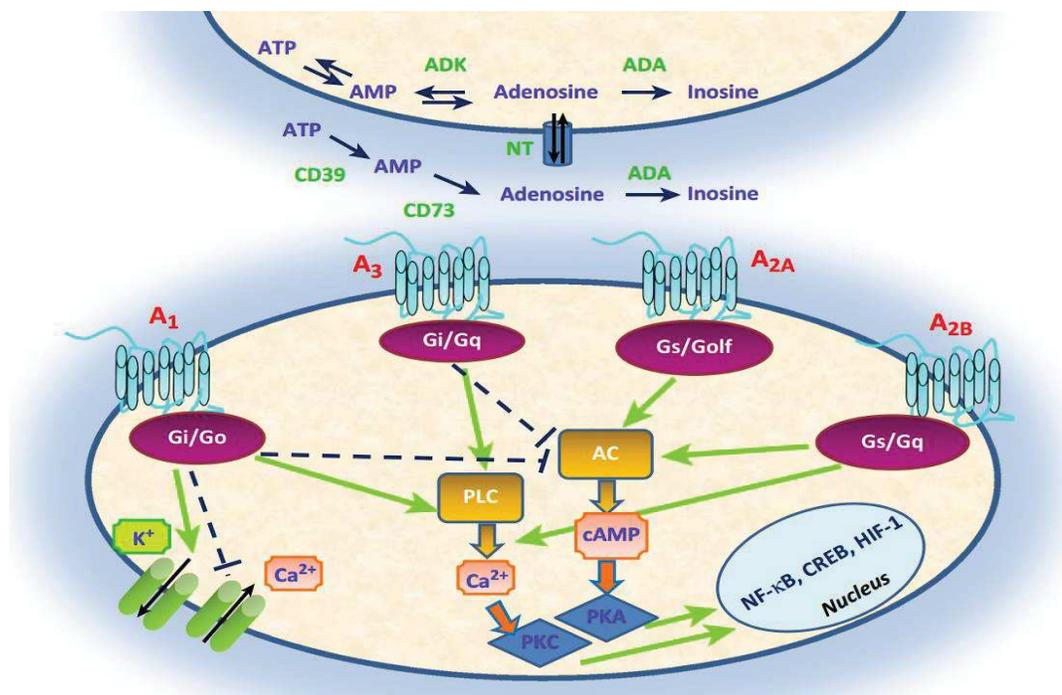
P1 receptors are metabotropic receptors, coupled to G proteins, and sensitive to adenosine, with different affinities. Four types of P1 receptors are known: A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> (Figure 1.6). These receptors have been implicated in numerous cellular responses, such as the regulation of neurotransmitter release (Burnstock, 2007). Interestingly, the A<sub>1</sub> and A<sub>3</sub> subtypes have an inhibitory effect on adenylyl cyclase (AC) activity, while A<sub>2A</sub> and A<sub>2B</sub> stimulate it, with a consequent modulation of cyclic AMP levels. Further, adenosine presents a higher affinity for A<sub>1</sub>, A<sub>2A</sub> and A<sub>3</sub> than for the A<sub>2B</sub> subtype (Borea et al., 2016).

Adenosine can be produced intracellularly, via hydrolysis of AMP or S-adenosyl-homo-cysteine, or extracellularly by ATP dephosphorylating (Figure 1.6). At physiological levels, adenosine acts as an endogenous homeostatic regulator of network activity and the extracellular concentrations are very low. However, under pathological conditions, such as epilepsy, ischemia and inflammation among others, the levels of adenosine increase (Borea et al., 2016).

Several studies have suggested that adenosine is an inhibitory modulator of brain activity, with anticonvulsive effects (Borea et al., 2016). These anticonvulsive effects are mainly associated with the activation of the A<sub>1</sub> receptor (Borea et al., 2016).

A<sub>1</sub> receptors are mainly found at pre-synapses in the excitatory neurons. Here, once activated, they decrease the release of neurotransmitters, including glutamate and inhibit the hyperpolarization of the postsynaptic cell membrane (Borea et al., 2016, Masino et al., 2014). Upregulation of the A<sub>1</sub> receptor has been reported as a

consequence of spontaneous seizures triggered by electrical stimulation (Hargus et al., 2012). Also, both upregulation of the A1 receptor and downregulation of the pro-convulsant A2A subtype have been shown in the cerebral cortex after hyperthermia-induced seizures, suggesting the existence of a specific neuroprotective mechanism (Leon-Navarro et al., 2015). On the other hand, the activation of A2A receptor has also been associated with the increase of cAMP and PKA activity, contributing to excitatory responses (Cunha et al., 1994). Further, a genetically modified mouse overexpressing astrocyte-expressed adenosine kinase (ADK), which is a key negative regulator of the brain inhibitory molecule adenosine, displayed an increase of seizure severity during status epilepticus, suggesting that overexpression of ADK without astrogliosis is sufficient to cause seizures (Li et al., 2008).



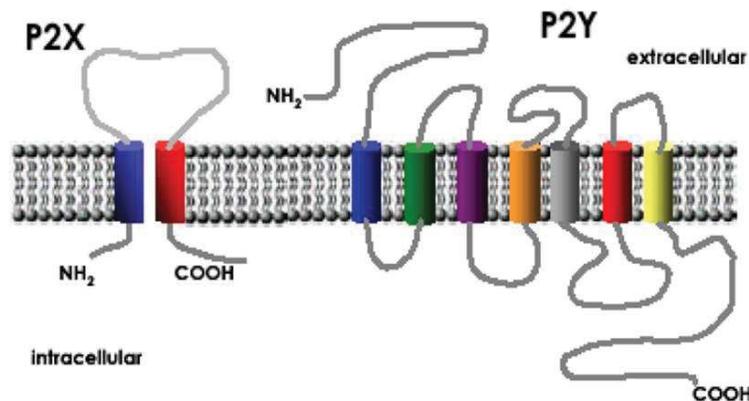
**Figure 1.6 – Activation of adenosine receptors and downstream pathways.**

Degradation of ATP results in an increase of adenosine. Adenosine can activate P1 receptors, which are metabotropic G protein coupled receptors. Four types of P1 are known: A1, A2A, A2B and A3. A1 and A3 subtypes are implicated in the inhibition of AC activity and stimulation of PLC; A1 receptors also modulate  $K^+$  and  $Ca^{2+}$  channels. A2A and A2B receptors stimulate AC, contributing to an increase in cAMP levels. A2B receptors also activate PLC, increasing  $Ca^{2+}$  levels. Regulation of PKA and PKC

triggers downstream signalling, which can modulate the transcription of genes involved in inflammation and cell regulation, such as NF $\kappa$ B, CREB, and HIF1. Adapted from (Borea et al., 2016).

### 1.9.2 P2 receptors

The P2 receptors are a family of cell membrane receptors comprising two subfamilies: P2X (P2XR) and P2Y (P2YR) receptors, which are structurally and functionally different from each other (Figure 1.7) (Abbracchio and Burnstock, 1994). P2 receptors are widely distributed throughout the CNS, and are expressed on neurons and glia cells, including astrocytes, microglia and oligodendrocytes (Stone et al., 2009) (Table 1.1).



**Figure 1.7 - P2 receptors family.**

P2X receptors are a family of seven cation-permeable ionotropic receptors. P2Y receptors are a group of eight metabotropic receptors with seven transmembrane domains, coupled to G proteins Adapter from (Volonte et al., 2003).

#### 1.9.2.1 P2X receptor family

P2XRs are a family of seven cation-permeable ionotropic receptors (P2X<sub>1-7</sub>) involved in fast excitatory neurotransmission in the brain (Burnstock, 2008). Structurally, P2X receptors comprise two transmembrane domains, a large extracellular loop and a intracellular N- and C-terminus (North, 2002). Functionally,

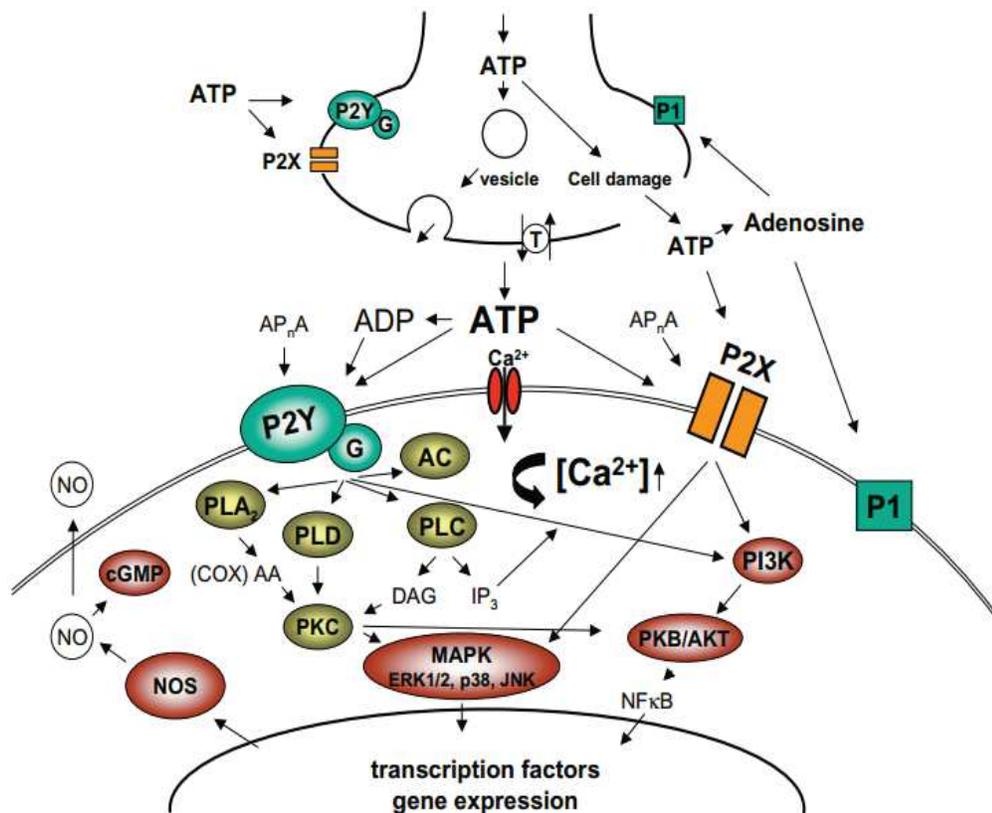
P2X receptors can form homomeric or heteromeric trimers with binding sites for ATP (orthosteric). All P2X receptors are sensitive to ATP, with different affinities. After ATP is released into the extracellular space, only a few milliseconds are required to activate the P2X receptors. With exception of the P2X7 receptor, all the other P2X receptors display a fast desensitization (North, 2002, Coddou et al., 2011, North and Jarvis, 2013). The P2X7 receptor, however, is activated only in the presence of high concentrations of ATP (North, 2002). This is one of the reasons why P2X7 receptors are frequently studied in some diseases. Additionally, P2X receptors are localized both pre and postsynaptically (Pankratov et al., 1998, Mori et al., 2001). In conclusion, the P2X receptor family has been widely studied in brain diseases and some subtypes of these receptors have been suggested in several pathological conditions, such as brain trauma and ischemia, neurodegenerative and neuroimmune disorders, neuroinflammation and epilepsy, among others (Burnstock, 2008, Burnstock and Knight, 2017, Burnstock, 2017).

### **1.9.2.2 P2Y receptor family**

While the P2X receptor family is made up of fast acting ligand-gated ion channels, the metabotropic P2Y receptor family consists of eight G-protein coupled slower-acting receptors: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub> (Burnstock, 2007, von Kugelgen, 2006).

In contrast to P2X channels, P2Y receptors can be activated by more than one substrate including the adenine nucleotides ATP (P2Y<sub>2</sub>, P2Y<sub>11</sub>) and ADP (P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub>), the uridine nucleotides UTP (P2Y<sub>2</sub> and P2Y<sub>4</sub>), UDP (P2Y<sub>6</sub> and P2Y<sub>14</sub>) and UDP-glucose (P2Y<sub>14</sub>). P2Y receptors contain the typical features of G-protein-coupled receptors which include an extracellular amino terminus, intracellular carboxyl terminus and seven transmembrane-spanning motifs (Jacobson et al., 2015). P2Y receptors can be further subdivided into groups based upon their coupling to specific G proteins. P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub> receptors are coupled to G<sub>q</sub> proteins,

which stimulate phospholipase C, ultimately resulting in the subsequent release of  $\text{Ca}^{2+}$  from intracellular stores and the activation of protein kinase C (PKC). Of these receptors, the  $\text{P2Y}_{11}$  receptor can also couple to  $\text{G}_s$ , stimulating adenylate cyclase and increasing the production of cyclic adenosine monophosphate (cAMP) (von Kugelgen, 2006).  $\text{P2Y}_{12}$ ,  $\text{P2Y}_{13}$  and  $\text{P2Y}_{14}$  are coupled to  $\text{G}_i$  proteins, inhibiting adenylate cyclase and thereby decreasing cAMP production (von Kugelgen, 2006) (Figure 1.8).



**Figure 1.8 – ATP Signalling.**

ATP release, metabolism, and the interaction of second messenger systems following the activation of ionotropic P2X and G protein (G)-coupled P2Y receptors. Extracellular ATP can be released after cell damage and is quickly converted to adenosine, acting through P1 receptors. The activation of P2 receptors usually leads to  $\text{Ca}^{2+}$  mobilization with and without  $\text{Ca}^{2+}$  entry from the extracellular space. P2Y receptors are implicated in several physiological functions and these functional responses may be regulated by a cross-talk of many second-messenger pathways: via the activation of phospholipase A2 (PLA<sub>2</sub>), phospholipase D (PLD), protein kinase C (PKC), adenylate cyclase (AC), phospholipase C (PLC) stimulating diacylglycerol (DAG) and inositol-(1,4,5)-trisphosphate (IP<sub>3</sub>), nitric oxide synthase (NOS),

phosphoinositide 3-kinase (PI3K)/serine-threonine kinase AKT, mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs), and cyclic guanosine monophosphate (cGMP). Adapted from (Franke and Illes, 2006).

### **1.10 P2Y receptors in brain inflammation and excitability**

P2Y receptors are involved in a myriad of different cellular functions and pathological processes pertinent to the process of epileptogenesis and epilepsy including neuroinflammation, neurodegeneration, synaptic reorganization and changes in neurotransmitter release (von Kugelgen, 2006, Jacobson and Boeynaems, 2010, Pitkanen et al., 2015, Guzman and Gerevich, 2016, Alves et al., 2018). This makes them an attractive antiepileptic therapeutic target.

Inflammatory processes in the brain have received much attention over recent years and are thought to play a major role in seizure-induced pathology and in the development of epilepsy (Vezzani et al., 2011). The principle ligands for P2Y receptors are the purine, ATP, the pyrimidine, UTP, and their metabolites, such as ADP and UDP (Burnstock, 2007). The role of each receptor in neuroinflammation is dictated by its affinity for different ligands and downstream targets. ATP is both released as a result of inflammation and promotes pro-inflammatory mechanisms. This circular causality can underpin a positive feedback loop whereby neuroinflammation becomes self-sustaining (Idzko et al., 2014). Less is known about the role of UTP in mediating neuroinflammation. The role of different P2Y receptors in mediating neuroinflammation and cell death seems to be divergent (Forster and Reiser, 2015), depending on downstream signalling pathways and mutually antagonistic, but is incompletely understood.

Activation of astrocytic P2Y<sub>2</sub> receptors promotes astrocyte activation and migration via an interaction with  $\alpha$ V-integrin (Wang et al., 2005). The P2Y<sub>2</sub> receptor has also been shown to play a protective role against chronic inflammation-induced neurodegeneration in a model of Alzheimer's disease (Kong et al., 2009). A role for the uridine-sensitive P2Y<sub>4</sub> receptor in mediating neuroinflammation has not been

established (Beamer et al., 2016), with progress hamstrung by a lack of specific tools for targeting this receptor. The P2Y<sub>6</sub> receptor promotes the activation of microglia and the adoption of a phagocytic phenotype following activation by the UTP metabolite UDP (Koizumi et al., 2007). This is dependent on downstream signalling involving phospholipase C and PKC. Other studies have suggested that P2Y<sub>12</sub> receptor plays a role in microglial activation (Ohsawa et al., 2010), showing that activation of integrin- $\beta$ 1 in microglia through P2Y<sub>12</sub> is involved in directional process extension by microglia in brain tissue. The effects of P2Y signalling are not limited to inflammatory processes and cellular survival alone. P2Y signalling also impacts directly on neuronal excitability, synaptic strength and synaptic plasticity (Guzman and Gerevich, 2016). P2Y receptor activation can lead to the inhibition of VACCs (Diverse-Pierluissi et al., 1991) thereby potentially influencing neuronal excitability and synaptic plasticity. P2Y receptors also block potassium channels (e.g. voltage-gated potassium channel subunit KvLQT2,3 (Filippov et al., 2006) or G protein-coupled inward rectifying channel 1, 2 & 4 (GIRK1,2 &4)) (Filippov et al., 2004), inhibiting membrane hyperpolarisation and thereby facilitating an increased frequency of neuronal firing (Brown and Passmore, 2009, Guzman and Gerevich, 2016). This subject will be presented in more detail further in chapter 4. In conclusion, while P2X receptors exert a mainly facilitatory effect on synaptic transmission (Khakh and North, 2012), the effects of P2Y receptors seem to be context-specific, either decreasing neuronal firing by inhibiting excitatory neurotransmitter release and receptor function or increasing neuronal firing by decreasing potassium conductance or increasing release of inhibitory neurotransmitters (Goncalves and Queiroz, 2008, Alves et al., 2018).

### **1.10.1 P2Y<sub>1</sub> receptor**

During our study, we have identified the P2Y<sub>1</sub> receptor subtype as a potential new target to treat status epilepticus. Therefore, this receptor will be introduced in more detail. The P2Y<sub>1</sub> receptor is encoded in humans by the *P2ry1* gene located on

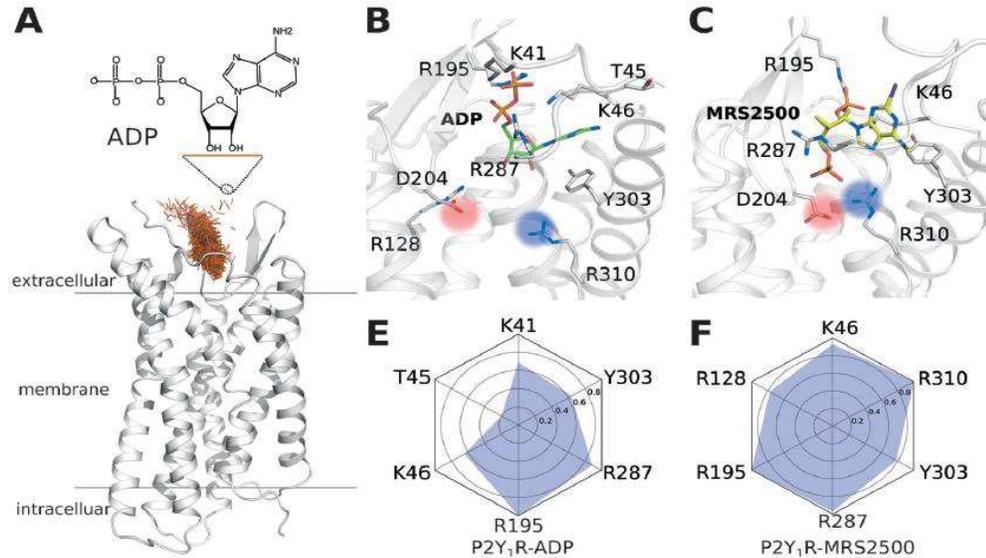
chromosome 3q25.2 (Ayyanathan et al., 1996a, Ayyanathan et al., 1996b). In 1996, Ayyanathan and collaborators isolated two types of human P2Y<sub>1</sub> cDNA clones. Both sequences coded for the same 373 amino acid peptide, differing only in the length of the 3'untranslated region (Ayyanathan et al., 1996b). The P2Y<sub>1</sub> receptor is activated preferentially by ADP. When ADP binds to the P2Y<sub>1</sub> receptor there is a conformational change in the receptor (exchange GDP for GTP), causing the activation of G proteins (Figure 1.9). The G $\alpha$  subunit, which is binding to GTP, is dissociated from the G $\beta\gamma$  subunit and both subunits will activate different pathways.

Advances in the understanding of the P2Y<sub>1</sub> receptor pharmacology have contributed to the development of potent and selective agonists and antagonists for the P2Y<sub>1</sub> receptor (von Kugelgen and Hoffmann, 2016).

ADP and ATP were both described as potent agonists of the P2Y<sub>1</sub> receptor, but experiments using highly purified nucleotides revealed that P2Y<sub>1</sub> receptors bind preferentially to ADP (100 fold greater than for ATP) whereas ATP is a weak partial agonist. MRS2365 is the most potent agonist of the P2Y<sub>1</sub> receptor, followed by 2-methylthio-ADP and ADP (MRS2365>2-methylthio-ADP>ADP) (von Kugelgen and Hoffmann, 2016).

Different inhibitors can block the P2Y<sub>1</sub> receptor. Suramin; Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (PPADS); reactive blue-2; 8,8'-[Carbonylbis (imino-3,1-phenylene carbonylimino)]bis(1,3,5-naphthalene-trisulfonic acid) hexasodium salt (NF023) are general antagonists for all P2YRs. Specific P2Y<sub>1</sub> receptor inhibitors are: 2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium (MRS2179);(1R\*,2S\*)-4-[2-Chloro-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester diammonium salt (MRS2279) and (1R\*,2S\*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500).

Affinity with P2Y<sub>1</sub> receptor (antagonists): MRS2500>MRS2279>MRS2179 (von Kugelgen and Hoffmann, 2016).



**Figure 1.9 - The ligand binding modes of P2Y<sub>1</sub> receptor.**

**(A)** The entrance pathway of ADP into the receptor represented as a superposition of the mass centers of ADP over a time period of 0.2 ms (brown points). **(B)** ADP binding mode. **(C)** MRS2500 binding mode. Adapted from (Yuan et al., 2016).

The P2Y<sub>1</sub> receptor is expressed in different regions of the brain, such as the cortex, hippocampus, caudate nucleus and putamen, confirmed by immunohistochemistry (Moore et al., 2000, Csolle et al., 2008, Engel et al., 2016). The P2Y<sub>1</sub> receptor is localized mainly in astrocytes, but has also been described to be expressed in neurons, microglia and oligodendrocytes ((Burnstock, 2007); Table 1), with physiological roles in neurotransmission and glial cell communication (Guzman and Gerevich, 2016). This receptor has been shown to be localized both pre- and postsynaptically and depending on the subcellular expression, P2Y<sub>1</sub> receptors might have different roles, including inhibition of neurotransmitter release and the facilitation of neuronal excitability (Guzman and Gerevich, 2016).

The P2Y<sub>1</sub> receptor is activated under conditions of oxidative stress, prompting the release of Interleukin-6 (IL-6) (Fujita et al., 2009). IL-6 has been shown to play an anti-inflammatory role during classical signalling involving the binding of IL-6 to the membrane-bound IL-6 receptor which induces the dimerization of the  $\beta$ -receptor

glycoprotein 130 (gp130). IL-6 has, however, also been described as critical for anti-inflammatory signalling in a process termed trans-signalling where IL-6 stimulates distant cells only expressing gp130 without expressing the IL-6 receptor (Rothaug et al., 2016). A more recent study has shown that in a chronic model of epilepsy, astrocytes from kindled rats show enhanced  $Ca^{2+}$ -dependent signalling and astroglial hyperexcitability, which requires the activation of the P2Y<sub>1</sub> receptor (Alvarez-Ferradas et al., 2015). P2Y<sub>1</sub> antagonism prevented cognitive deficits and neuronal damage in a model of ischemia in mice (Carmo et al., 2014). A recent study also showed improved histological and cognitive outcomes in a model of traumatic brain injury in mice provided by P2Y<sub>1</sub> receptor antagonism (Choo et al., 2013).

### **1.11 P2 receptors in status epilepticus and epilepsy**

P2 receptors have been suggested to be implicated in status epilepticus and in epilepsy. **Table 1** outlines a summary of all studies carried out to date for P2X and P2Y receptors in status epilepticus and epilepsy (Engel et al., 2016, Alves et al., 2018).

#### **1.11.1 Role of the P2X receptors in status epilepticus and epilepsy**

Most of the studies analysing purinergic signalling during seizures have focused on the contribution of P2X receptors for the treatment of epilepsy, in particular the P2X7 subtype (Engel et al., 2016). P2X7 receptor showed an increased expression in the hippocampus and cortex after status epilepticus in experimental mouse models (Avignone et al., 2008, Engel et al., 2012, Jimenez-Pacheco et al., 2013, Jimenez-Pacheco et al., 2016) and is up-regulated in patients suffering from temporal lobe epilepsy (Jimenez-Pacheco et al., 2016). In addition, specific P2X7 receptor agonists and antagonists were used in functional studies confirming the recruitment of the P2X7 receptor during seizure generation and seizure-induced cell death (Engel et al., 2012). Our group reported an up-regulation of hippocampal P2X7 receptor achieved

through the inhibition of a P2X7-suppressing micro-RNA resulting in a more severe epileptic phenotype (Jimenez-Mateos et al., 2015). In the same study, it was also shown that increased expression of P2X7 receptor was followed by an increase of cytokine levels (IL1 $\beta$  and TNF $\alpha$ ), astrogliosis and cognitive impairment (Jimenez-Mateos et al., 2015).

A functional role of the P2X7 receptor has also been suggested during chronic epilepsy. Different studies reported that an inhibition of the P2X7 receptor ameliorated the epileptic phenotype in mice, showing a reduction in the amount of seizures, astrogliosis and microgliosis (Jimenez-Pacheco et al., 2016, Amorim et al., 2017). Most recently, Fischer et al, (2016), also showed that inhibition of the P2X7 receptor significantly delayed the progression of kindling development. They also reported a significant reduction of gliosis (GFAP and Iba1) in the hippocampus (Fischer et al., 2016).

Apart from the P2X7 receptor, only P2X2 and P2X4 receptors have been studied in epilepsy. In relation to the others subtypes of the P2X receptors very little is known. P2X2 receptor has been reported to be down-regulated after status epilepticus (Engel et al., 2012). On the other hand, P2X4 receptor is up-regulated in the hippocampus after status epilepticus and is mostly localized in activated microglial cells (Ulmann et al., 2013). In addition, mice lacking the P2X4 receptor showed that CA1 subfield of the hippocampus was in part protected from status epilepticus-induced neuronal death (Ulmann et al., 2013). P2X receptors might have an important role in pathophysiology of status epilepticus and these receptors can be a potential candidate for seizure control.

### **1.11.2 P2Y receptor in status epilepticus and epilepsy**

As mentioned above, most of the studies performed to investigate the role of P2 receptors in epilepsy have been carried out on P2X receptors (Engel et al., 2016,

Alves et al., 2018). The role of P2Y receptors in status epilepticus and during epilepsy is poorly studied. This topic will be presented in more detail in chapter 3.

#### **1.11.2.1 P2Y<sub>1</sub> receptor in status epilepticus and epilepsy**

This topic will be discussed in more detail in chapter 4 and chapter 5. However, it is important to mention that the P2Y<sub>1</sub> receptor has been suggested to be implicated in status epilepticus and in chronic epilepsy. For example, P2Y<sub>1</sub> receptor has been shown to be increased in neuronal progenitor cells after pilocarpine-induced status epilepticus (Rozmer et al., 2017) and also in patients with intractable epilepsy associated with focal cortical dysplasia (FCD) (Sukigara et al., 2014).

In a chronic model of epilepsy, astrocytes from kindled rats show an enhanced spontaneous Ca<sup>2+</sup>-dependent signalling and astroglial hyperexcitability, which requires the activation of P2Y<sub>1</sub> receptors (Alvarez-Ferradas et al., 2015). More recently, a functional role of the P2Y<sub>1</sub> receptor to seizure-induced cell death has been shown in rats. Here, the authors reported that in a rat model of kainic acid-induced status epilepticus (i.p), P2Y<sub>1</sub> receptor antagonism protected from seizure-induced cell death without interfering in seizure severity (Simoes et al., 2018).

**Table 1.1 – P2 receptor family after status epilepticus and epilepsy**

Receptor	Cell type expression	Agonist	Receptor expression in:		Effect of agonists/antagonists or knockout
			Status Epilepticus:	Epilepsy:	
<b>P2X1</b>	Neurons, astrocytes, oligodendrocytes, microglia <sup>1</sup>	ATP <sup>2</sup>	No change (W) (mouse) <sup>10</sup> Up-regulated (qPCR) (mouse) <sup>11</sup>		Not studied
<b>P2X2</b>	Neurons, astrocytes, oligodendrocytes, microglia <sup>1</sup>	ATP <sup>2</sup>	Decreased (W) (mouse) <sup>10</sup> No change (W) (rat) <sup>13</sup>	Decreased (ISH) (gerbil) <sup>12</sup> No change (W) (rat) <sup>13</sup>	Not studied
<b>P2X3</b>	Neurons, astrocytes, oligodendrocytes, microglia <sup>1</sup>	ATP <sup>2</sup>	No change (W) (mouse) <sup>10</sup>	Increased (WB, qPCR) (mouse, human) <sup>31</sup>	Not studied
<b>P2X4</b>	Neurons, astrocytes, oligodendrocytes, microglia, endothelial cells <sup>1</sup>	ATP <sup>2</sup>	Increased (W, IH) (mouse) <sup>14</sup> No change (W) (mouse) <sup>10</sup> , (rat) <sup>13</sup> Up-regulated (qPCR) (mouse) <sup>11</sup>	Decreased (ISH, W) (gerbil) <sup>12</sup> , (rat) <sup>13</sup>	KO-mice: Decreased seizure-induced cell death (i.p. KA) (mouse) <sup>14</sup> No effect on seizures (i.p. KA) (mouse) <sup>14</sup> Decreased inflammation and microglia density (i.p. KA) (mouse) <sup>14</sup> No change in IL-1 $\beta$ levels (i.p. KA) (mouse) <sup>14</sup>
<b>P2X5</b>	Neurons, astrocytes, endothelial cells <sup>1</sup>	ATP <sup>2</sup>	No change (W) (mouse) <sup>10</sup>		Not studied
<b>P2X6</b>	Neurons, astrocytes, microglia <sup>1</sup>	ATP <sup>3</sup>	Not studied		Not studied

**Table 1.1 – P2 receptor family after status epilepticus and epilepsy - continuation**

Receptor	Cell type expression	Agonist	Receptor expression in:		Effect of agonists/antagonists or knockout
			Status Epilepticus:	Epilepsy:	
P2X7	Neurons, astrocytes, oligodendrocytes, microglia, endothelial cells <sup>1</sup>	ATP <sup>2</sup>			<p>BzATP (Agonist):                      Increased seizures (i.a. KA) (mouse)<sup>10</sup>                      No effect on seizures (Pilo) (mouse)<sup>19</sup>                      Increased microglia activation (Pilo) (rat)<sup>20</sup>                      Increase in astrocyte loss (Pilo) (rat)<sup>21</sup>                      Increased TNF-<math>\alpha</math> immunoreactivity (Pilo) (rat)<sup>22</sup>                      Decreased seizure-induced cell death (Pilo) (rat)<sup>22</sup>                      Increase seizure severity and number of seizure (Pilo) (rat)<sup>29</sup></p>
					<p>KO-mice:                      Decreased seizures (i.a. KA) (mouse)<sup>10</sup>                      Increased seizures (Pilo) (mouse)<sup>19</sup>                      No effect on seizures (i.p. KA and i.p. Pic) (mouse)<sup>19</sup>                      Decrease spontaneous seizure (i.a, KA) (mouse)<sup>26</sup></p>
			<p>Increased (W) (mouse)<sup>10</sup>, (rat)<sup>13</sup>, (rat)<sup>15</sup>                      Up-regulated (qPCR) (mouse)<sup>11,26</sup>                      Increased (IH) (rat)<sup>15</sup>, (rat)<sup>16</sup>                      Decrease (W) (IH) (mouse)<sup>26</sup></p> <p>Cortex:                      Increased (W, GFP) (human, mouse)<sup>17</sup></p>	<p>Increased (W) (rat)<sup>13</sup> (Human)<sup>27,28</sup>                      Increased (qPCR) (Human)<sup>28</sup></p>	<p>A-43, A-74, BBG, OxATP, IgG-P2X<sub>7</sub>, JNJ-47965567, JNJ-42253432, AFC-5128 (Antagonists):                      Decreased seizures (i.a. KA) (mouse)<sup>10</sup>, (rat)<sup>15</sup>, (mouse)<sup>17</sup>                      Decreased kindling score (PTZ) (rat)<sup>23</sup>                      Increased seizures (Pilo) (mouse)<sup>19</sup>                      Decreased seizure-induced cell death (i.a. KA) (mouse)<sup>10</sup>, (rat)<sup>15</sup>                      Increased seizure-induced cell death (Pilo) (rat)<sup>22</sup>                      Decreased microglia activation (i.a. KA and Pilo) (mouse)<sup>10</sup>, (rat)<sup>20</sup>                      Decreased II-1<math>\beta</math> levels (i.a. KA) (mouse)<sup>10</sup>                      Decreased astrocyte loss (Pilo) (rat)<sup>21</sup>                      Suppressed epileptic seizure (i.a, KA) (mouse)<sup>27</sup>                      Reduction of gliosis (i.a, KA) (mouse)<sup>27</sup>                      Reduction in seizure severity; no changes in number of seizures or microglia activation (s.c, KA) (mouse)<sup>30</sup>                      Delay the progression of kindling development, and significantly reduce gliosis (GFAP and Iba1) (i.p, KA) (rat)<sup>32</sup></p>

**Table 1.1 – P2 receptor family after status epilepticus and epilepsy - continuation**

Receptor	Cell type expression	Agonist	Receptor expression in:		Effect of agonists/antagonists or knockout
			Status Epilepticus:	Epilepsy:	
<b>P2Y<sub>1</sub></b>	Neurons, astrocytes, oligodendrocytes, microglia, endothelial cells <sup>1</sup>	ADP>ATP <sup>4</sup>	Increased (mouse) <sup>29</sup> (IH) Up-regulated (mouse) <sup>34</sup> (W)	Increased (human) <sup>18,34</sup> (W) (mouse) <sup>34</sup>	MRS2179 (antagonist), 2-methylthioADP (agonist): No effect on SLEs (rat) <sup>24</sup> MRS2500 (antagonist): Decreased neuronal death, but no changes in seizures behaviour <sup>33</sup>
<b>P2Y<sub>2</sub></b>	Neurons, astrocytes, oligodendrocytes, microglia, endothelial cells <sup>1</sup>	UTP/ATP <sup>5</sup>	Up-regulated (mouse) <sup>34</sup> (W)	Increased (human) <sup>18,34</sup> (W) (mouse) <sup>34</sup>	Not studied
<b>P2Y<sub>4</sub></b>	Neurons, astrocytes, oligodendrocytes, microglia <sup>1</sup>	UTP>ATP5	Up-regulated (mouse) <sup>34</sup> (W)	Increased (human) <sup>18</sup> (W) Non-significant increase (W) (mouse) <sup>34</sup> (human) <sup>34</sup>	Not studied
<b>P2Y<sub>6</sub></b>	Neurons, astrocytes, oligodendrocytes, microglia, endothelial cells <sup>1</sup>	UDP>UTP>>A TP5	Up-regulated (qPCR) (mouse) <sup>11</sup> Non-significant increase (W) (mouse) <sup>34</sup>	No change (W) (mouse) (human) <sup>34</sup>	Not studied
<b>P2Y<sub>11</sub></b>	Neurons, oligodendrocytes, endothelial cells <sup>1</sup>	ATP <sup>6</sup>	Not studied		Not studied
<b>P2Y<sub>12</sub></b>	Neurons, astrocytes, oligodendrocytes, microglia, endothelial cells <sup>1</sup>	ADP>ATP <sup>7</sup>	Up-regulated (qPCR) (mouse) <sup>11</sup> Down-regulated (WB) (mouse) <sup>34</sup>	Increased (mouse) <sup>34</sup> (W) Non-significant increase (W) (human) <sup>34</sup>	KO-mice: Reduced microglial response (mouse) <sup>25</sup> Worsened seizures (i.p KA) (mouse) <sup>25</sup> Increased seizure phenotype (i.c.v KA) (mouse) <sup>25</sup> Intense seizures (i.c.v KA) (mouse) <sup>25</sup> Lower increase in hippocampal microglial process number (i.p and i.c.v KA) (mouse) <sup>25</sup>
<b>P2Y<sub>13</sub></b>	Neurons, oligodendrocytes, microglia <sup>1</sup>	ADP>>ATP <sup>8</sup>	Up-regulated (qPCR) (mouse) <sup>11</sup> No change (WB) (mouse) <sup>34</sup>	Non-significant increased (W) (mouse) <sup>34</sup> Reduced (W) (human) <sup>34</sup>	Not studied
<b>P2Y<sub>14</sub></b>	Neurons, astrocytes, microglia <sup>1</sup>	UDP-sugars <sup>9</sup>	No change (WB) (mouse) <sup>34</sup>	No change (W) (mouse) (human) <sup>34</sup>	Not studied

<sup>1</sup>Burnstock G, 2007; <sup>2</sup>Burnstock G, 2003; <sup>3</sup>Collo et al., 1996; <sup>4</sup>Waldo and harden, 2004; <sup>5</sup>Nicholas et al., 1996; <sup>6</sup>Communi et al., 1999; <sup>7</sup>Takasaki et al., 2001; <sup>8</sup>Communi et al., 2001; <sup>9</sup>Chambers et al., 2000; <sup>10</sup>Engel et al., 2012; <sup>11</sup>Avignone et al., 2008; <sup>12</sup>Tae-Cheon et al., 2003; <sup>13</sup>Dona et al., 2009; <sup>14</sup>Ulmann et al., 2013; <sup>15</sup>Mesuret et al., 2014; <sup>16</sup>Rappold et al., 2006; <sup>17</sup>Jimenez-Pacheco et al., 2013; <sup>18</sup>Sukigara et al., 2014; <sup>19</sup>Kim and Kang, 2011; <sup>20</sup>Choi et al., 2012; <sup>21</sup>Kim et al., 2011a; <sup>22</sup>Kim et al., 2011b; <sup>23</sup>Soni et al., 2015; <sup>24</sup>Lopatár et al., 2011; <sup>25</sup>Eyo et al., 2014; <sup>26</sup>Jimenez-Mateos et al., 2016; <sup>27</sup>Jimenez-Pacheco et al., 2016; <sup>28</sup>Wei et., 2016; <sup>29</sup>Rozmer et al., 2016; <sup>30</sup>Amhaoul et al., 2016; <sup>31</sup>Zhou et al., 2016; <sup>32</sup>Fischer et al., 2017; <sup>33</sup>Simoes et al., 2018; <sup>34</sup>Alves et al., 2018.

## **1.12 Hypothesis**

P2Y receptors are activated during seizures and contribute to seizure-induced pathology and the development of epilepsy.

## **1.13. Objectives**

### **1. Characterize the induction and expressional changes of P2Y receptors in the brain after seizures and epilepsy**

1.1.1. Analyse mRNA and protein level changes of the different P2Y receptors subtypes following status epilepticus and during epilepsy.

1.1.2. Analyse protein levels of P2Y receptors in brain tissue from human TLE patients.

1.1.3. Determine the impact of the broad-spectrum P2Y agonists ADP and UTP on status epilepticus.

### **2. Establish the contribution of P2Y<sub>1</sub> receptors to status epilepticus**

2.1. Determine the contribution of the P2Y<sub>1</sub> receptor to seizure generation and seizure-induced pathology (e.g. inflammation, cell death) during and following status epilepticus using specific P2Y<sub>1</sub> receptor agonists, antagonists and P2Y<sub>1</sub> receptor knock-out mice.

### **3. Evaluate the impact of P2Y<sub>1</sub> receptor modulation on the epileptic phenotype**

3.1. Determine the contribution of the P2Y<sub>1</sub> receptor to the development of epilepsy.

3.2. Establish the contribution of the P2Y<sub>1</sub> receptor to chronic epilepsy.

## Chapter 2 - Material and Methods

## **2.1 Material**

### **Chemical and General Reagents**

**Table 2.1** contains the list of chemicals and reagents including catalogue number and manufacturers.

### **Equipment**

**Table 2.2** contains the list of equipment including manufacturers.

### **Primers**

**Table 2.3** contains the list of primers used in this study, including the respective sequences.

### **Antibodies**

**Table 2.4** contains the list of antibodies used in this study, including catalogue number and manufacturers.

## 2.2 Methods

### 2.2.1 Intra-amygdala kainic acid mouse model of status epilepticus and epilepsy

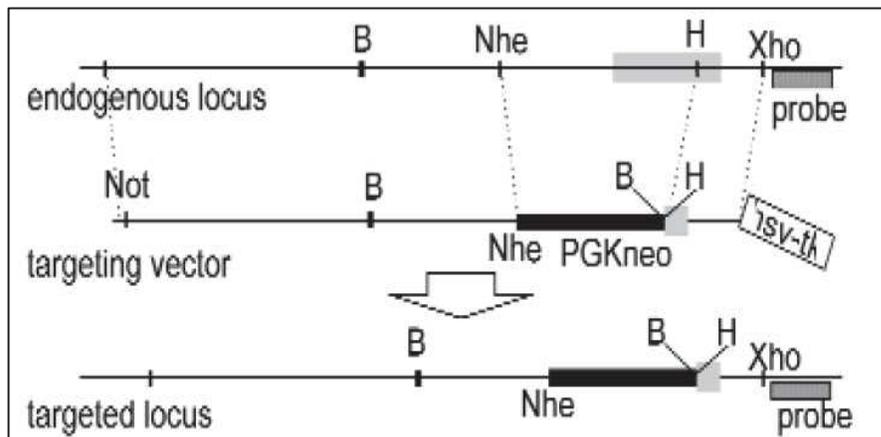
#### 2.2.1.1 Animals and husbandry

All animal experiments were performed in accordance with the principles of the European Communities Council Directive (2010/63/EU). Procedures were reviewed and approved by the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC 1322) and HPRA (AE19127/P038; AE19127/P001). All efforts were maximised to reduce the number of animals used in this study.

Mice used in these experiments were 8-12 weeks old male C57BL/6, obtained from Harlan Laboratories (Bicester, UK) and from the Biomedical Research Facility (BRF), Royal College Surgeons in Ireland (Dublin, Ireland), or P2Y<sub>1</sub> knockout mice, obtained from Jackson laboratories. Animals were housed in a controlled biomedical facility on a 12 hours light / dark cycle at 22±1 °C and humidity of 40-60% with food and water provided *ad libitum*.

##### 2.2.1.1.1 P2Y<sub>1</sub> knockout mice

P2Y<sub>1</sub> knockout mice (B6.129P2-P2ry1<sup>tm1Bhk</sup>/J) were obtained from Jackson laboratories. These mice result from a target mutation in the P2Y<sub>1</sub> allele (Fabre et al., 1999). B6.129P2-P2ry1<sup>tm1Bhk</sup>/J mice were generated using a targeting vector containing a PGKneo cassette to disrupt a large part of the coding region. The targeting vector was prepared from genomic clones containing the *P2ry1* gene. The targeting vector is identical to the corresponding region in the mouse genome, however in a specific location (4.7-kb fragment of DNA extending from the HindIII site located 3' of the gene to the NheI site), *P2ry1* locus was disrupted and replaced by the *neo* gene (**Figure 2.1**) (Fabre et al., 1999). In-house, P2Y<sub>1</sub> knockout heterozygous male and female obtained from Jackson were bred with C57BL/6 mice (WT) to obtain P2Y<sub>1</sub> knockout homozygous mice.



**Figure 2.1 – Restriction map of the P2Y<sub>1</sub> targeting construct.**

A vector containing the PGKneo cassette was used to disrupt a large part of the coding region. The targeting vector was prepared from genomic clones containing *P2ry1* gene and is identical to the corresponding region in the mouse genome, except that a 4.7-kb fragment of DNA extending from the HindIII site located at 3' of the gene to the NheI site has been replaced by the *neo* gene. Adapted from Fabre et al. (1999).

### **Genotyping protocol**

#### **DNA extraction**

Mice were marked for identification using the ear punch method. The ear tissue was collected and used to genotype the animals. 500 µl of buffer (50 mM Tris-HCl pH 8; 5 mM EDTA pH 8; 0.05 % SDS and dH<sub>2</sub>O) and 5 µl of proteinase K were added to the ear tissue. Samples were incubated at 55 °C overnight at 700 rpm. On the following day, 143 µl of 6 M NaCl was added to the samples, which were centrifuged for 10 minutes at 13200 rpm at 4 °C. Precipitation of DNA was performed using 350 µl of isopropanol. Samples were centrifuged 10 minutes at 13200 rpm at 4°C. Then, the pellet was washed in 250 µl of 75% ethanol and centrifuged for 2 minutes at 13200 rpm at 4°C. Supernatant was discarded and the pellet was re-suspended in dH<sub>2</sub>O and DNA quantification was measured using a nanodrop spectrophotometer.

## DNA amplification

The protocol used to perform polymerase chain reaction (PCR) was adapted from Jackson laboratory. The PCR protocol is presented in **Tables 2.5A** and **2.5B**.

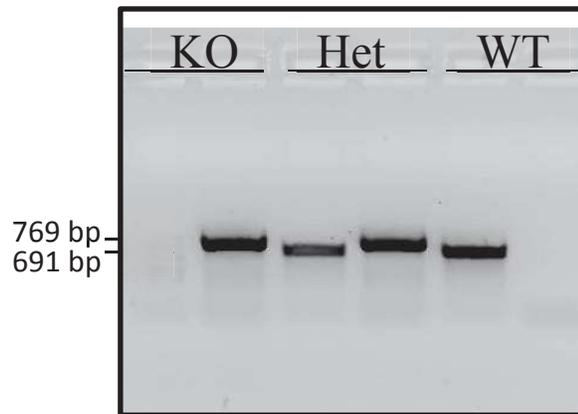
**Table 2.5A – Protocol for P2Y<sub>1</sub> knockout genotyping**

PCR mix	P2Y <sub>1</sub> knockout
	Volume (μl)
5 X KAPA 2 Fast Hot Start Genotyping Mix (Sigma-Aldrich)	6.25
Primer common (5'-TCTTCTACTCTGGCACTGGGACTC-3')	0.625
Primer Wild-Type (5'-ATTTTTTAGACTCACGACTT-3')	0.625
Primer Mutant (5'-GCTTCCTCGTGCTTTACGGTAT-3')	0.625
dH <sub>2</sub> O	2.8
DNA (10 ng / μl)	1

**Table 2.5B – PCR program for P2Y<sub>1</sub> genotyping**

Steps #	Time	WT program	knockout program
1	2min	94°C	94°C
2	20s	94°C	94°C
3	15s	<b>55°C</b>	<b>65°C</b>
4	10s	<b>58°C</b>	<b>68°C</b>
5		2-4 for 10 cycles	2-4 for 10 cycles
6	15s	94°C	94°C
7	15s	<b>55°C</b>	<b>65°C</b>
8	10s	72°C	72°C
9		6-8 for 28 cycles	6-8 for 28 cycles
10	2min	72°C	72°C
11	∞	10°C	10°C

DNA product was run on a 2% agarose gel and the gel was imaged under ultraviolet light on a Fuji Las 3000. The WT band corresponds to 691 base pairs and knockout band to 769 base pairs (**Figure 2.2**).



**Figure 2.2 – Genotype of the P2Y<sub>1</sub> receptor knockout mice.**

Agarose gel showing the bands corresponding to a knock-out (769 bp), heterozygous (691 and 769 bp) and wild-type (691 bp) P2Y<sub>1</sub> mouse.

### 2.2.1.2 Surgery

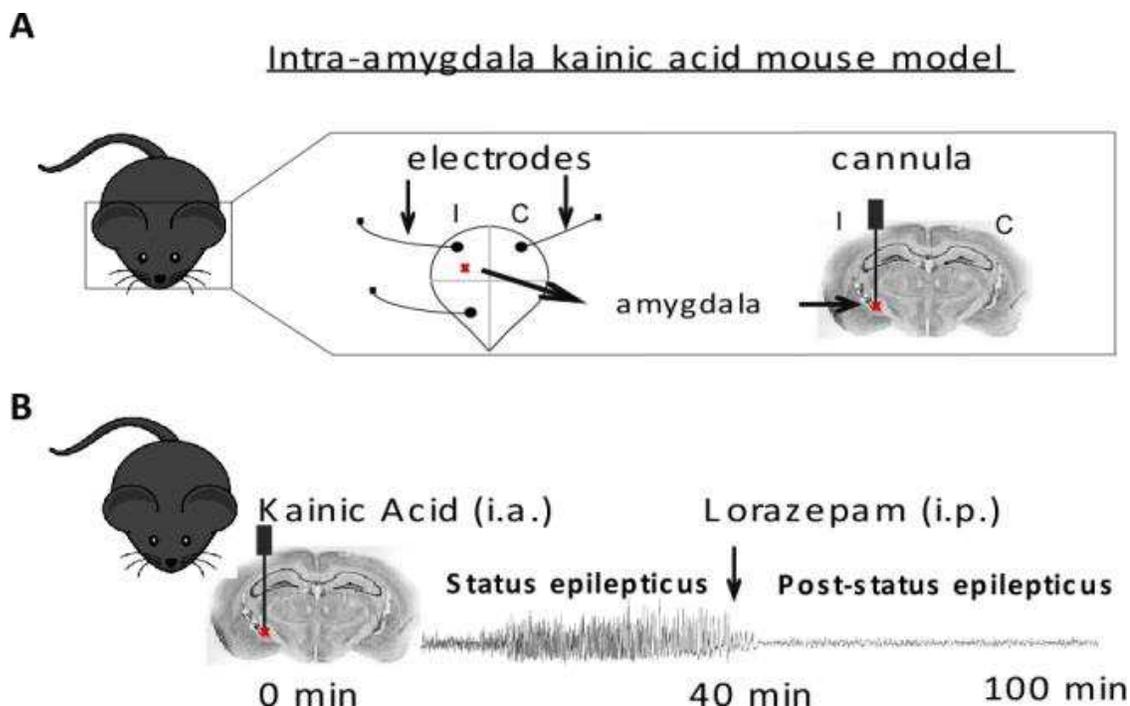
Surgical procedures were performed by Dr. Tobias Engel. Mice were anesthetized using isoflurane (5% induction, 1-2% maintenance) and maintained normothermic by means of a feedback-controlled heat blanket. Anesthetized mice were placed in a stereotaxic frame. A midline scalp incision was performed to expose the skull and then using a drill, three partial craniotomies were prepared: one above the frontal cortex and two above the hippocampi to insert the screw electrodes for EEG recording. A guide cannula to inject the kainic acid was placed following the Bregma coordinates: AP = 0.94 mm, L = 2.85 mm, for the intra-amygdala injection (Engel et al., 2010). Dental cement was used to fix the entire assembly on top of the skull (**Figure 2.3A**). Following surgery, mice were moved to an incubator at 25°C to recover.

### 2.2.1.3 Status epilepticus induction

Status epilepticus was induced by a microinjection of kainic acid (0.3 µg kainic acid in 0.2 µl phosphate-buffered saline (PBS)) into the right baso-lateral amygdala by

an insertion of a 31-gauge internal cannula into the guide cannula. This cannula is inserted 3.5 mm below the cortical surface to target the central and baso-lateral amygdala nucleus. Vehicle-injected control animals received 0.2  $\mu$ l of PBS (PBS, pH=7.4). EEG was recorded for 40 minutes using the Xltek EEG system. After 40 minutes, lorazepam (6mg/kg) was administered intraperitoneal (i.p.) to attenuate seizures and reduce morbidity and mortality. Mice were killed at different time-points (1 hour, 4 hours, 8 hours, 24 hours or 14 days) after status epilepticus or housed in a climate-controlled biomedical facility for 14 days (**Figure 2.3B**). All mice develop epilepsy after a short latency period (3-5days) with 2-5 seizures/day (Mouri et al., 2008).

A second mouse model of status epilepticus was used in this project. Here, status epilepticus was induced by a subcutaneous injection of pilocarpine 30 minutes after the injection of methyl-scopolamine (1 mg/kg). EEG was recorded for the following 90 minutes (Engel et al., 2013) followed by lorazepam (6mg/kg) injection. At the time of death, deeply anesthetized mice were perfused with saline and killed at different time-points after status epilepticus.



**Figure 2.3 – Intra-amygdala kainic acid mouse model.**

(A) Schematic diagram showing the placement of the electrodes on the mouse skull. In each hemisphere, one electrode was placed above the hippocampus. EEG electrodes will allow the recording of seizure activity during the experiment. In the ipsilateral side, the red **x** is the location of the amygdala. (B) Schematic diagram representing a coronal image of the mouse brain, showing as well the amygdala, region where kainic acid is injected to induced status epilepticus. After 40 minutes of status epilepticus, mice are injected with lorazepam (i.p.), a strong anti-convulsive drug, to attenuate status epilepticus and reduce morbidity and mortality.

#### 2.2.1.4 In vivo drug administration

Mice were assigned randomly to receive either vehicle or drug. All P2Y1 receptor drugs were delivered by an intracerebroventricular (i.c.v.) microinjection (2  $\mu$ l) (coordinates from Bregma: AP = - 0.4 mm; L = - 0.95 mm) 15 minutes prior to KA injection or 15 minutes after KA injection and 60 minutes post-KA injection. In long term experiment, mice were also treated 10 days following the induction of status epilepticus, twice daily for 5 days (2  $\mu$ l/ injection). Control mice were injected with 2  $\mu$ l

of vehicle solution (sterile H<sub>2</sub>O). Minocycline was delivered by an intraperitoneal (i.p) injection (200 µl) 4 and 24 hours prior to KA injection.

1 - **P2Y<sub>1</sub>R inhibitor MRS2500**: 1 nmol (chemical name: (1*R*\*,2*S*\*)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt) (Tocris Biosciences). In the vehicle group, animals were injected with 2 µl of vehicle (sterile H<sub>2</sub>O).

2 - **P2Y<sub>1</sub>R agonist MRS2365**: 0.3 nmol and 1 nmol (chemical name: [(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl)diphosphoric acid mono ester trisodium salt (Tocris Bioscience). In the vehicle group, animals were injected with 2 µl of vehicle (sterile H<sub>2</sub>O).

3 - **ADP or UTP**: 9 nmol resulting in approximately 300 µM ADP or UTP in the ventricle (ventricle volume was calculated as 30 µl) (Sigma-Aldrich). In the vehicle group, animals were injected with 2 µl of vehicle (sterile H<sub>2</sub>O).

4 – **Minocycline**: 30 mg/Kg (Sigma-Aldrich). In the vehicle group, animals were injected with 200 µl of vehicle (PBS).

## 2.2.2 Quantification of EEG

### 2.2.2.1 Quantification of EEG during status epilepticus

As mentioned before in section 2.2.1.3, EEG was recorded using an Xltek EEG system. Baseline was recorded before KA injection, followed by 40 minutes of status epilepticus and 1 hour post-status epilepticus. EEG recordings were analysed by uploading EEG into Labchart7 software to calculate amplitude and total seizure power of the EEG signal (Engel et al., 2013). The duration of high-frequency (>5 Hz) and high-

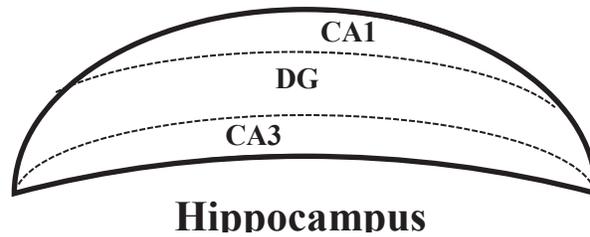
amplitude (>2 times baseline) polyspike discharges of  $\geq 5$  s duration, synonymous with injury-causing electrographic activity (Shinoda et al., 2004), was also counted manually by a reviewer blind to treatment.

#### **2.2.2.2 Quantification of EEG in long term telemetry recording**

Telemetry devices (DSI) were used to record spontaneous seizures during chronic epilepsy. These devices were implanted under the skin between the shoulders in the back of the animals after electrode cranial surgery. Skin then was sewn and wiped with iodine solution to avoid infections. Animals were placed in cages on top of the receivers (RPC-1) and the devices activated. EEG was recorded in freely-moving mice and transferred to a computer via Data Exchange Matrix. During this study, EEG was recorded 24h / day during 14 or 20 days after induction of status epilepticus and spontaneous seizures were confirmed by analysis of the EEG. Dr. Tobias Engel performed the surgeries for telemetry device implantation.

#### **2.2.3 Microdissection of the brain**

Anesthetized animals were euthanized by cervical dislocation at different time points: 1, 4, 8, 24 hours and 14 days after status epilepticus. Then, mice were decapitated using sharp laboratory scissors and the brain removed. Brains were placed into a petri dish on top of a cold board and using a scalpel the cerebellum was removed and brain hemispheres divided in two equal parts. Mid brain and cortical structures were removed and the hippocampi from both hemispheres were collected for analysis. In some cases, hippocampi were dissected in subfields (CA1, CA3 and DG) using tweezers (**Figure 2.4**). Samples were stored at  $-80^{\circ}\text{C}$ .



**Figure 2.4 – Microdissection of hippocampal subfields in mouse.**

Mouse hippocampus was removed from the brain. Using tweezers, hippocampal tissue was dissected into three subfields: CA1, CA3 and DG.

#### **2.2.4 Temporal lobe epilepsy patient brain tissue**

This study was approved by the Ethics (Medical Research) Committee of Beaumont Hospital, Dublin (05/18), and written informed consent was obtained from all patients. TLE patients (n = 6) were referred for surgical resection of the temporal lobe for the treatment of intractable TLE. After temporal lobe resection, hippocampal tissue was obtained and frozen in liquid nitrogen and stored at -80°C until use. A pathologist assessed the hippocampal tissue and confirmed the presence of hippocampal sclerosis and other pathological changes. Control (autopsy) temporal hippocampus tissue (n = 6) was obtained from individuals from the NIH NeuroBioBank Brain at the University of Maryland, Baltimore, MD, U.S.A. Samples were processed for Western blot analysis. Full details of control and patient pathology and clinical data are presented in **Table 2.6**.

##### **2.2.4.1 Autopsy control experiment**

Hippocampi were extracted from adult mice (C57Bl/6) after deep pentobarbital anaesthesia and decapitation and either frozen immediately (“surgical” control) or

frozen 4 or 8 hours after being left at room temperature, simulating a post-mortem interval (Jimenez-Pacheco et al., 2013). Samples were then processed for Western blotting.

**Table 2.6 – Human sample information**

Identifier	Gender	Age, years	Tissue	Cause of death	PMI*, hours
C1	F	24	Hippocampus	Accident; Head injuries	7
C2	F	42	Hippocampus	HASCVD**	4
C3	M	32	Hippocampus	Morphine intoxication	7
C4	M	41	Hippocampus	Mixed drugs intoxication	6
C5	M	29	Hippocampus	Acute pancreatitis; hepatic steatosis	8
C6	F	35	Hippocampus	Multiple injuries	2
TLE1	M	38	Hippocampus	Mesial hippocampal sclerosis moderate	n.a.
TLE2	M	58	Hippocampus	Hippocampal gliosis moderate	n.a.
TLE3	M	53	Hippocampus	Gliosis	n.a.
TLE4	F	37	Hippocampus	Hippocampal sclerosis moderate	n.a.
TLE5	F	34	Hippocampus	Mesial hippocampal sclerosis moderate	n.a.
TLE6	F	38	Hippocampus	Hippocampal gliosis moderate	n.a.

\*PMI- post-mortem interval

\*\*Hypertensive Atherosclerotic Cardiovascular Disease

## **2.2.5 RNA extraction and quantitative PCR**

### **2.2.5.1 RNA extraction**

#### **Tissue homogenisation**

The extraction of mRNA was performed using the Trizol method, as previously described (Jimenez-Mateos et al., 2012, Jimenez-Mateos et al., 2008). 800 µl of trizol was added to the hippocampal tissue following homogenization using a dounce homogeniser. Then, samples were centrifuged at 12000 g for 10 minutes at 4°C and the supernatant transferred to a new tube. The pellet containing membranes and debris was discarded.

#### **Separation phase**

After 5 minutes at RT, 200 µl of chloroform was added to each sample and the tube was vigorously shaken by hand for 15 seconds. Samples were then centrifuged at 13000 rpm for 15 minutes at 4°C.

#### **RNA precipitation**

Lower phase was discarded and 450 µl of isopropanol was added to the upper phase to precipitate the RNA overnight at - 20°C.

#### **RNA purification**

The following day, samples were centrifuged at 13000 rpm for 15 minutes at 4°C. Supernatant was removed and discarded. 1 ml of ethanol (75%) was added to each of the samples and vortexed until the pellet had been dislodged from the tube. Samples were centrifuged at 12000 g for 5 minutes at 4°C. Ethanol was removed and pellets were dried for 20 minutes at room temperature.

### Re-dissolving RNA

Pellet was dissolved in 25  $\mu$ l of DEPC water, followed by incubation at 60°C for 10 minutes. RNA concentration was determined using a nanodrop spectrophotometer and only samples with an absorbance ratio of 260/280 between 1.8–2.2 were considered acceptable.

### 2.2.5.2 Reverse Transcriptase PCR

500  $\mu$ g of total RNA was used to produce complementary DNA (cDNA) by reverse transcription using SuperScript III reverse transcriptase enzyme. 1  $\mu$ l of 10x DNase I reaction buffer and 1  $\mu$ l of DNase I was added to the RNA samples (volume of 8  $\mu$ l) to perform a final volume reaction of 10  $\mu$ l, followed by 15 minutes of incubation at room temperature. Then, 1  $\mu$ l of the EDTA (25 mM) was added to stop the reaction and the samples were incubated at 65 °C for 10 minutes. 1  $\mu$ l of random primers (doxi NTD polyT=dN6 primers) was added to the samples to perform the reverse transcription to cDNA and placed on the thermocycler using the following program (Table 2.7):

**Table 2.7 RT-PCR cycles program**

Step	Target temperature	Incubation time
1	65 °C	5 min
2	4 °C	1 min
3	25 °C	10 min
4	42 °C	50 min
5	72 °C	15 min
6	4 °C	5 min

On step 3, at minute 8, PCR reaction was paused momentarily to add 8  $\mu$ l of Master mix containing 4  $\mu$ l of 5x buffer RT, 2  $\mu$ l of 0.1 M DTT, 1  $\mu$ l of 10  $\mu$ M dNTP's, 0.5

μl of RNase OUT inhibitor enzyme and 0.5 μl of Reverse Transcriptase III. Then, the PCR cycle was continued.

### 2.2.5.3 Quantitative PCR

Quantitative PCR (qPCR) was performed using a LightCycler 1.5 using the Quantitect SYBR Green PCR Kit. Each capillary contained 2 μl of cDNA sample, 10 μl SyBR green Quantitect Reagent, 7 μl of RNase/DNase free water and 1 μl of 10 mM primer pair to a final volume of 20 μl. Capillaries were capped and placed in the LightCycler 1.5 (**Table 2.8**). Data was analysed and normalised to the expression of β-actin. Primer3 was used to design the primers for the selected target genes used in this study (**Table 2.3**).

**Table 2.8 qPCR cycles program**

Cycle	Target temperature	Incubation time	Temperature trans. Rate
<b>Pre- incubation</b>	95°C	15 min	20.00
<b>Amplification (50x)</b>	94°C	15 sec	20.00
	55°C	20 sec	20.00
	72°C	40 sec	20.00
<b>Melting curve</b>	94°C	0	20.00
	65°C	15 sec	20.00
	95°C	0	0.10
<b>Cooling</b>	40°C	30 sec	20.00

## **2.2.6 Protein extraction and Western Blot analysis**

### **2.2.6.1 Protein extraction and quantification**

To perform protein extraction, hippocampal tissue was homogenised in lysis buffer (150 mM NaCl, 50 mM Tris HCl pH8.0, 1 % NP40, 1 mM EDTA pH 8.0) containing phosphatase (Vanadate 1:1000) and protease (Aprotinin 1:500; Leupeptin 1:1000 and PMSF 1:1000) inhibitors. 150 µl or 300 µl of lysis buffer was used to homegenise hippocampal subfields or whole hippocampus, respectively. Protein quantification was performed using the Micro BCA kit, following the instructions of the supplier. Following protein quantification, 1x loading buffer (10 % SDS; 0.5 mM Tris-HCl pH 6.8; 5% β-mercaptoethanol; 0.012 % bromophenol blue and 30 % glycerol) was added to each sample. Samples were boiled at 95 °C for 10 minutes and then stored at - 80°C.

### **2.2.6.2 Synaptoneurosomes preparation for western blot analysis**

Synaptoneurosomes extraction for western blot analysis was performed as previously described (Nagy and Delgado-Escueta, 1984). A pool of 2 hippocampi per group was homogenised on ice in 2 ml of homogeniser buffer (1mM EDTA; 0.32 M sucrose; 1 mg/ml BSA and 5 mM HEPES pH 7.4) containing phosphatase (Vanadate 1:1000) and protease (Aprotinin 1:500; Leupeptin 1:1000 and PMSF 1:1000) inhibitors, followed by centrifugation at 3000 x g for 10 minutes at 4 °C. Supernatant (synaptoneurosomes and cytoplasm) was recovered and centrifuged at 14000 x g for 12 minutes at 4 °C. The supernatant (cytoplasm) was removed and stored in a tube and the pellet (synaptoneurosomes) was re-suspended in 220 µl of Krebs-Ringer buffer (140 mM NaCl; 5 mM KCl; 5 mM glucose; 1 mM EDTA and 10 mM HEPES pH 7.4). 180 µl of Percoll (45% v/v) was added to the tube and was centrifuged at 14000 x g for 2 minutes at 4 °C. Synaptoneurosomes were recovered from the top layer with a sterile Pasteur pipette and re-suspended in 1 ml of Krebs-Ringer buffer. Then, synaptoneurosomes were centrifuged at 14000 x g for 30 seconds at 4 °C and the

pellet re-suspended in the same lysis buffer used for protein extraction containing phosphatase (Vanadate 1:1000) and protease (Aprotinin 1:100; Leupeptin 1:1000 and PMSF 1:500) inhibitors. BCA assay kit was used to quantify the synaptoneurosomes samples. After quantification, 1x loading buffer (10 % SDS; 0.5 mM Tris-HCl pH 6.8; 5%  $\beta$ -mercaptoethanol; 0.012 % bromophenol blue and 30 % glycerol) was added to each sample. Samples were boiled at 95 °C for 10 minutes and stored at - 80°C.

### **2.2.6.3 Western blot analysis**

To study protein levels sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Protein samples were separated by size using polyacrylamide gels. These gels are constituted by two different layers: stacking gel (top layer) and resolving gel (bottom layer). The stacking gel has a low concentration of acrylamide and the pH is 6.8, which is very important to slow down the movement of the protein samples through this gel. In the resolving gel (pH 8.8), glycine is negatively charged and migrates much faster than the proteins; this gel has increased concentration of acrylamide allowing the proteins to separate by molecular weight.

The resolving gel is composed of dH<sub>2</sub>O; 1 M Tris-HCl pH 8.8; 10 % SDS; 10 % ammonium persulfate (APS); 40 % bis-acrylamide and tetramethylethylenediamine (TEMED). The percentage of the gel was chosen according with the size of the protein: 15% gel is used to study 10-30 KDa proteins, 12% gel for 30-65 KDa proteins, 10% gel for 66-100 KDa proteins and 8% gel for 100-130 KDa proteins.

### **Electrophoresis**

Gels are placed in the mini-protean tetra electrophoresis system container filled with 1x electrophoresis buffer (250 mM glycine; 25 mM Tris-base; 10% SDS; pH 8.3). Protein samples were boiled at 95°C for 5 minutes and 30  $\mu$ g of protein was loaded into each well. The protein size marker (PageRuler™ Prestained Protein Ladder

Plus) was also loaded into one well of the gel. 120 volts were applied to the system to perform electrophoresis.

### **Protein transfer**

Proteins were transferred from the gel to a nitrocellulose membrane using a semi-dry transfer system. Whatman paper and nitrocellulose membranes were soaked in the semi-dry transfer buffer (25 mM Tris base; 192 mM glycine; 20% methanol). Then, a layer of two Whatman paper was placed in the system, followed by the nitrocellulose membrane, gel with proteins and two more Whatman paper on top. The transfer was performed for 90 minutes at 18 V. 1x Ponceau was used to confirm the transference of the proteins to the membrane. Membranes were washed in Tris-buffer saline solution with Tween 20 (1 mM Tris-base; 15 mM NaCl; 0.05 % Tween 20; pH 7.5) (TBS-T) and blocked in 5%-milk-TBS-T solution for 30 minutes at room temperature.

### **Immunoblotting**

Membranes were incubated in 5% milk-TBS-T with the primary antibody overnight at 4°C. On the second day, membranes were washed 3 times with TBS-T 5 minutes each and then incubated in 5% milk-TBS-T with the secondary antibody (dilution 1:1000) for 2 hours at room temperature. The secondary antibodies were Horse-radish peroxidase-conjugated goat anti-rabbit or anti-mouse. Membranes were washed for 30 minutes in TBS-T, incubated for 1 minute with Imombilon western HRP substrate. Protein bands were visualized using Fujifilm LAS-4000 system with chemiluminescence. The primary antibodies used in this study are listed in **Table 2.4**.

### **Quantification of the protein signal**

AlphaEaseFC4.0 software was used to quantify proteins. Spot dense option was used to evaluate the optical density of each protein band. The background corresponding to each band was also evaluated and removed from the protein band. Protein quantity was normalised to the loading control ( $\beta$ -actin or  $\alpha$ -tubulin). Then, all

control samples were set to 1 and the rest of the samples were normalized to this value. N-fold of the proteins was presented in graphics designed by GraphPad Prism 5 program.

## **2.2.7 Histological study**

### **2.2.7.1 Fresh frozen tissue**

#### **2.2.7.1.1 Brain extraction and tissue preparation**

Mice were sacrificed and a transcardial perfusion with sterile PBS was carried out. Brains were removed and frozen in 2-methylbutane for 5 minutes in dry ice. CM1900 cryostat (Leica) was used to slice the brain and coronal cryosections of 12  $\mu\text{m}$  thickness were obtained from the hippocampus.

#### **2.2.7.1.2 Fluoro-Jade B staining (FjB)**

FjB staining was used to analyze neuronal death. The tissue sections were selected and kept at room temperature for 5 minutes. Then, tissue samples were fixed in formalin for 30 minutes, followed by immersion in 100% ethanol for 3 minutes, 70% ethanol for 1 minute and washed in  $\text{dH}_2\text{O}$  for 1 minute. Sections were transferred to a solution of 0.06% permanganate potassium ( $\text{KMnO}_4$ ) and incubated for 15 minutes, followed by 2 washes with  $\text{dH}_2\text{O}$  for 1 minute each. After this step, sections were kept in darkness and incubated with 0.001% FjB solution for 30 minutes with shaking and then washed 9 times for 1 minute each. Sections were left at 37° C for 30 minutes until dry completely and then immersed in histoclear solution 3 times for 2 minutes each. Sections were removed from histoclear solution and mounted with DPX mounting medium. Slices were analyzed using a Nikon 2000s epifluorescence microscope. Cell counts were the average of two adjacent sections with a 40x lens by an observer blind to treatment.

### **2.2.7.1.3 Immunofluorescence staining**

Samples were immersed in 4% formalin for 30 minutes, followed by incubation with 3% Triton-X-100/PBS for 20 minutes. Sections were rinsed in 0.1% Triton-X-100/PBS 3 times for 5 minutes and incubated in 5% goat serum blocking solution for 1 hour, followed by incubation with primary antibody (GFAP, 1:400; NeuN, 1:400; Ibal, 1:400 (**Table 2.4**) overnight at 4°C. The following day, slides were washed 4 times with 0.1% Triton-X-100/PBS for 5 minutes and then incubated with AlexaFluor 488 or 568 fluorescent secondary antibodies (1:400) at room temperature in darkness for 2 hours. Sections were washed 6 times with 0.1% Triton-X-100/PBS for 5 minutes and incubated with DAPI (1:500) solution for 10 minutes and mounted with Fluorosave reagent. An epifluorescent microscope, Nikon 2000s, was used to capture images. Cell counts were the average of two adjacent sections with a 40x lens by an observer blind to treatment.

### **2.2.7.2 Free floating tissue**

#### **2.2.8.2.1 Brain extraction and tissue preparation**

Animals were sacrificed and perfused with 10 ml of sterile PBS followed by 15 ml of 4% paraformaldehyde (PFA). Brains were removed and kept in 4% PFA for 24 hours at 4°C. Then, brains were transferred to a solution of PBS and immersed into a 4% agarose solution. Once solidified, sagittal sections of 30 µm thickness were taken from the hippocampus using the VT1000S vibratome. Tissue was placed in cryoprotective solution (30% ethylene glycol, 30% glycol in PBS, 2x phosphate borate and deionized water in a ratio of 3:3:3:1) and stored at -20°C.

#### **2.2.7.2.2 Immunofluorescence staining**

Slices were washed in PBS to remove the cryoprotective solution for 5 minutes, followed by treatment with 0.1% Triton X-100 in PBS for 15 minutes. Slices were

incubated in 1 M glycine for 30 minutes to remove the free aldehyde groups prevented from PFA solution and then washed in PBS 3 times for 5 minutes. 1% BSA-PBS was used as a blocking solution for 1 hour, followed by incubation with primary antibody (in 1% BSA-PBS) overnight at 4°C (**Table 2.4**). On the second day, slices were washed 2 times for 5 minutes each with PBS and incubated with secondary antibody raised in goat conjugated with Alexa Fluor 488 and Alexa Fluor 568 at room temperature for 2 hours. Sections were stained with DAPI (1:500) and mounted into glass slides with FluoroSave reagent. Sections were examined using the epifluorescence microscope Nikon 2000s or confocal microscopy.

## **2.2.8 Primary cell culture**

### **2.2.8.1 Preparation of 6-well and 24-well plates for primary cell culture**

24-well plates were coated with Poly-L-lysine and laminin to help the cell adhesion. Coverslips, previously sterilised in 100% ethanol, were placed in the 24-well plates followed by addition of 300 µl of Poly-L-Lysine (1.5 µg/ml) and plates were incubated overnight at 37°C. Then, wells were rinsed 3 times with sterile H<sub>2</sub>O. 300 µl of laminin (2.5 µg/ml) was then added to each well. 24-well plates were incubated at 37°C for 3-4 hours, and then, laminin was removed and wells were rinsed 3 times with neurobasal medium before plating hippocampal neurons.

### **2.2.8.2 Primary Hippocampal cell preparation**

Primary cultures of hippocampal neurons were prepared from E18 embryonic mice as described (Jimenez-Mateos et al., 2012). Pregnant females were euthanized by cervical dislocation and the uterus removed by hysterectomy. Then, the amniotic sacs were placed in Hanks Balanced Salt Solution (HBSS). Embryos were removed from the amniotic sacs and sacrificed. For dissection, the head was removed and placed in a dish containing HBSS. The skin and the skull were removed carefully to avoid damage

to the brain. The cerebellum was discarded and the brain was split in half along the midline. Using curved scissors, the meninges were peeled off and the hippocampus removed. Then, hippocampi were placed in a falcon containing neurobasal medium and homogenised by briefly pipetting up and down. For the complete break-down of the tissue, trypsin-EDTA was added to the falcon containing the hippocampi and incubated in a water bath for 15 minutes at 37°C. Neurobasal medium was added to the falcon containing the hippocampi and pipetted up and down to completely homogenise the tissue followed by centrifugation for 5 minutes at 1500 rpm at room temperature. Supernatant was discarded and the pellet resuspended in 1 ml of neurobasal medium + 10% horse serum (containing 10% Horse Serum, 2 mM Glutamine, 2 mM pyruvate and 100 U/ml penicillin/ streptomycin in Neurobasal medium). Then, cells (200 µl of the solution) were added to each well (plate prepared previously as explained in section 2.2.9.1 section) and incubated for 3 hours at 37°C to allow the cells to adhere to the coverslip lining the bottom of the well. After 3 hours, neurobasal medium + 10% horse serum was removed completely and replaced by N2/B27/Neurobasal medium (N2; B27; 2 mM Glutamine; 2 mM pyruvate and 100 U/ml penicillin/streptomycin in Neurobasal medium). Cells were incubated at 37°C in a humidified atmosphere with 5% (v/v) CO<sub>2</sub> for 7–10 days (every 2 days 50% of the N2/B27/Neurobasal medium was replaced by a fresh medium).

### **2.2.8.3 Transfection of hippocampal neurons by calcium precipitation**

1 µg of plasmid DNA (P2Y<sub>1</sub> or GFP) was added to a sterile tube (reaction A). In a second tube, a second reaction, B, containing 2 µl of CaCl<sub>2</sub> 2.5M and H<sub>2</sub>O was prepared producing a total volume (A and B) of 20 µl. Reaction B was added to the reaction A and mixed thoroughly by pipetting. 20 µl of BBS 2x was then added to the final reaction and mixed creating bubbles.

Culture medium was removed from the wells and stored to be used later. 260 µl of transfection buffer was added to the cells, followed by addition of 40 µl of

DNA/BBS mixture. Plate was incubated at 37°C without CO<sub>2</sub> for 45 minutes. Then, each well containing cells was washed 3 times with experimental buffer (1.2 mM CaCl<sub>2</sub>; 7.2 mM glucose; 1.2 mM MgCl<sub>2</sub>). The culture medium removed before was added to the wells (500 µl / well) and the plate incubated at 37°C for 24 hours.

#### **2.2.8.4 Cell death assay in transfected hippocampal neurons**

Two plates with hippocampal neurons transfected with GFP or GFP-P2Y<sub>1</sub> were treated with 0.3 µM of kainic acid and incubated for 5 hours at 37°C. A third plate containing hippocampal neurons transfected with GFP-P2Y<sub>1</sub> was treated with 10 µM of P2Y<sub>1</sub> antagonist MRS2500 30 minutes prior to kainic acid treatment. This plate was also incubated for 5 hours at 37°C like the other two plates. After 5 hours of incubation, cells were incubated with propidium iodide (PI) marker (1/1000) and DAPI (1/1000) for 20 minutes. Then, cells were imaged with an Eclipse TE 300 inverted microscope (Nikon) with 10x, 0.43 numerical aperture (NA) phase-contrast objective. The number of PI-positive cells was expressed as a percentage of total cells in the field. Resultant images were processed using ImageJ.

#### **2.2.8.5 Microglia cell culture**

Microglia cells were obtained from pups at postnatal day 5 (P5). P5 pups were decapitated and the skin and the skull were removed carefully to avoid damage to the brain. The cerebellum was discarded and the cortex and hippocampus were transferred to 10% FBS/DMEM media (DMEM containing 10% FBS (v/v), 100 U/ml penicillin and 100 mg/ml streptomycin). A total of 8 brains (16 hemispheres) were pooled together and cut into small pieces, followed by incubation in 5 ml of trypsin-EDTA in a water bath at 37°C for 15 minutes. Then, trypsin was neutralized by adding 20 ml of DMEM, containing 10% FBS. Then, tissue was centrifuged at 1500 rpm/min for 3 minutes. The supernatant media was removed and 10 ml of fresh 10%

FBS/DMEM media was added. Using a 1 ml pipette, the tissue was triturated until all the tissue was dissociated. The cells were then counted using a haemocytometer. Cells were plated at 100.000 cell/cm<sup>2</sup> into different T75 flasks (75 cm<sup>2</sup>). The flasks containing the cells were incubated at 37°C with 5% CO<sub>2</sub> and every 2-3 days the medium was removed and fresh medium was added. After 12-14 days, astrocytes were confluent and overlaying microglia. The isolation of microglia was performed by orbital shaking, placing the T75 flasks on a plate shaker at 250 rpm for 2 hours. The media containing the floating microglia was centrifuged at 200 g for 5 minutes, and the pellet re-suspended in DMEM + 10% FBS medium. Cells were counted and plated in a density of 10.000 cell/cm<sup>2</sup> in a 6-well plate.

#### **2.2.8.6 Treatment of microglia cells with P2Y<sub>1</sub> antagonist**

Microglia cells were treated with 100 µM of glutamate or 100 µM of glutamate / 10 µM of P2Y<sub>1</sub> antagonist MRS2500 for 2 hours. After 2 hours, cells were fixed with 4% PFA and immunohistochemistry was carried out. Cells were incubated with primary antibody against P2Y<sub>1</sub> receptor and Iba1. The number of microglia positive cells (Iba-1) was counted in both treatments. The activation stage of the microglia cells was also analysed in both treatments and the ratio of resting/reactive was established. ImageJ was used to count the number of microglia cells.

#### **2.2.9 Data analysis**

Statistical analysis of data was performed using Graph Pad Prism software and STATVIEW software. Data was presented as means ± standard error of the mean (SEM). Three or more group data were analysed with ANOVA parametric statistics with post hoc Fisher's protected least significant difference test. For two-group comparison, Student's t-test (STATVIEW software) was used to determine statistical differences between groups. Significance was accepted at \* p < 0.05, \*\* p < 0.01 \*\*\* p < 0.001.

**Table 2.1 – List of reagents**

<b>Animal work</b>		
<b>Reagents name</b>	<b>Supplier</b>	<b>Cat number / Reference number</b>
Dental cement	J.B. Tough Dental Ttd	-
Kainic acid (KA)	Sigma-Aldrich	K0250
Phosphate buffered saline (PBS)	Sigma-Aldrich	SLBV2666
Lorazepam (Lz)	Pfizer (Ativam)	-
<b>MRS2500</b>		
(P2Y1R antagonist)	Tocris Bioscience	2159
<b>MRS2365</b>		
(P2Y1R agonist)	Tocris Bioscience	2157
ADP	Sigma-Aldrich	A7699
UTP	Sigma-Aldrich	U6875
Isoflurane	Isoflurin	-
2-methylbutane	Sigma-Aldrich	M32631
<b>Genotyping</b>		
Tris-HCl	Sigma-Aldrich	A258148
Proteinase K	Bioscience	AM2542
NaCl	Sigma-Aldrich	7647-14-5
Isopropanol	Sigma-Aldrich	19-076-4
Taq DNA polimerase	Invitrogen	10342-020
<b>Kappa 2 Fast Hot Start genotyping mix</b>		
	Kappa Biosystems	KK5608
<b>RNA extraction</b>		
TRIzol reagent	Ambican	15596018
Chloroform	Sigma-Aldrich	372978
<b>Superscript III reverse transcriptase enzyme</b>		
	Invitrogen	18080044
DNaseI	Invitrogen	18068015
EDTA (pcr)	Invitrogen	P/N Y02353
Random hexamer primers	Thermo scientific	S0142
5xRT buffer	Invitrogen	P/N Y02321
dNTP's	Qiagen	201912

RNA extraction		
<b>RNase OUT</b>	Invitrogen	10777019
<b>Quantitech SYBR Green</b>	Qiagen	204143
<b>DTT</b>	Invitrogen	P/N Y00147
Western blot		
<b>Micro BCA Protein Assay kit</b>	Thermo Scientific	23235
<b>Bovine serum albumin (BSA)</b>	Fisher Scientific	BP9700-100
<b>Aprotinin</b>	Sigma-Aldrich	A1153
<b>Leupeptin</b>	Sigma-Aldrich	L9783
<b>Vanadate</b>	Sigma-Aldrich	S6508
<b>PMSF</b>	Sigma-Aldrich	P7626
<b>Percoll</b>	Sigma-Aldrich	E0414
<b>30 % Acrylamide/Bisacrylamide</b>	Sigma Aldrich	A7168
<b>Tetramethylethylenediamine (TEMED)</b>	Sigma Aldrich	T9281
<b>Tris (Trizma base)</b>	Sigma Aldrich	T1503
<b>Glycine</b>	Sigma Aldrich	G8898
<b>Sodium dodecyl sulphate (SDS)</b>	Sigma Aldrich	L3771
<b><math>\beta</math>-mercaptoethanol</b>	Sigma Aldrich	M6250
<b>Bromophenol blue</b>	Sigma Aldrich	B8026
<b>Glycerol</b>	BDH Chemicals	444482V
<b>Ammonium persulfate</b>	Sigma Aldrich	215589
<b>Methanol</b>	Sigma Aldrich	32213
<b>Sodium chloride</b>	Fischer Scientific	7647-14-5
<b>Tween-20</b>	Sigma Aldrich	274348
<b>Milk powder</b>	Marvel	-
<b>Hydrochloric Acid</b>	Sigma Aldrich	7102
<b>Immobilon western HRP substrate</b>	Millipore	WBKLS0500
<b>Page ruler<sup>TM</sup> Prestained protein ladder</b>	Thermo scientific	26619
<b>Ponceau S solution</b>	Sigma-Aldrich	P7170
<b>Horse-radish peroxidase-conjugated goat anti-rabbit</b>	Millipore	AP132P

<b>Western blot</b>		
<b>Horse-radish peroxidase-conjugated goat anti-mouse</b>	Millipore	AP124P
<b>Immunohistochemistry</b>		
<b>Fluoro-jade B</b>	Millipore	AG310
<b>Permanganate potassium</b>	Fluka	60459
<b>Histoclear solution</b>	National diagnostics	HS-202
<b>DPX mounting medium for histology</b>	Sigma-Aldrich	06522
<b>Triton X</b>	Sigma-Aldrich	SLBS6421
<b>Alexa Fluor 488 mouse</b>	Invitrogen	A11029
<b>Alexa Fluor 488 rabbit</b>	Invitrogen	A1008
<b>Alexa Fluor 568 mouse</b>	Invitrogen	A11031
<b>Alexa Fluor 568 rabbit</b>	Invitrogen	A11011
<b>Fluorosave</b>	Millipore	345789
<b>Paraformaldehyde solution 4% in PBS</b>	Thermo Fisher Scientific	AAJ19943K2
<b>Hoechst Stain solution (Dapi)</b>	Sigma-Aldrich	H6024
<b>3.3'-Diamino-benzidin tablets (DAB)</b>	Sigma-Aldrich	D4293-50 set
<b>Vectastain kit (rabbit)</b>	VectorLabs	PK6101
<b>Goat serum</b>	Sigma-Aldrich	G9023
<b>Cell culture</b>		
<b>Hanks Balanced Salt Solution (HBSS)</b>	Sigma-Aldrich	H8264
<b>Trypsin-EDTA</b>	Sigma-Aldrich	T4049
<b>Neurobasal medium</b>	Bioscience	21103049
<b>B27</b>	Bioscience	17504044
<b>N2</b>	Bioscience	A1370701
<b>P2ry1 plasmid</b>	Origene technologies	MG226766
<b>Propidium Iodide (PI)</b>	Sigma-Aldrich	113486
<b>Dulbecco's Modified Eagle's Medium (DMEM) low glucose</b>	Sigma-Aldrich	D5546

**Cell culture**

**Fetal bovine serum (FBS)**

Sigma-Aldrich

035M3285

**Table 2.2 – List of equipment and general material**

<b>Animal work</b>		
<b>Reagents name</b>	<b>Supplier</b>	<b>Cat number / Reference number</b>
<b>Screw electrode 1.6mm</b>	Bilaney Consultants Ltd	E363/20/1.6
<b>Stainless steel tubing 26 gauge cut to 7.65mm</b>	Bilaney Consultants Ltd	SST-26/SPC
<b>Internal cannulas cut to 9mm (i.c.v.)</b>	Bilaney Consultants Ltd	C315I/10-19
<b>Internal cannula cut to 10.75mm (i.a)</b>	Bilaney Consultants Ltd	C315I/SPC
<b>Telemetry devices</b>	Data Science International	-
<b>Heat blanket</b>	Harved Apparatus Ltd	-
<b>Stereotaxic frame</b>	Kopf instruments	-
<b>Xltek EEG system digital</b>	Medivent Ltd	-
<b>Labchart7 software</b>	AD instruments, Ltd	-
<b>DNA / RNA work</b>		
<b>Nanodrop spectrophotometer</b>	Thermo Scientific	-
<b>LightCycler 1.5</b>	Roche Diagnosis	-
<b>Capillaries</b>	Roche Diagnosis	4929292001
<b>Western blot</b>		
<b>Tecan plate reader</b>	Tecan	-
<b>Mini-Protean tetra cell</b>	BioRad	1658004
<b>Nitrocellulose membranes</b>	GE Health care	A10243838
<b>Gel blotting paper (Whatman paper)</b>	Fisher Scientific	10426994
<b>Semi-dry transfer</b>		-
<b>Immunohistochemistry</b>		
<b>CM1900 cryostat</b>	Leica	-
<b>Glass insert</b>	Leica microsystems	14047742497
<b>Nikon 2000s epifluorescence microscope</b>	Micron Optical	-
<b>Vibratome VT1000S</b>	Leica	-
<b>LSM Confocal microscopy</b>	Leica	-

**Table 2.3 - Primer sequences used in qPCR experiments**

<b>Primer</b>	<b>Forward sequence</b>	<b>Reverse sequence</b>	<b>Supplier</b>
<i>P2ry1</i>	GTAGGTAGTACGCCAGGGTC	AAGTAGTTCGGCTGTTCCCA	Sigma Aldrich
<i>P2ry2</i>	GGCCTTGAACCTCTTACCCT	ACTGTCATGCATTCCAGGGA	Sigma Aldrich
<i>P2ry4</i>	AGCTGTCTTGATCCAGTGCT	GCAGTTAGTCCCCTTCCTGT	Sigma Aldrich
<i>P2ry6</i>	AAGCCTGGTACTCTTGGGAC	CACCTTGGGAAGCCATG	Sigma Aldrich
<i>P2ry12</i>	ATCACAGAGGGCTTTGGGAA	ATGGGAAGAGAACCTGGGTG	Sigma Aldrich
<i>P2ry13</i>	CAGCTCCGAGGATTTGTGTG	AGAACATCAGGGACCAGACG	Sigma Aldrich
<i>P2ry14</i>	AACAACCTCCACCACACAGA	TCTTGGAGCTGGGCACATAA	Sigma Aldrich
<i><math>\beta</math>-actin</i>	GGTTGGCCTTAGGGTTCAGG	GGGTGTGATGGTGGGAATGG	Sigma Aldrich
<i>GFAP</i>	AGAAAACCGCATCACCATTCC	TCACATCACCACGTCCTTGT	Sigma Aldrich
<i>cFos</i>	GGAATTAACCTGGTGCTGGA	CATTCAGACCACCTCGACAA	Sigma Aldrich
<i>Neo</i>	GCAGTGTTTGGGGTCAGAAT	GGGGAACCTCCTGACTAGGG	Sigma Aldrich
<i>Iba1</i>	TGGAGGGGATCAACAAGCAA	ACCCAAGTTTCTCCAGCAT	Sigma Aldrich

**Table 2.4 - Antibodies used in western blot and Immunostaining**

<b>Antibody name</b>	<b>Classification</b>	<b>Supplier</b>	<b>WB dilution</b>	<b>Immunostaining dilution</b>
<b>P2Y<sub>1</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>1</sub>R</b>	Mouse	Santa Cruz	-	1:100
<b>P2Y<sub>2</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>4</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>6</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>11</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>12</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>13</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>P2Y<sub>14</sub>R</b>	Rabbit	Alomone labs	1:200	-
<b>β-Tubulin</b>	Mouse monoclonal	Santa Cruz	1:1000	-
<b>β-Actin</b>	Mouse monoclonal	Sigma	1:1000	-
<b>c-Fos</b>	Mouse	Santa Cruz	1:400	-
<b>GFAP</b>	Mouse	Cell signalling	1:1000	1:400
<b>NeuN</b>	Rabbit	Merck Millipore	-	1:400
<b>NeuN</b>	Mouse	Merck Millipore	-	1:400
<b>Iba I</b>	Rabbit	Wako	-	1:400
<b>GAPDH</b>	Rabbit	Cell signalling	1:1000	-
<b>P2X7R</b>	Rabbit	Alomone labs	1:200	-
<b>GluR6/7</b>	Mouse	Merck Millipore	1:1000	-
<b>GABA<sub>A</sub></b>	Mouse	Merck Millipore	1:1000	-

WB- western blot; Immunostaining- Immunofluorescence and DAB

**Chapter 3 - Expression and function of the metabotropic purinergic P2Y receptor family in experimental seizure models and drug-refractory epilepsy patients**

### 3.1 Introduction

An increasing recognition of the importance of neuroinflammation in epileptogenesis has spurred interest in targeting inflammatory pathways for developing AEDs with a non-classical mechanism of action (Vezzani et al., 2011). A contribution of P2Y receptors to seizure pathology has recently been suggested (Engel et al., 2016). Early studies showed changes in the induction of a subset of P2Y receptors after kainic acid-induced status epilepticus (Avignone et al., 2008). More recently, the P2Y<sub>1</sub> receptor has been shown to be activated in neuronal progenitor cells after pilocarpine-induced status epilepticus (Rozmer et al., 2016). Furthermore, P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>4</sub> protein levels are up-regulated in astrocytes in brain tissue from patients suffering from cortical dysplasia (Sukigara et al., 2014). P2Y<sub>12</sub> appears to promote the formation of microglial extension after seizures and P2Y<sub>12</sub> deficiency results in an aggravated seizure phenotype during status epilepticus (Eyo et al., 2014). To date, however, no systematic attempt has been undertaken to determine the expression profile of the complete P2Y receptor family after status epilepticus and chronic experimental and human epilepsy. Here we used two different mouse models of status epilepticus and brain tissue from TLE patients to examine the expression responses of the P2Y receptor family. We also investigated the *in vivo* effects of P2Y receptor agonists ADP and UTP on acute seizures and hippocampal pathology.

### 3.2 Chapter objectives

**Hypothesis:** P2Y receptors are altered in the hippocampus after status epilepticus and during epilepsy in experimental models and in brain tissue from TLE patients. Broad-spectrum agonists of P2Y receptors, ADP and UTP, have an effect on seizure severity and on seizure-induced pathology.

**Objectives:**

1. Characterize the induction and expressional changes of P2Y receptors in the brain after status epilepticus and in chronic epilepsy;
2. Characterize the expressional changes of P2Y receptors in the brain of TLE patients;
3. Evaluate the functional role of P2Y receptors in epilepsy using the broad-spectrum agonists ADP and UTP.

### 3.3 Results

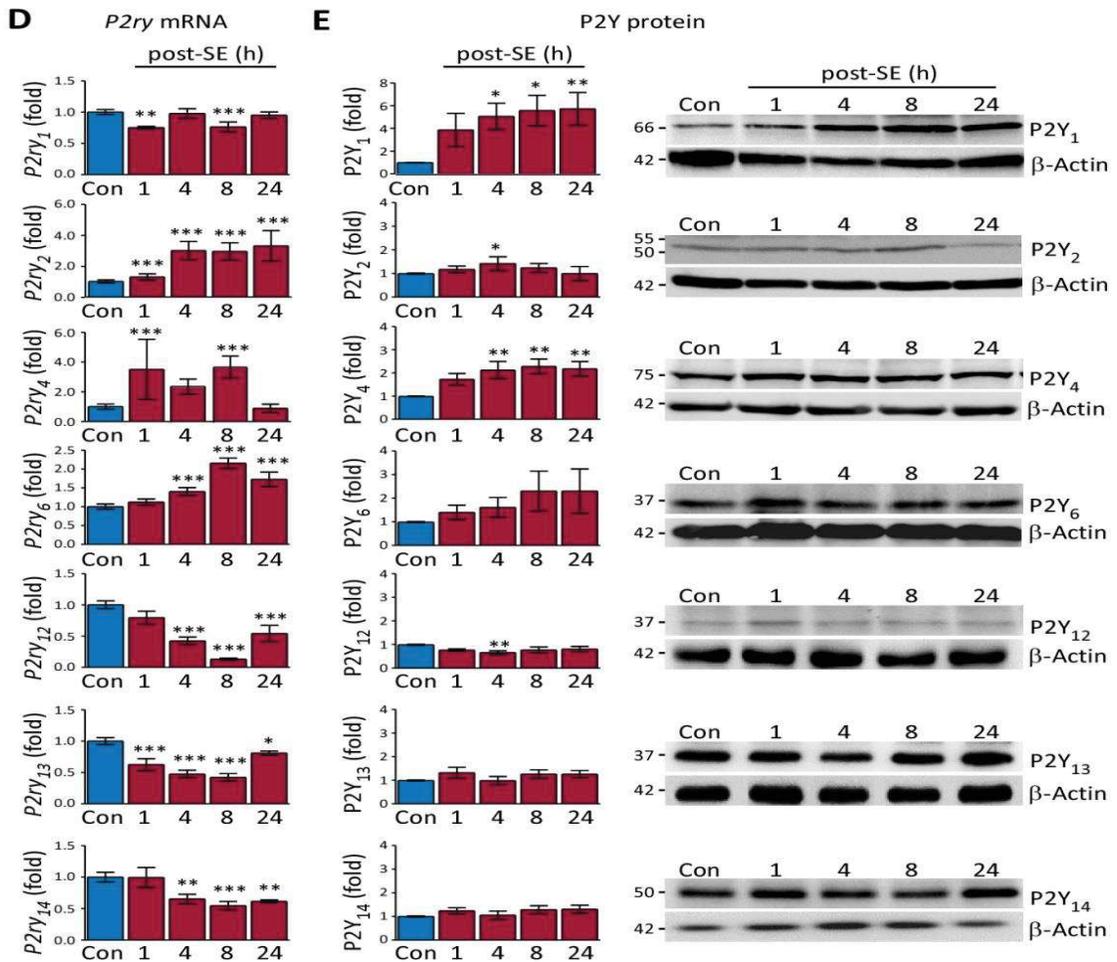
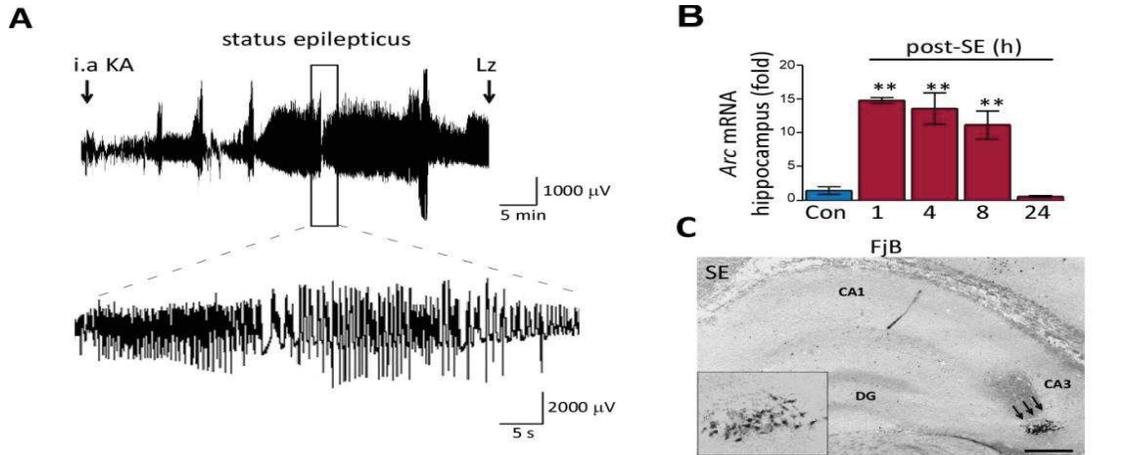
#### 3.3.1 Status epilepticus evoked by intra-amygdala kainic acid leads to select changes to P2Y receptor expression

To determine the induction and expression profile of the P2Y receptor family after status epilepticus, we used the well characterized mouse model of intra-amygdala kainic acid-induced status epilepticus (Mouri et al., 2008) (**Figure 3.1A**). Increased hippocampal transcript levels of the activity-regulated gene *Arc* confirmed recruitment of the ipsilateral hippocampus (**Figure 3.1B**). In this model, kainic acid-induced neurodegeneration is most prominent in the ipsilateral CA3 subfield. Diffuse cell death is, however, also evident within the ipsilateral dentate gyrus (DG) and CA1 region (**Figure 3.1C**) (Mouri et al., 2008).

P2Y receptor expression was analysed in the ipsilateral hippocampus at different time-points following intra-amygdala kainic acid (**Figure 3.1D, E**). Transcript levels of *P2ry<sub>2</sub>*, *P2ry<sub>4</sub>* and *P2ry<sub>6</sub>* were all increased following status epilepticus whereas mRNA levels were lower after status epilepticus for *P2ry<sub>1</sub>*, *P2ry<sub>12</sub>*, *P2ry<sub>13</sub>* and *P2ry<sub>14</sub>* (**Figure 3.1D, F**). Interestingly, transcript down-regulation was the predominant response among the P2Y receptors known to be sensitive to ATP/ADP (P2Y<sub>1</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub>), whereas transcript up-regulation was the predominant response among P2Y receptors sensitive to UTP/UDP (P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>) with the exception of the P2Y<sub>14</sub> receptor which is activated by UDP-glucose (**Figure 3.1D, F**) (von Kugelgen, 2006). *P2ry<sub>11</sub>* transcript and expression levels were not analysed due to the lack of a *P2ry<sub>11</sub>* gene orthologue in the murine genome (Dreisig and Kornum, 2016).

Western blotting revealed a strong increase of P2Y<sub>1</sub> and P2Y<sub>4</sub> protein levels following status epilepticus. A modest increase in protein levels could be observed for P2Y<sub>2</sub> and a non-significant increase for P2Y<sub>6</sub> (**Figure 3.1E, F**). In contrast, P2Y<sub>12</sub> receptor expression decreased and no changes in expression could be observed for P2Y<sub>13</sub> and P2Y<sub>14</sub> (**Figure 3.1E, F**). Therefore, P2Y receptor protein changes track closely with changes in P2Y receptor transcript levels following status epilepticus, with the only

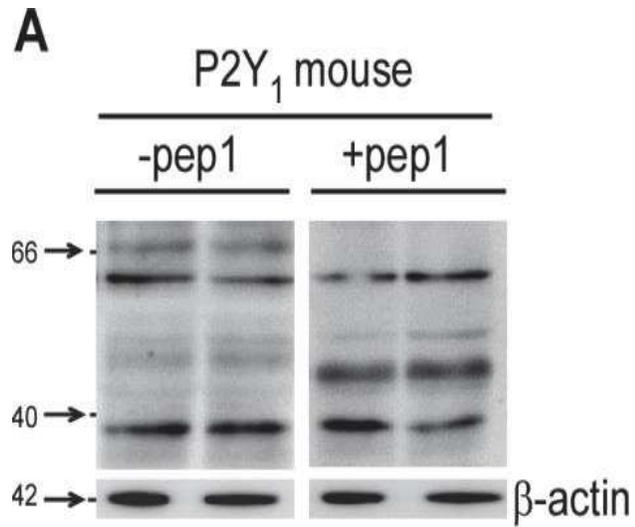
exception being P2Y<sub>1</sub>. Antibody specificity for P2Y<sub>1</sub> was confirmed by pre-absorption tests (**Figure 3.2A**). These results further suggest that status epilepticus leads to select changes to the expression of the P2Y receptor family, with P2Y receptors coupled to Gq being increased (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub>) and P2Y receptors coupled to Gi being down-regulated or not changed (P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>) (**Figure 3.1F**).



Receptor	Agonist	Induction	G-coupling	Expression
P2Y <sub>1</sub>	ADP>ATP	decreased	Gq	increased
P2Y <sub>2</sub>	UTP/ATP	increased	Gq	increased
P2Y <sub>4</sub>	UTP>ATP	increased	Gq	increased
P2Y <sub>6</sub>	UDP>UTP>>ADP	increased	Gq	increased
P2Y <sub>12</sub>	ADP>ATP	decreased	Gi	decreased
P2Y <sub>13</sub>	ADP>>ATP	decreased	Gi	not changed
P2Y <sub>14</sub>	UDP-Glucose	decreased	Gi	not changed

**Figure 3.1 – P2Y receptor induction and expression in the hippocampus following intra-amygdala kainic acid-induced status epilepticus.**

**(A)** Representative EEG traces showing high amplitude and high frequency spiking following intra-amygdala kainic acid injection. Status epilepticus develops shortly after intra-amygdala kainic acid injection and mice are treated with intraperitoneal lorazepam (Lz) 40 minutes later. **(B)** Increased *Arc* mRNA transcription in the ipsilateral hippocampus after intra-amygdala kainic acid-induced status epilepticus (n = 8 (control (Con)) and 4 (post-status epilepticus)). **(C)** Representative image (5 x lens) of FjB staining showing seizure-induced cell death mainly localized to the ipsilateral CA3 subfield of the hippocampus 24 hours following intra-amygdala kainic acid injection (arrows and insert). Note, image invert reveals these as black-stained cells. The ipsilateral CA1 and DG hippocampal subfields are mainly spared from damage. CA, cornu ammonis; DG, dentate gyrus; Scale bar = 500  $\mu$ m. **(D)** Graphs showing changes in *P2ry* mRNA levels post-status epilepticus in the ipsilateral hippocampus (n = 12-14 (control (Con)) and 6-8 (post-status epilepticus)). **(E)** Graphs and representative Western blots (n=1 per lane) showing expression changes of P2Y receptor family after intra-amygdala KA-induced status epilepticus in the ipsilateral hippocampus (n = 8 (control (Con)) and 4 (post-status epilepticus)).  $\beta$ -actin is shown as guide to loading. **(F)** Table summarizing P2Y receptor induction and expression following intra-amygdala status epilepticus. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001



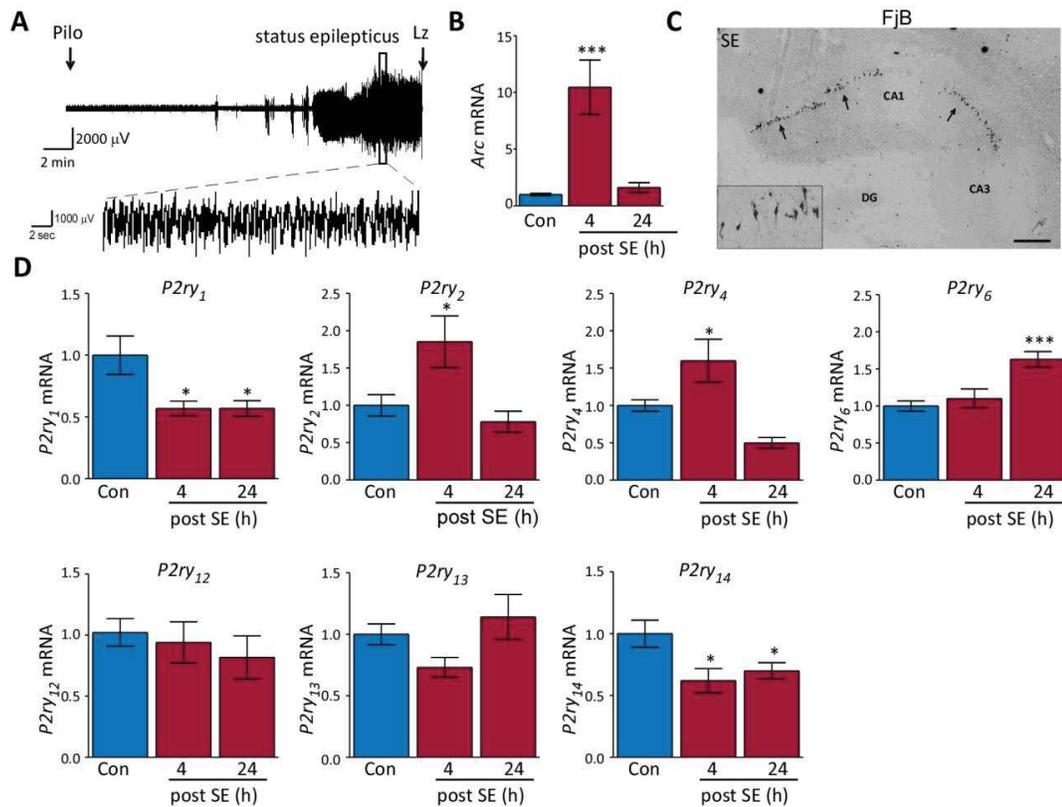
**Figure 3.2 - Specificity of P2Y<sub>1</sub> antibody.**

**(A)** Duplicated hippocampal samples (8 hours post-status epilepticus) were run on the same gel and then transferred to a nitrocellulose membrane. Next, membrane was cut and one membrane incubated with P2Y<sub>1</sub> antibody without anti-P2Y<sub>1</sub> peptide (-pepP2Y<sub>1</sub>) and the other membrane with P2Y<sub>1</sub> antibody containing anti-P2Y<sub>1</sub> peptide (+pepP2Y<sub>1</sub>). n=1 per lane. Note, band disappearing at molecular weight of 66 kDa (arrow) in membrane incubated with +pepP2Y<sub>1</sub>. β-actin was used as loading control (42 kDa).

### 3.3.2 Conservation of seizure-induced changes in P2Y receptor in other models

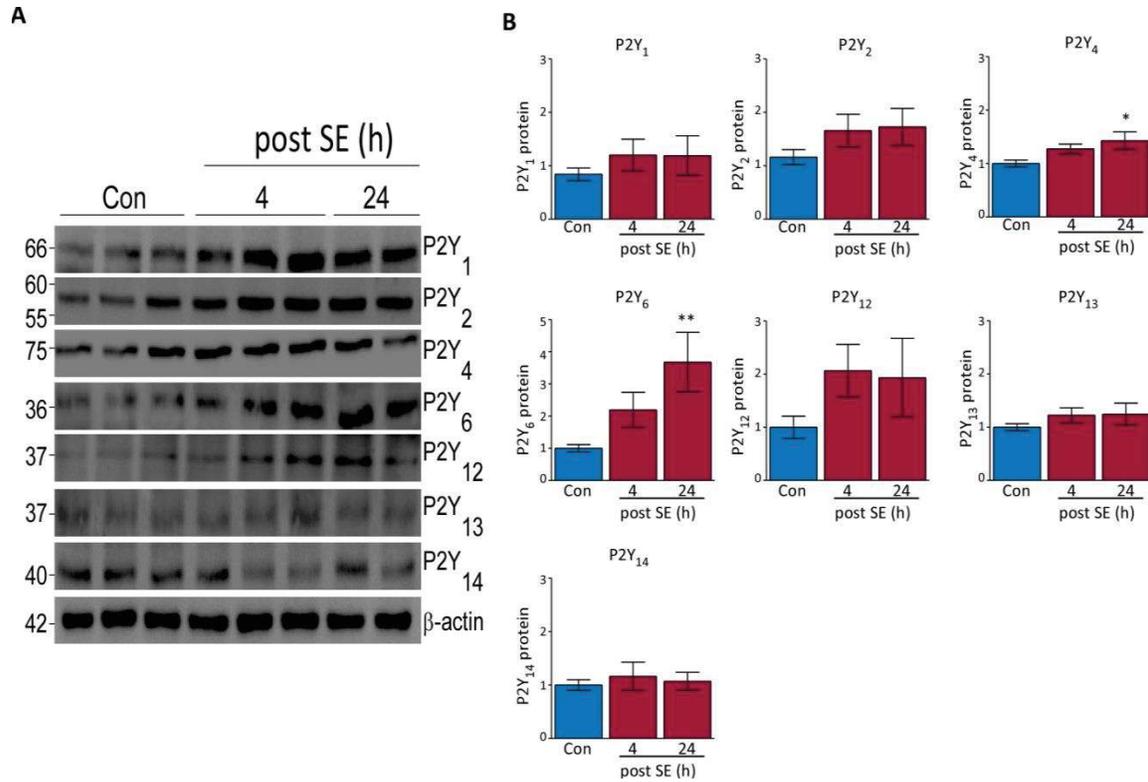
To assure that the observed changes in P2Y induction and expression are a shared response to status epilepticus rather than model-specific, we used a second commonly used mouse model where status epilepticus is induced by a systemic injection of the muscarinic receptor agonist pilocarpine (Curia et al., 2008) (**Figure 3.3A**). Increased *Arc* mRNA levels confirmed recruitment of the hippocampus during pilocarpine-induced status epilepticus (**Figure 3.3B**). Seizure-induced cell death was mainly restricted to the CA1 subfield of the hippocampus (**Figure 3.3C**) (Engel et al., 2013). As seen before for the intra-amygdala kainic acid mouse model, *P2ry<sub>1</sub>* and *P2ry<sub>14</sub>* transcript levels were decreased in the pilocarpine model with *P2ry<sub>12</sub>* and *P2ry<sub>13</sub>* showing a non-significant minor tendency towards a reduction in mRNA transcription (**Figure 3.3D**). Further in line with results from the intra-amygdala kainic acid mouse model, *P2ry<sub>2</sub>*, *P2ry<sub>4</sub>* and *P2ry<sub>6</sub>* mRNA levels were increased (**Figure 3.3D**). These data suggest the bi-directional P2Y receptor changes are a common response to experimental status epilepticus in mice and are not unique to the intra-amygdala kainic acid model.

In relation to the protein level, as seen before for the intra-amygdala kainic acid mouse model, P2Y<sub>4</sub> and P2Y<sub>6</sub> were increased, mainly at 24 hours after status epilepticus (**Figure 3.4A, B**). Also, P2Y<sub>1</sub> and P2Y<sub>2</sub> showed a tendency towards an up-regulation in the protein levels (**Figure 3.4A, B**). P2Y<sub>12</sub> is the exception. In contrast to what was observed before in the intra-amygdala kainic acid mouse model, P2Y<sub>12</sub> showed a tendency to be increased after status epilepticus and P2Y<sub>13</sub> and P2Y<sub>14</sub> protein levels were unchanged (**Figure 3.4A, B**).



**Figure 3.3 - Hippocampal *P2ry* transcript levels after pilocarpine-induced status epilepticus.**

(A) Representative EEG traces showing high amplitude and high frequency spiking after systemic pilocarpine (Pilo) injection. Lorazepam (Lz) was administered 90 minutes following pilocarpine injection. (B) Graph showing increased *Arc* mRNA levels after pilocarpine injection in the hippocampus (n = 6 (control (Con)) and 6 (post-status epilepticus)). (C) Representative image (5x lens) showing cell death mainly localized to the CA1 subfield of the hippocampus 24 hours following pilocarpine-induced status epilepticus as shown by FjB-positive cells (arrows and insert). Scale bar 500  $\mu$ m (D) Graphs showing *P2ry* mRNA changes in the hippocampus following pilocarpine-induced status epilepticus (n = 6 (control (Con)) and 6 (post-status epilepticus)).  $\beta$ -Actin is shown as guide to loading. (E) Western blot and (F) correspondent graphs showing P2Y receptors protein levels changes in the hippocampus after status epilepticus (n = 6 (control (Con)) and 6 (post-status epilepticus)). \*p<0.05, \*\* \*p<0.001.



**Figure 3.4- Hippocampal P2ry transcript levels after pilocarpine-induced status epilepticus.**

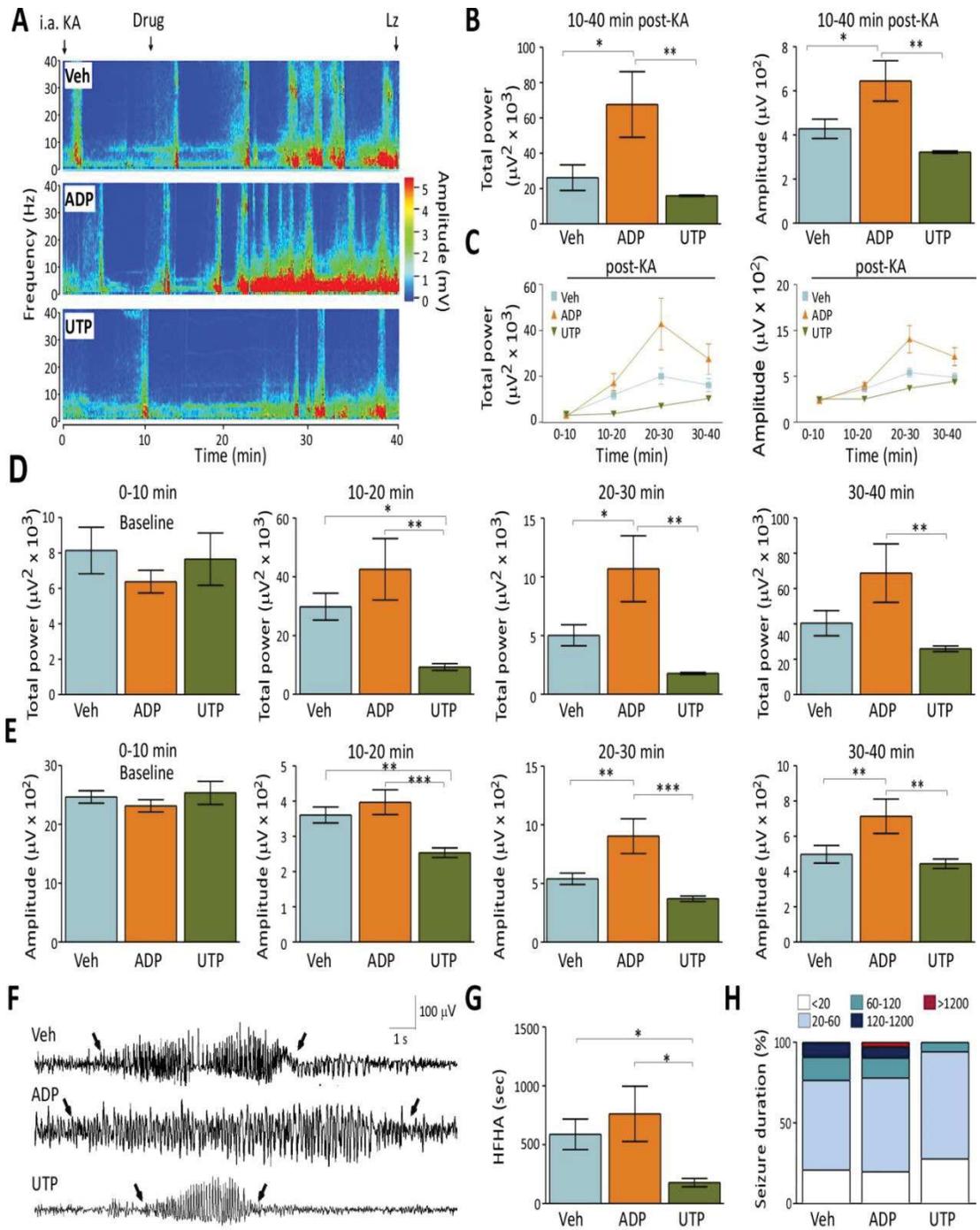
(A) Western blot (n=1 per lane) and (B) corresponding graphs showing P2Y receptors protein levels changes in the hippocampus after status epilepticus (n = 6 (control (Con)) and 6 (post-status epilepticus)). \*p<0.05, \*\* p<0.01.

### 3.3.3 Opposing effects of the P2Y receptor agonists ADP and UTP on seizure severity during status epilepticus

The changes in the expression of P2Y receptors observed following status epilepticus would suggest there may be agonist-specific responses of the P2Y receptors during seizures. Therefore, we injected the two well-known P2Y agonists ADP and UTP (Burnstock, 2007) into the ventricle of mice 15 minutes after the induction of status epilepticus by intra-amygdala kainic acid. Injection of mice with 9 nmol ADP resulted in an increase in total EEG power and amplitude during a 30 minutes recording period until the time of anticonvulsant injection (**Figure 3.5B, C**). This increase was most evident 20 minutes following treatment with ADP. In contrast, injection of UTP into mice during status epilepticus decreased seizure severity (**Figure 3.5B, C**). The effect was only significant, however, during the initial 10 minutes post-UTP injection (**Figure 3.5D, E**). No difference in EEG total power and amplitude was observed during the 10 minutes post-kainic acid injection before drug treatment (baseline) (**Figure 3.5D, E**).

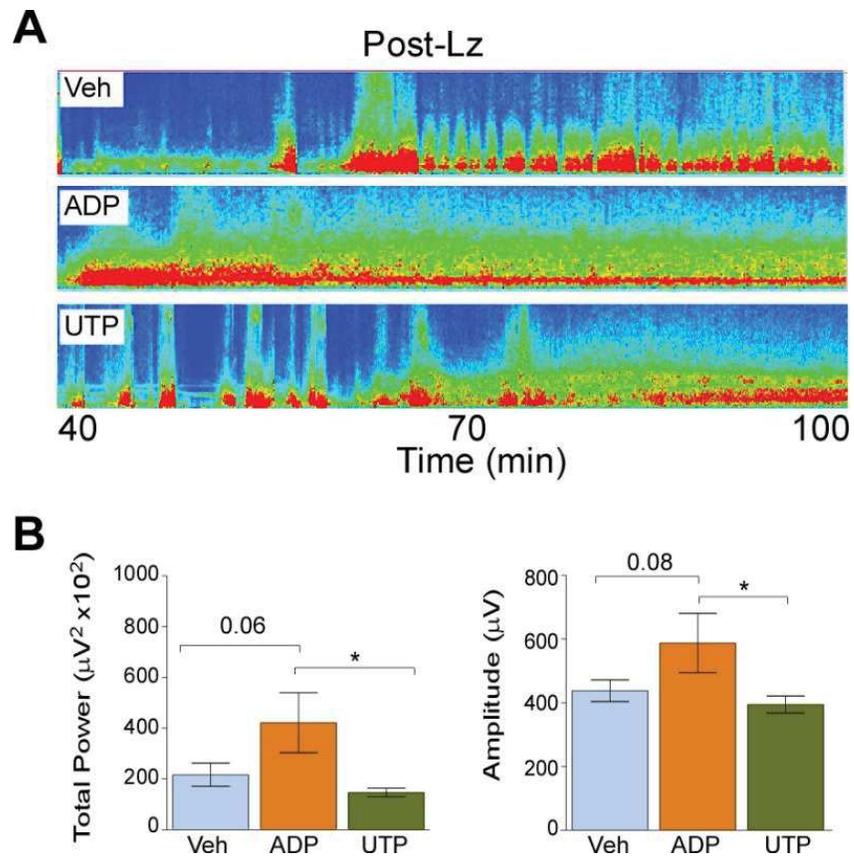
EEG recordings were also analysed for an additional 60 minutes following lorazepam. Although no significant differences between treatment groups were observed, the same trend continued with ADP increasing EEG power and UTP showing a tendency toward lower EEG power (**Figure 3.6A, B**).

Previous work in the intra-amygdala kainic acid model of status epilepticus has shown that HFHA paroxysmal discharges correlate with seizure-induced brain pathology (Shinoda et al., 2004). HFHA analysis revealed that ADP injected mice showed a trend toward longer durations of HFHA spiking while UTP-treated mice a reduction in HFHA spiking (**Figure 3.5F, G**). Interestingly, only ADP-treated mice showed continuous HFHA spiking for more than 20 minutes. In contrast, HFHA burst time remained below 60 seconds in UTP-treated mice (**Figure 3.5H**) suggesting ADP promoted and UTP attenuated status epilepticus.



**Figure 3.5 - P2Y agonist treatment alters seizure severity during status epilepticus and seizure-induced damage.**

**(A)** Representative heat maps depicting EEG frequency and amplitude of mice treated with i.c.v. vehicle, ADP (300  $\mu$ M) or UTP (300  $\mu$ M) during a 40 minutes recording period, starting at the time-point of intra-amygdala kainic acid injection until the administration of lorazepam (Lz). Drug (Vehicle, ADP or UTP) was injected 10 minutes following intra-amygdala kainic acid. **(B)** Graphs showing increased total power and amplitude after the injection of ADP in mice subjected to intra-amygdala kainic acid during a 30 minutes recording period post-drug injection. No significant changes in total power and amplitude could be observed in the UTP-treated group when compared to vehicle-injected mice (n = 10 (vehicle (Veh)) and 7 (ADP and UTP)). **(C)** Graphs showing an increase in seizure severity (total power and amplitude) in mice treated with ADP and a decrease in seizure severity (total power and amplitude) in mice when treated with UTP and compared to vehicle-injected animals. EEG was analysed in 10 minutes segments (n = 10 (vehicle (Veh)), 8 (10 -20 minutes and 20 -30 minutes (ADP and UTP)) and 7 (30 – 40 minutes (ADP and UTP))). EEG total power **(D)** and amplitude **(E)** of 10 minutes segments analysed separately starting at injection of intra-amygdala kainic acid until treatment with lorazepam. Note, increased seizure severity after ADP treatment and decreased seizure severity after treatment with UTP when compared to control (n = 10 (vehicle (Veh)), 8 (10 -20 minutes and 20 -30 minutes (ADP and UTP)) and 7 (30 – 40 minutes (ADP and UTP))). **(F)** Representative images showing examples of high frequency and high amplitude spiking on the EEG during the 30 minutes recording period after drug treatment (see arrows). **(G)** Graph showing a decrease in high frequency and high amplitude spiking (HFHA) in mice treated with UTP (n = 10 (vehicle (Veh)) and 7 (ADP and UTP)). **(H)** Graph showing percentage of different seizure times during the 30 minutes recording period from drug injection until the administration of lorazepam. Note, only ADP-treated mice develop seizures lasting longer than 1200 seconds (20 minutes). UTP-treated mice do not develop seizures lasting longer than 60 seconds (n = 10 (vehicle (Veh)), 8 (10 -20 minutes and 20 -30 minutes (ADP and UTP)) and 7 (30 – 40 minutes (ADP and UTP))). \*p<0.05, \* \*p<0.01.

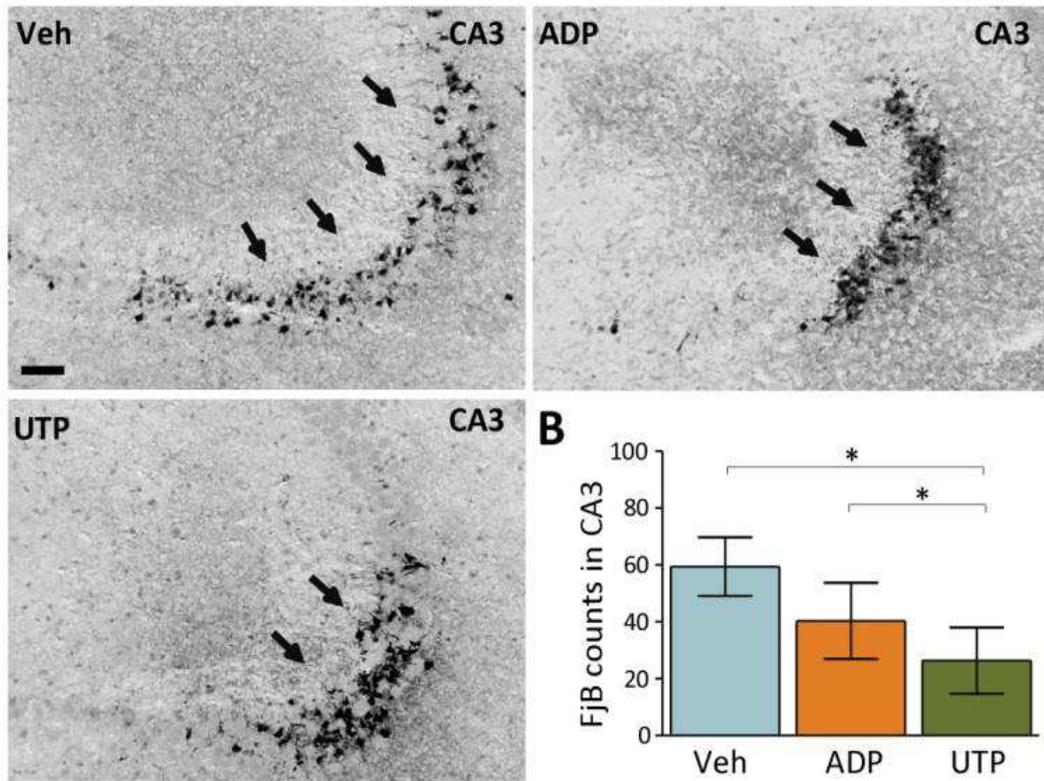


**Figure 3.6 - Enduring effects of P2Y agonists on seizure severity following lorazepam treatment.**

**(A)** Representative heat maps of EEG amplitude and frequency showing increased seizure severity in mice treated with ADP and decreased seizure severity in mice treated with UTP during a 60 minutes recording period from the time of lorazepam treatment. **(B)** Graphs showing a tendency towards increased seizure total power and amplitude in mice treated with ADP when compared to vehicle-injected control mice (n = 8 (vehicle (Veh), 7 ADP and 6 UTP). \*p<0.05, \* \*p<0.01.

### **3.3.4 UTP treatment reduces status epilepticus-induced brain pathology**

We next assessed hippocampal pathology in mice treated with P2Y receptor agonists during status epilepticus. As reported before, vehicle-injected mice subjected to status epilepticus showed the typical CA3 lesion as evidenced by FjB-positive cells (**Figure 3.7A, B**) (Mouri et al., 2008). Mice treated with ADP during status epilepticus showed a non-significant reduction in FjB-positive cells in the CA3 subfield while mice injected with UTP displayed significantly less FjB-positive neurons in the CA3 subfield when compared to vehicle-injected (**Figure 3.7A, B**).

**A**

**Figure 3.7 - UTP treatment protects the brain from seizure-induced damage.**

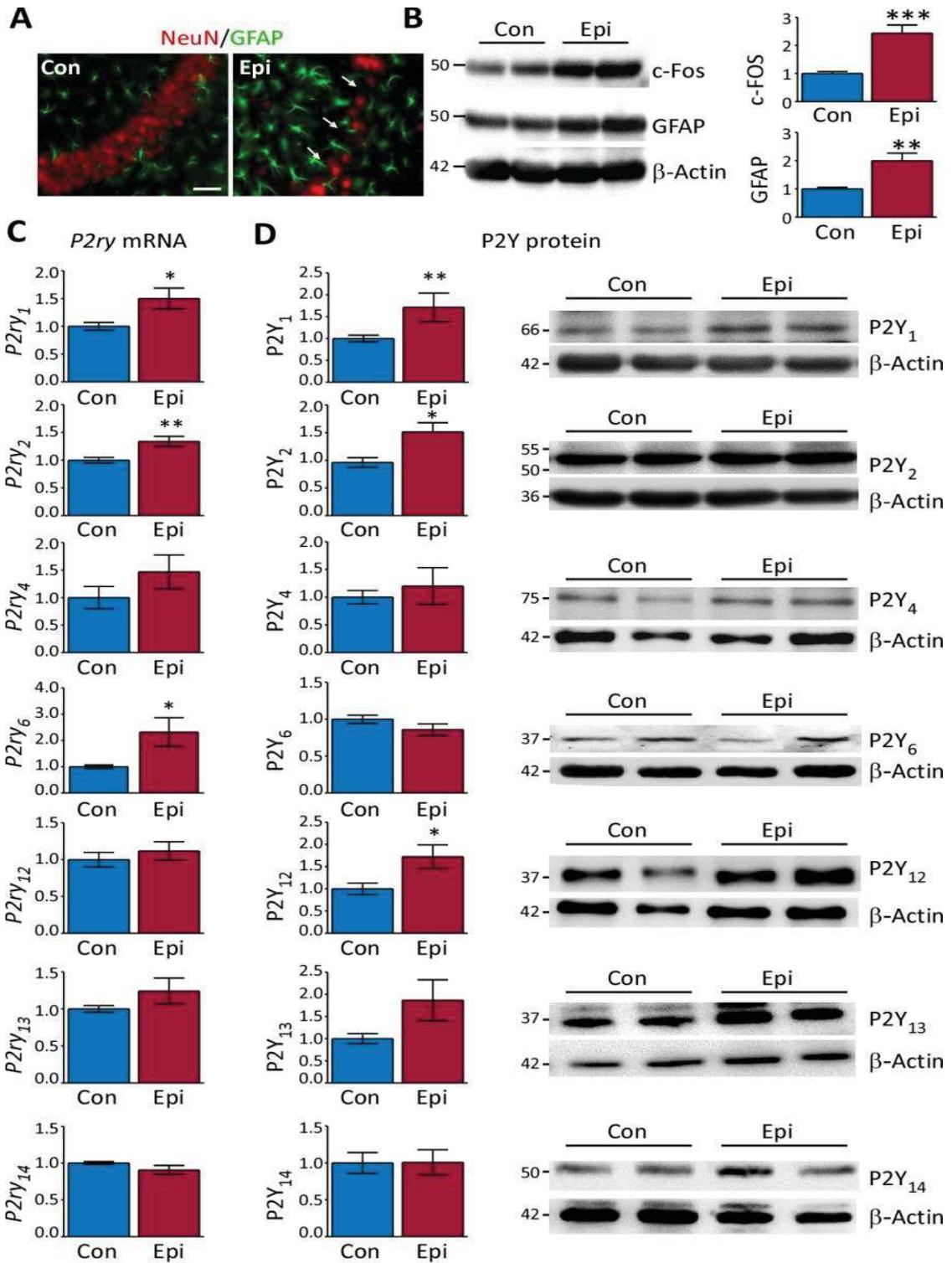
Representative images (A) and graph (B) showing less FjB-positive cells 24 hours post- status epilepticus in mice treated with UTP (n = 8 (Veh), 6 (ADP) and 6 (UTP)). Scale bar = 100  $\mu$ m; \*p<0.05.

### 3.3.5 P2Y receptor expression in experimental epilepsy

To investigate whether P2Y receptor expression is also altered in epilepsy, we analysed hippocampal samples from epileptic mice killed 14 days post-status epilepticus induced by intra-amygdala kainic acid (Mouri et al., 2008, Jimenez-Mateos et al., 2012). Tissue sections showed a visible lesion within the ipsilateral CA3 subfield of the hippocampus involving neuron loss and astrogliosis (**Figure 3.8A**), similar to neuropathological changes found in human TLE (Pitkanen et al., 2015). Increased hippocampal levels of the neuronal activity-regulated gene *c-Fos* further confirmed involvement of the hippocampus during chronic epilepsy (**Figure 3.8B**). Also, as expected, GFAP levels were increased in the hippocampus of epileptic mice (**Figure 3.8B**).

Analysis of P2Y receptor expression determined increased *P2ry<sub>1</sub>*, *P2ry<sub>2</sub>* and *P2ry<sub>6</sub>* transcript levels in the hippocampus of epileptic mice. *P2ry<sub>4</sub>* transcription showed a non-significant tendency to be increased and no changes were observed for *P2ry<sub>12</sub>*, *P2ry<sub>13</sub>* and *P2ry<sub>14</sub>* transcripts (**Figure 3.8C**). Thus, the main transcriptional response of the P2Y receptor family in this model of experimental epilepsy is increased expression.

Western blotting using ipsilateral hippocampi from epileptic mice revealed an increase in P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>12</sub> protein levels (**Figure 3.8D**). In line with a general increase in *P2ry* transcription observed during epilepsy, P2Y receptor expression levels were also mainly increased.

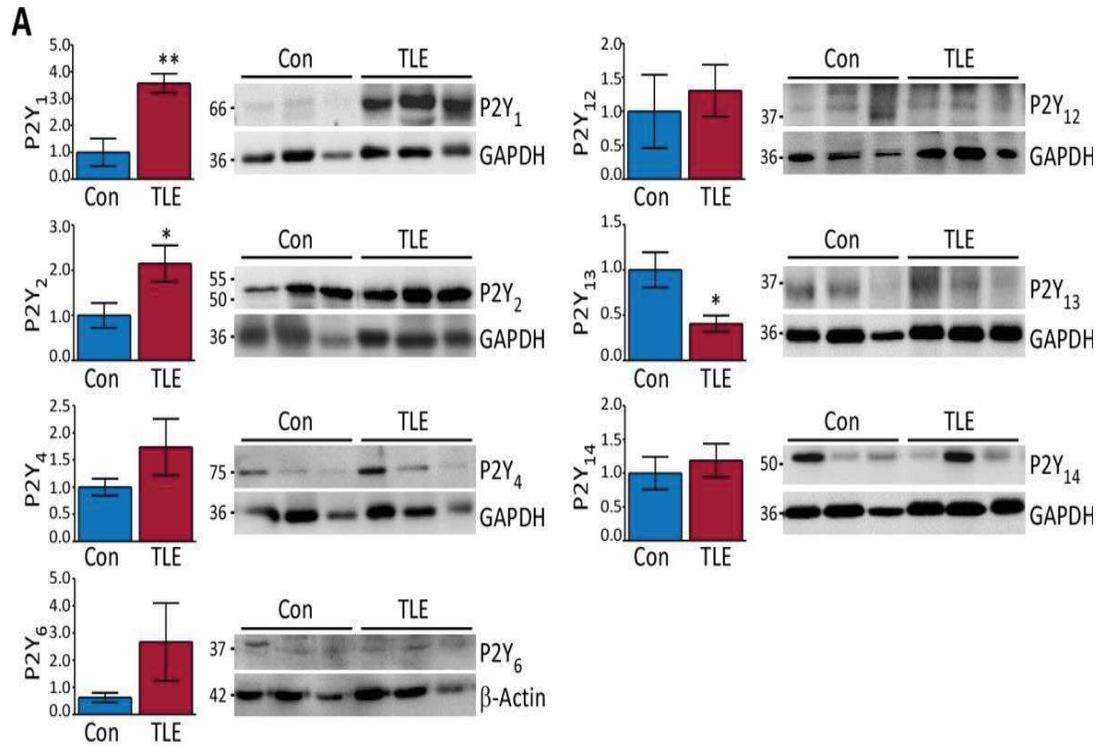


**Figure 3.8 - P2Y receptor expression changes in experimental epilepsy.**

**(A)** Immunofluorescence showing reactive astrocytes (GFAP, green) and decreased neuronal density (NeuN; red, arrows) in the ipsilateral CA3 subfield in epileptic mice 14 days following intra-amygdala-kainic acid induced status epilepticus. Scale bar = 50  $\mu$ m. **(B)** Representative Western (n = 1 per lane) and graphs showing increased c-Fos and GFAP levels in hippocampal tissue from epileptic mice analysed 14 days post- status epilepticus when compared to vehicle-injected control mice (n = 10 control (Con), 10 epilepsy (Epi)). **(C)** Graphs showing *P2ry* mRNA levels in the ipsilateral hippocampus from epileptic mice 14 days post- status epilepticus (n = 10 control (Con), 10 epilepsy (Epi)). **(D)** Graphs and representative Western blot (n = 1 per lane) showing P2Y receptor expression changes in the ipsilateral hippocampus 14 days post- status epilepticus (n = 10 control (Con), 10 epilepsy (Epi)).  $\beta$ -actin is shown as guide to loading. \* $p < 0.05$ , \*\* $p < 0.01$ .

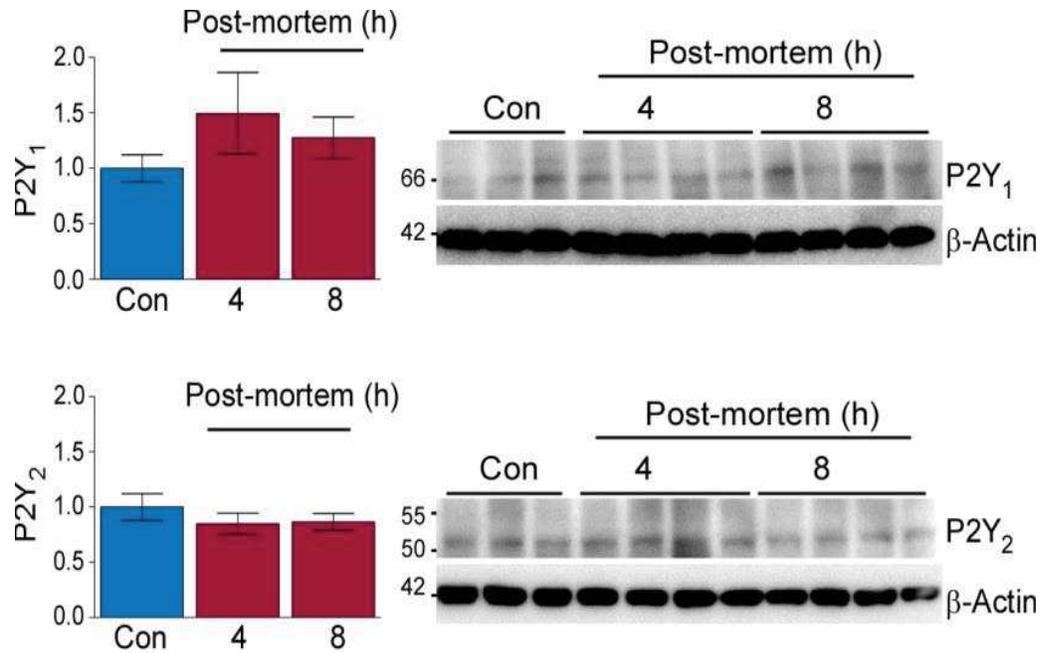
### 3.3.6 P2Y receptor expression in human temporal lobe epilepsy

Finally, to establish the clinical relevance of our findings we analysed P2Y receptor levels in surgically-resected hippocampi from drug-refractory TLE patients, comparing to age- and gender-matched autopsy controls. Pathology reports confirmed neuronal loss and gliosis, consistent with hippocampal sclerosis (**Table 2.6**). Western blot analysis revealed higher levels of P2Y<sub>1</sub> and P2Y<sub>2</sub> in patient samples compared to controls whereas levels of P2Y<sub>13</sub> were lower in patients than controls. The remaining P2Y receptors including P2Y<sub>6</sub>, P2Y<sub>12</sub> and P2Y<sub>14</sub> showed no significant differences except for P2Y<sub>4</sub> which showed a modest, non-significant, tendency to higher levels (**Figure 3.9A**). Post mortem autopsy delay experiments confirmed P2Y receptor levels being stable over the period corresponding to the maximal delay in the human control subjects (**Figure 3.10**). These findings support the data from our experimental model showing an increase in P2Y receptor levels within the damaged hippocampus in epilepsy.



**Figure 3.9 - P2Y receptor expression in the hippocampus of TLE patients.**

**(A)** Representative Western blot (n=1 per lane) and corresponding graph showing increased GFAP protein levels in the hippocampus of TLE patients when compared to control (Con) (n=6/group). **(B)** Representative western blot (n=1 per lane) and corresponding graphs showing P2Y receptor expression levels in the hippocampus of TLE patients when compared to control (Con) (n=6/group). GAPDH is shown as guide to loading. \*p < 0.05, \*\*p < 0.01.



**Figure 3.10 - Absence of post-mortem effect on hippocampal P2Y<sub>1</sub> and P2Y<sub>2</sub> receptor levels.**

Graphs and Western blot (n = 1 per lane) showing no changes in P2Y<sub>1</sub> and P2Y<sub>2</sub> receptor expression levels in hippocampal tissue 4 hours and 8 hours post-mortem. β-actin is used as guide to loading (n = 4 control (Con), 4 4hours post-mortem, 4 8hours post-mortem).

### 3.4 Discussion

The main findings in this chapter are that experimental status epilepticus causes a selective acute down-regulation of adenine-sensitive P2Y receptor transcription and up-regulation of uracil-sensitive P2Y receptor transcription. Suggesting opposing functional roles for both receptor subtypes during seizures, central injection of mice with ADP increases seizure severity whereas UTP treatment decreases seizure severity. We also report select up-regulation of P2Y receptors in experimental and human epilepsy. These findings support a functional role for P2Y receptors in status epilepticus and potentially new therapeutic targets for seizure control or disease-modification in epilepsy.

While the expression and function of P2X receptors has been well-established in experimental status epilepticus and epilepsy, the P2Y receptor subfamily has received minimal prior attention (Engel et al., 2016). This has been partly due to a lack of suitable tools (e.g. drugs) and animal models (Jacobson and Boeynaems, 2010). Only few studies have attempted to determine seizure-induced P2Y receptor changes in the brain and a possible causal impact of these receptors in seizure generation and during epilepsy. One of the first studies describing changes of the P2Y receptor family after seizures used the intraperitoneal kainic acid mouse model of status epilepticus. Results from these studies are in line with our results showing an early increase in *P2ry<sub>6</sub>* transcription and decrease in *P2ry<sub>12</sub>* and *P2ry<sub>13</sub>* transcripts following status epilepticus (Avignone et al., 2008). However, in contrast to our results showing increased *P2ry<sub>6</sub>* receptor levels with no changes in *P2ry<sub>12</sub>* and *P2ry<sub>13</sub>* at later time-points following status epilepticus, the transcription of all three receptors was up-regulated in the intraperitoneal kainic acid model (Avignone et al., 2008). Differences in seizure-induced neurodegeneration or post-seizure inflammation responses might account for the observed discrepancies (Levesque and Avoli, 2013). During chronic epilepsy, the main response was an up-regulation of P2Y receptors. This is in line with a recent study showing increased levels of P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>4</sub> in brain tissue from patients with

epilepsy due to cortical dysplasia (Sukigara et al., 2014). We have now extended these data, showing no significant changes for the remaining receptors in a TLE patient cohort, with the only exception being P2Y<sub>13</sub> displaying lower levels. Interestingly, P2Y<sub>13</sub> receptor activation has been shown to exert neuroprotective effects *in vitro* (Ortega et al., 2011, Morente et al., 2014), therefore the reduction in P2Y<sub>13</sub> levels could contribute to increased vulnerability to seizure-induced neurodegeneration. We did observe some differences between our mouse model of epilepsy and TLE patient brain. In particular, while P2Y<sub>13</sub> expression was decreased in human TLE P2Y<sub>13</sub> levels were increased in the mouse model. Furthermore, P2Y<sub>12</sub> showed only a minor, non-significant increase in TLE patient samples, but was significantly up-regulated in experimental epilepsy. These discrepancies may be due to differences in seizure severity and/or seizure frequency (animal model vs. patients), time of disease stage (early disease stage in our experimental animal model (14 days post-status epilepticus) vs. rather late stage of disease in TLE patients (years of epilepsy pathology) or treatment effects (e.g. AEDs). Regardless of these differences, most P2Y receptors seemed to be up-regulated during epilepsy.

A key observation in the present study is that the direction of changes in P2Y receptor subtypes after status epilepticus correlates closely with the known receptor agonist profile and G protein-coupling of the receptors. Most strikingly, whereas adenine-sensitive receptors were down-regulated, the majority of uracil sensitive receptors, with the UDP-glucose sensitive P2Y<sub>14</sub> receptor being the only exception, are up-regulated following status epilepticus. NF- $\kappa$ B has been shown to drive the transcription of the P2Y<sub>2</sub> receptor during inflammatory conditions (Degagne et al., 2009). However, to date, we do not know what drives or blocks transcription of P2Y receptors during seizures. Nevertheless, the clear distinction in the induction pattern of these receptors points towards common pathways from receptor activation to transcriptional control. The fact that ADP treatment increases seizure pathology and UTP treatment protects the brain from seizure damage further suggest these being intracellular adaptations leading to the distinct post-status epilepticus P2Y receptor

induction profile. Why does ADP increase seizure-pathology and why does UTP treatment protect the brain from damage? Previous data has shown that UTP reduces the neuronal firing rate of cortical and thalamic neurons (Kovacs et al., 2013). This would be consistent with our EEG observations. It is however difficult to narrow down our results to a specific P2Y receptor subtype with all uracil-sensitive P2Y receptors being up-regulated after status epilepticus. In contrast to the uracil-sensitive P2Y receptors, it is tempting to speculate that the pro-convulsive effects of ADP are mainly mediated through the P2Y<sub>1</sub> receptor subtype. The P2Y<sub>1</sub> receptor is the only adenine-sensitive receptor showing increased protein levels following status epilepticus, despite a decrease in mRNA transcript levels. The reason for this remains elusive; however, we have shown a seizure-induced inhibition of the ubiquitin-proteasome system (Engel et al., 2017). Seizure-induced changes in receptor stability or internalization may also account for the increase in P2Y<sub>1</sub> receptor levels (Dores et al., 2016). Although, to date, P2Y<sub>1</sub> has not been studied in the setting of epilepsy, several lines of evidence point towards a possible role of this receptor in epileptic pathology. P2Y<sub>1</sub> activation has been associated with changes in the release of neurotransmitters including glutamate and gamma-aminobutyric acid (GABA) (Heinrich et al., 2008, Jacob et al., 2014). P2Y<sub>1</sub> has also been shown to interfere with synaptic transmission by modulating neurotransmitter receptor function (Guzman and Gerevich, 2016) and to mediate the propagation of Ca<sup>2+</sup> waves within astrocyte networks (Alvarez-Ferradas et al., 2015). The P2Y<sub>12</sub> receptor represents a second adenine-sensitive receptor potentially having a causal role during seizure pathology. However, P2Y<sub>12</sub> knockout mice were found to display an exacerbated seizure phenotype in two kainate models (Eyo et al., 2014), suggesting P2Y<sub>12</sub> activation has anti-convulsive rather than pro-convulsive effects.

Another key finding of the present study is the increased expression of Gq-coupled P2Y receptors and the decreased expression of Gi-coupled P2Y receptors. The canonical Gq signalling pathway leads to an increase in Phospholipase C (PLC) activation, increased intracellular inositol 3 phosphate (IP<sub>3</sub>) levels resulting in the

release of  $\text{Ca}^{2+}$  from intracellular calcium stores and the formation of diacylglycerol (DAG) with the subsequent activation of protein kinase C (PKC) (von Kugelgen, 2006). Notably, both PLC and PKC deficiency have been associated with a more severe epileptic phenotype and therefore the changes in P2Y signalling may represent adaptive changes to limit epileptogenic changes (Kim et al., 1997, Terunuma et al., 2008). On the other hand, Gi signalling inhibits adenylate cyclase, thereby decreasing cAMP production and PKA activation (von Kugelgen, 2006). Again, in good agreement with pro-survival mechanisms driving P2Y expression, elevated cAMP has been reported to be pro-epileptogenic and PKA inhibition reduced epileptiform activity (Boulton et al., 1993, Kurada et al., 2014).

Probably the most likely cause for the observed neuroprotection via UTP is a reduction in seizure severity. However, UTP or its metabolites have been frequently described to provide neuroprotective effects in the brain against various pathological insults such as ischemia or Alzheimer's disease (Lecca and Ceruti, 2008, Woods et al., 2016). It is unclear why ADP treatment did not translate into increased neurodegeneration despite exacerbating seizures. A possible explanation is the conversion of ADP into adenosine by extracellular ectonucleotidases (Zimmermann, 2006). Adenosine is a known anticonvulsive and neuroprotective molecule (Boison, 2012). Whether treatment with ADP or UTP influences the development of epilepsy or the epileptic phenotype has not been investigated. A causal role is, however, likely with different P2Y receptors being up- or down-regulated during chronic epilepsy. In particular, the strong anticonvulsive and neuroprotective effects provided by UTP during status epilepticus strongly suggests that drugs, antagonising uracil-sensitive P2Y receptors, may represent a new treatment strategy not only for status epilepticus but also during epileptogenesis and during epilepsy possibly with disease modifying potential. However, the extremely short half-life of ADP or UTP in the brain (Zimmermann, 2006) makes studies using this class of agonists extremely difficult and more stable and centrally available specific P2Y agonists/antagonists will have to be used in future studies.

In conclusion, the data in this chapter demonstrates a specific induction and expression profile of the P2Y receptor family after prolonged, damaging seizures and during epilepsy and identifies P2Y receptors as possible new targets for the treatment of status epilepticus and drug-refractory epilepsy.

**Chapter 4 – P2Y<sub>1</sub> receptor antagonism reduces seizure severity and hippocampal damage during status epilepticus**

## 4.1 Introduction

Studies in Chapter 3 demonstrated that hippocampal P2Y<sub>1</sub> receptor expression is consistently upregulated in both experimental and human epilepsy. What are the functional consequences of this change? Previous work would suggest that targeting the receptor could alter the pathophysiology of status epilepticus. For example, the P2Y<sub>1</sub> receptor has been shown to promote calcium waves (Alvarez-Ferradas et al., 2015) and a recent study has suggested neuroprotective properties of P2Y<sub>1</sub> antagonism during seizure induced cell death in a rat model of status epilepticus (Simoes et al., 2018). The focus of the following chapter was to explore whether targeting of the P2Y<sub>1</sub> receptor alters seizures and neurodegeneration and to investigate the underlying mechanisms.

The role of different P2Y receptors in mediating neuroinflammation and cell death seems to be divergent (Forster and Reiser, 2015), depending on downstream signalling pathways, and remains incompletely understood. A key cell population expressing P2Y receptors are glia (Burnstock, 2007) and some subtypes, such as the P2Y<sub>1</sub> receptor, have been implicated in microglia function (De Simone et al., 2010). The P2Y<sub>1</sub> receptor is also expressed on astrocytes and activated under conditions of oxidative stress, contributing to the release of IL-6 (Fujita et al., 2009). Since IL-6 mediates predominantly anti-inflammatory effects, this may oppose inflammatory mechanisms following epilepsy. Thus, blocking the P2Y<sub>1</sub> receptor could have deleterious effects (Rothaug et al., 2016). In contrast, activation of the P2Y<sub>1</sub> receptor on astrocytes may enhance Ca<sup>2+</sup>-dependent signalling and promote hyperexcitability (Alvarez-Ferradas et al., 2015). These apparently contradictory actions make it difficult to predict how the elevated P2Y<sub>1</sub> receptor levels will affect status epilepticus.

The effects of P2Y signalling are not limited to inflammatory processes and cellular survival alone. P2Y signalling also impacts directly on neuronal excitability, synaptic strength and synaptic plasticity (Guzman and Gerevich, 2016). Presynaptic P2Y receptors have been shown to affect the release of different neurotransmitters

including glutamate, noradrenaline and GABA (Guzman and Gerevich, 2016), most likely by reducing presynaptic  $\text{Ca}^{2+}$  influx (Fischer et al., 2009b). Activation of the P2Y<sub>1</sub> receptor inhibits the release of glutamate in the hippocampus (Mendoza-Fernandez et al., 2000, Rodrigues et al., 2005, Koizumi et al., 2003), possibly through the inhibition of voltage-activated  $\text{Ca}^{2+}$  channels (VACCs) (Gerevich et al., 2004). P2Y<sub>1</sub> activation also blocks the release of noradrenaline in the hippocampus, likely through the same mechanism (Csolle et al., 2008). P2Y receptor activation can also alter the expression/function of other membrane receptors and voltage-gated ion channels. P2Y<sub>1</sub> activation triggers the desensitization or internalization of the metabotropic glutamate receptor 1 (mGluR1) (Mundell, et al., 2004) and inhibits NMDA receptor channels (Luthardt, et al., 2003). P2Y<sub>1</sub> also increases the sensitivity of the GABA<sub>A</sub> receptor (Saitow et al., 2005) and inhibits P2X receptors (Gerevich et al., 2007). Together, these presynaptic actions favour a role for the P2Y<sub>1</sub> receptor in opposing excessive neuronal activity.

Activation of the P2Y<sub>1</sub> may also indirectly change neurotransmitter release via actions on glia. P2Y<sub>1</sub>-receptor activation on astrocytes results in an increase in inhibitory-postsynaptic currents (IPSCs) in pyramidal neurons (Bowser and Khakh, 2004). In a more recent study, Jacob et al. (2014) showed that astrocytic P2Y<sub>1</sub> activation increases extracellular concentrations of GABA by inhibiting  $\text{Ca}^{2+}$  signalling dependent GABA transport (Jacob et al., 2014).

Taken together, the findings reported above, and results presented in Chapter 3 suggest P2Y<sub>1</sub> receptor targeting may alter seizure severity or neuropathological outcomes in status epilepticus. A range of pharmacological and genetic tools exist to test this hypothesis, including specific agonists and antagonists for the P2Y<sub>1</sub> receptor, and P2Y<sub>1</sub> receptor knock-out mice. These allow for a complete functional study of the P2Y<sub>1</sub> receptor in experimental status epilepticus *in vitro* and *in vivo*. The main focus of this Chapter was to study the functional role of P2Y<sub>1</sub> during status epilepticus. Using the available pharmacologic and genetic tools, we explored how modulating P2Y<sub>1</sub>

receptor activity affects seizures and seizure-induced damage in two different *in vivo* models; the intra-amygdala kainic acid and systemic pilocarpine model, in mice.

## 4.2 Chapter objectives

**Hypothesis:** P2Y<sub>1</sub> receptor modulation protects the brain from seizures and seizure-induced pathology.

**Objectives:**

1. Determine the specific cellular location and functional contribution of the P2Y<sub>1</sub> receptor to status epilepticus: evaluate the contribution of P2Y<sub>1</sub> to seizures, seizure-induced cell death and inflammation during status epilepticus.
2. Determine the mechanism by which P2Y<sub>1</sub> receptor activation alters seizures and seizure-induced pathology during status epilepticus.

### 4.3 Results

#### 4.3.1 Hippocampal subfield-specific changes in P2Y<sub>1</sub> expression following status epilepticus

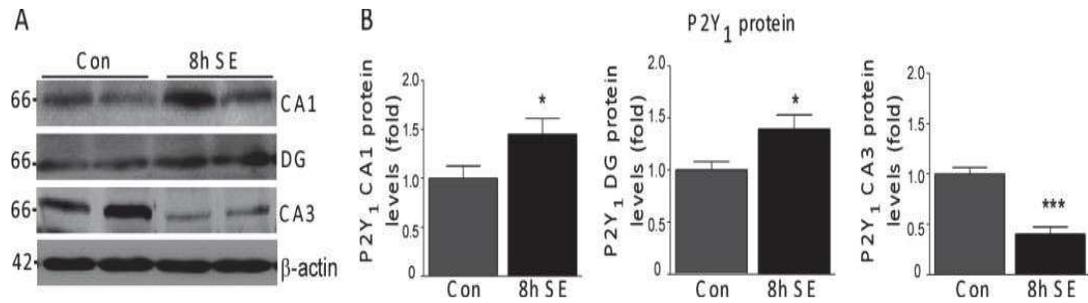
It is well established that the P2Y<sub>1</sub> receptor is expressed in different regions of the brain, including the hippocampus (Webb et al., 1998, Burnstock, 2007, Moore et al., 2000). As mentioned in Chapter 3, expression of the P2Y<sub>1</sub> receptor increases in the mouse hippocampus following status epilepticus in the kainic acid model (Alves et al., 2017). However, there have been no studies to date that explored expression of the P2Y<sub>1</sub> receptor in the different subfields of the hippocampus following status epilepticus. This is important because each subfield contains a unique mix of neuronal and glial cells and is differentially vulnerable to the effects of status epilepticus. Therefore, mapping how P2Y<sub>1</sub> receptor expression changes can be informative in establishing whether changes occur in regions of damage or survival. To investigate the subfield-specific expression of the P2Y<sub>1</sub> receptor (CA1, CA3 and DG) following status epilepticus, mice were killed 8 hours after status epilepticus, a time-point corresponding to when expression was increased in Chapter 3. Hippocampal subfields were microdissected and analyzed by Western blot. This analysis showed that protein levels of the P2Y<sub>1</sub> receptor increased in the CA1 and DG subfields of the hippocampus (**Figure 4.1A, B**). In contrast, P2Y<sub>1</sub> receptor expression was significantly decreased in the CA3 region (**Figure 4.1A, B**).

Under physiological conditions, P2Y<sub>1</sub> receptors are expressed in different cell types in the brain, including glial cells and neurons (Webb et al., 1998, Burnstock, 2008). To investigate in what cell types P2Y<sub>1</sub> is expressed following status epilepticus, we carried out immunohistochemistry to verify if the P2Y<sub>1</sub> receptor is co-localized with specific markers for microglia (Iba1), neurons (NeuN) and astrocytes (GFAP) at different time-points after status epilepticus.

In the normal hippocampus, the P2Y<sub>1</sub> receptor was mainly present on NeuN-positive cells with the morphological appearance of neurons (**Figure 4.2 B**). However,

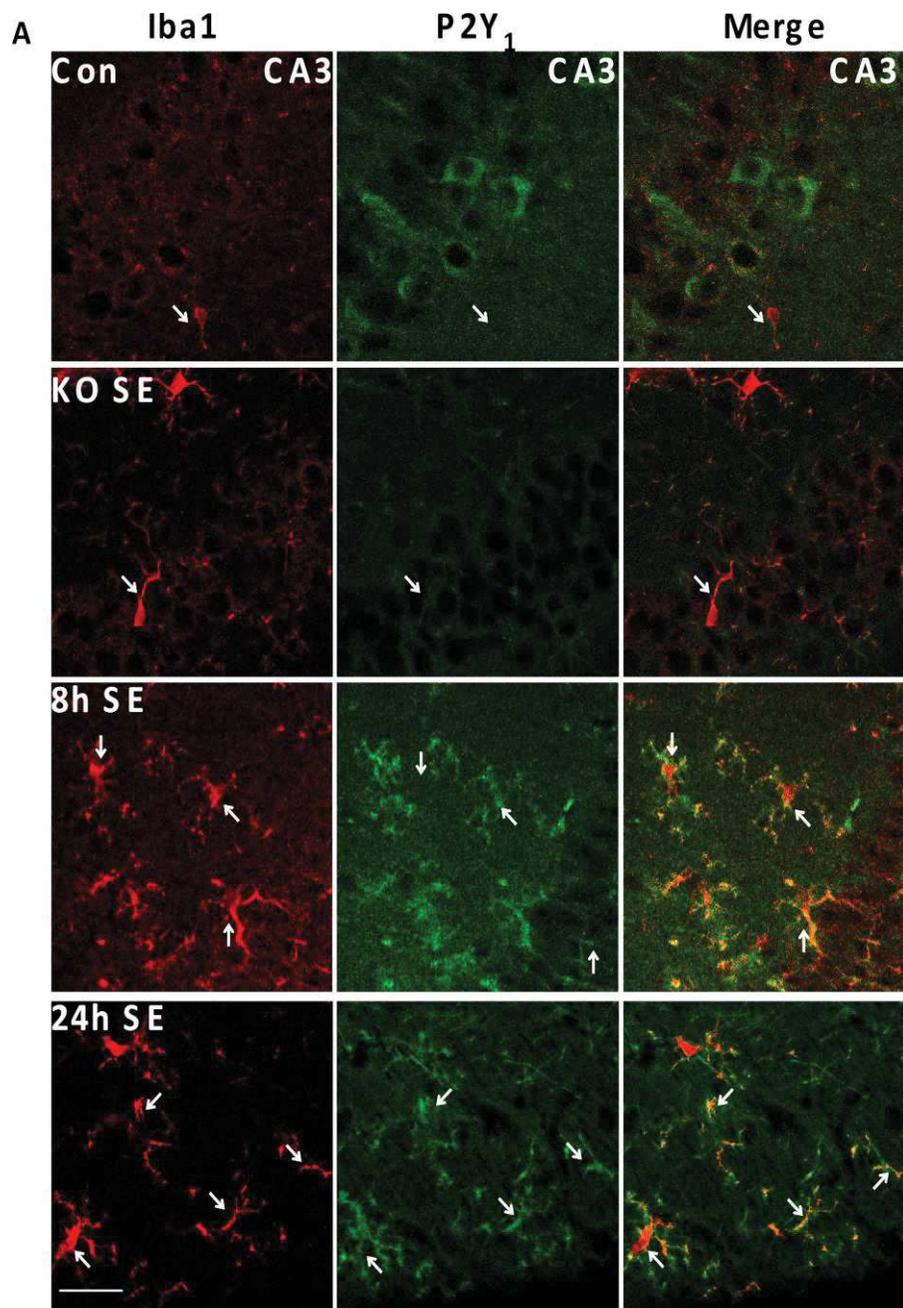
at 8 hours and 24 hours after kainic acid induced-status epilepticus, P2Y<sub>1</sub> expression was detected on microglia (**Figure 4.2 A**) as well as neurons (**Figure 4.2B**). Surprisingly, no co-localization was observed with astrocytes (**Figure 4.2C**). The specificity of staining was confirmed using tissue sections from P2Y<sub>1</sub> knock-out mice (**Figure 4.2A-C**).

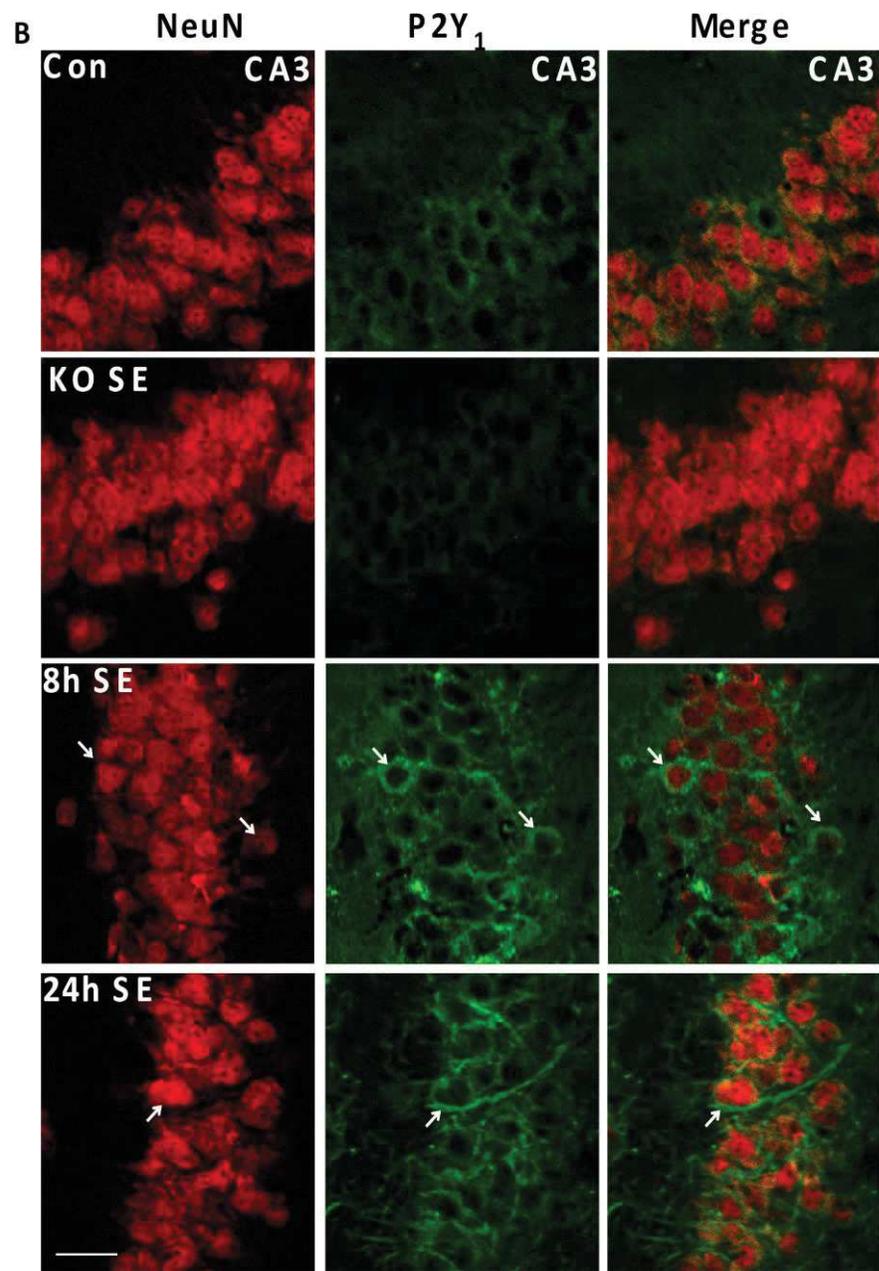
Since the P2Y<sub>1</sub> receptor has been implicated in regulation of neurotransmitter release at synapses, we also investigated whether there were any changes in this population of receptors after status epilepticus. For this, we prepared synaptoneurosomes from mice subjected to status epilepticus at different time-points. This experiment revealed levels of the P2Y<sub>1</sub> receptor significantly increased in hippocampal synaptoneurosomes at 8 hours and 24 hours after status epilepticus (**Figure 4.3A**).

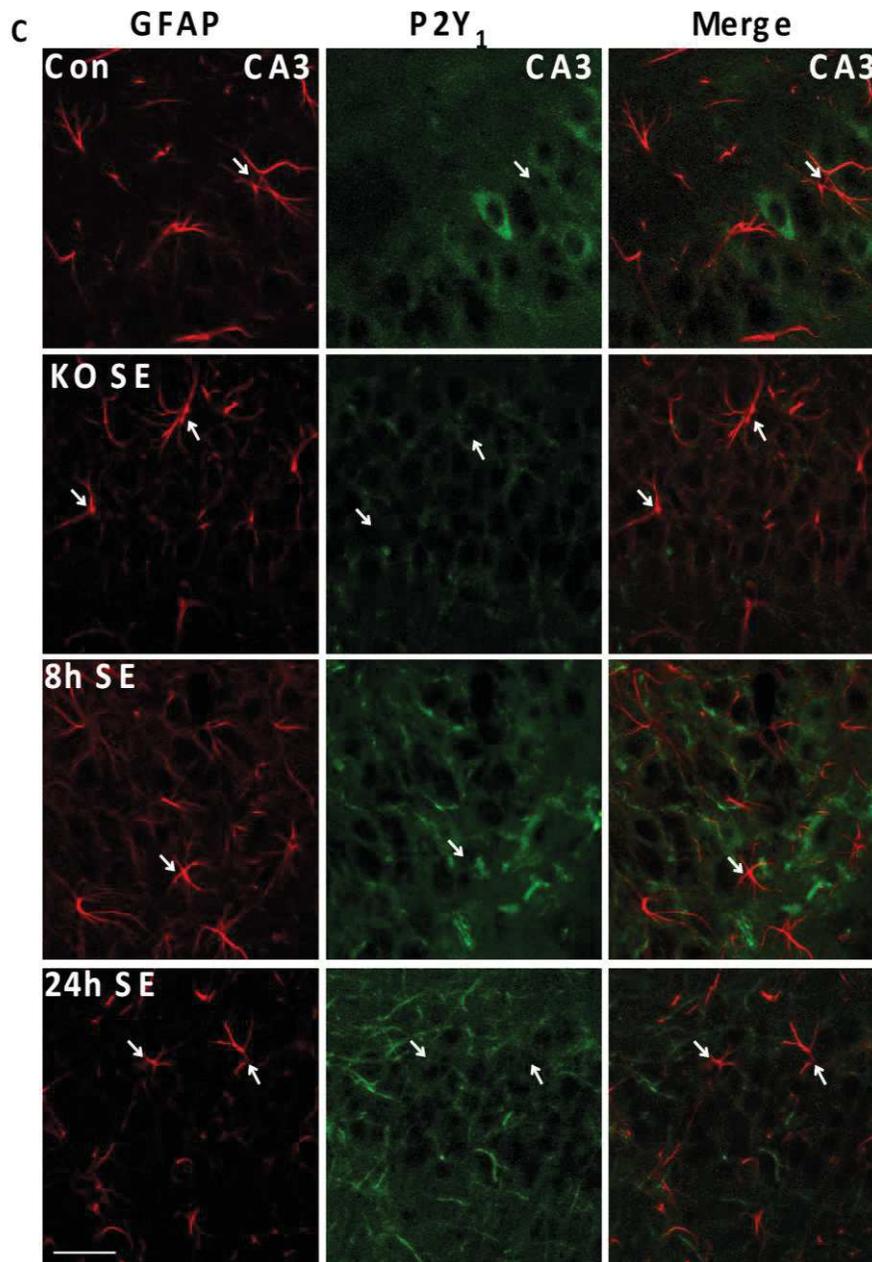


**Figure 4.1 – P2Y<sub>1</sub> receptor expression in hippocampal subfields in the intra-amygdala kainic acid mouse model of status epilepticus**

(A, B) Representative western blot (n=1 per lane) and graphs showing expressional changes of P2Y<sub>1</sub> receptor in the hippocampal subfields 8 hours after status epilepticus. In CA1 and DG, P2Y<sub>1</sub> receptor protein levels were significantly up-regulated. In CA3, however, P2Y<sub>1</sub> was down-regulated. CA, cornu ammonis; DG, dentate gyrus (n= 8 (control (Con)) and 9 (post-SE)). β-actin is shown as guide to loading. \*p<0.05, \*\* \*p<0.001.



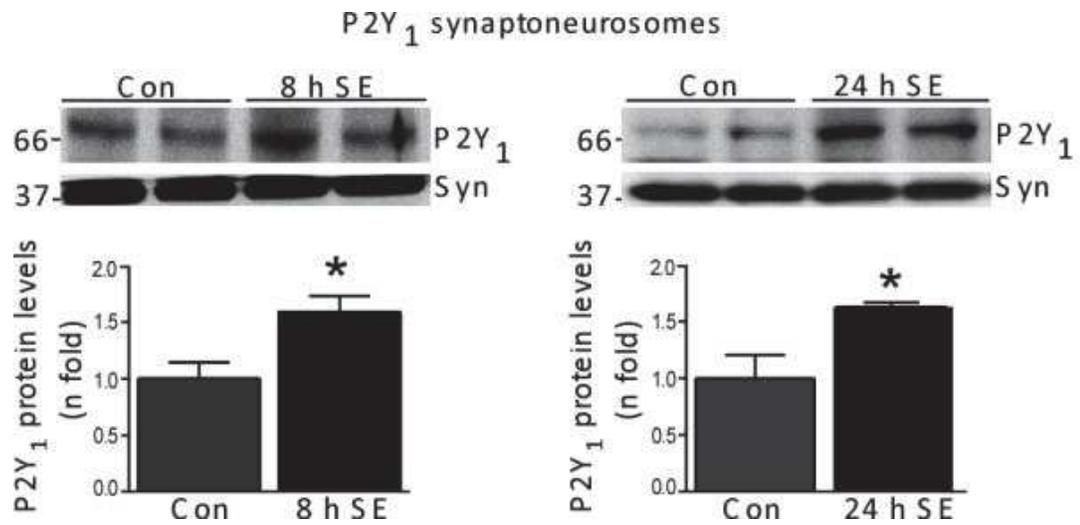




**Figure 4.2 – P2Y<sub>1</sub> receptor is expressed in microglia and neurons following status epilepticus.**

(A) Photomicrograph showing the co-localization of the P2Y<sub>1</sub> receptor (Green) with Iba1 (red), specific marker of microglia cells, at 8 hours and 24 hours after status epilepticus in the CA3 region of the hippocampus. The specificity of the P2Y<sub>1</sub> receptor antibody was confirmed using P2Y<sub>1</sub> knockout tissue. White arrows indicate the co-localization of the P2Y<sub>1</sub> receptor with microglia. (B) Immunofluorescent staining revealing that P2Y<sub>1</sub> receptor (green) is co-localized with NeuN (red), which is a specific marker

to neurons. P2Y<sub>1</sub> knockout was used to verify the specificity of the P2Y<sub>1</sub> receptor antibody. White arrows indicate the co-localization of the P2Y<sub>1</sub> receptor with neurons in the CA3 region of the hippocampus. **(C)** Immunofluorescent staining reporting that P2Y<sub>1</sub> receptor (green) is not co-localized with astrocytes (red). As shown above, absence of staining in P2Y<sub>1</sub> knockout tissue. Scale bar= 50μm.



**Figure 4.3 – P2Y<sub>1</sub> receptor is increased in synaptoneurosomes after status epilepticus.**

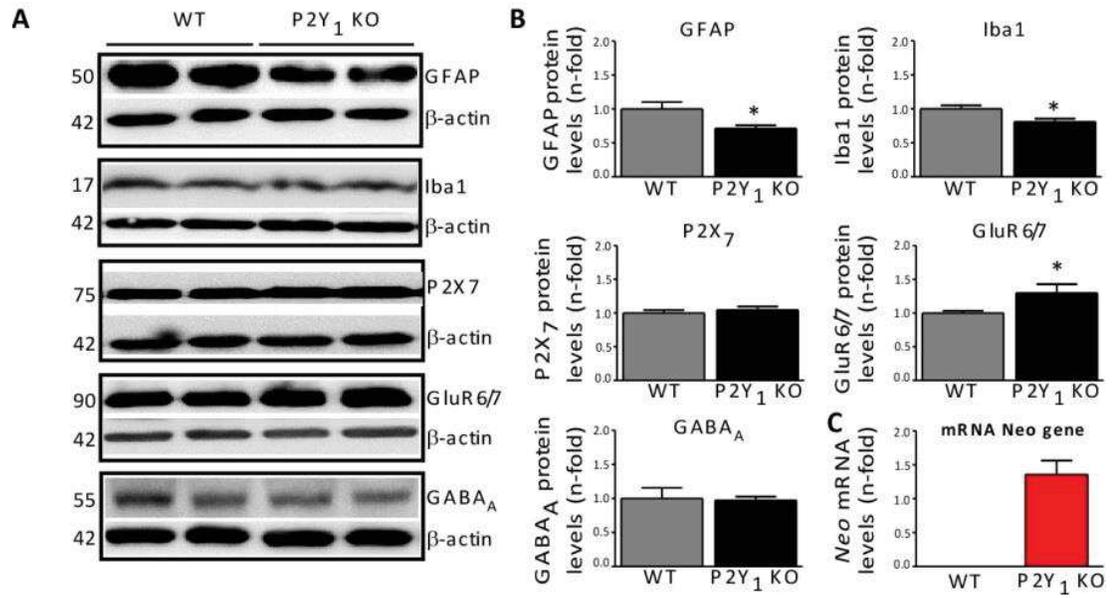
(A) Western blot (each lane is from n= 2 hippocampi) and respective graphs showing a significant increase of the P2Y<sub>1</sub> receptor in synaptoneurosomes at 8 hours and 24 hours after status epilepticus (n= 3 (control (Con)) and 3 (post-SE)). Synaptophysin is shown as guide to loading. \*p<0.05.

### 4.3.2 Functional contribution of the P2Y<sub>1</sub> receptor to seizures and seizures-induced pathology in the kainic acid model

#### 4.3.2.1 Loss of P2Y<sub>1</sub> receptor alters expression of glial and excitatory neurotransmitter receptor expression in mice

To obtain genetic evidence that P2Y<sub>1</sub> receptor signalling can affect seizures or seizure-induced pathology we studied the outcome of status epilepticus in mice deficient in the P2Y<sub>1</sub> receptor. P2Y<sub>1</sub> receptor knock-out was confirmed by the presence of the *neo* gene, which was used to replace the *P2ry1* gene (**Figure 4.4C**).

First, we investigated whether loss of the P2Y<sub>1</sub> receptor altered basal expression of key glial and neurotransmitter markers. Western blot analysis of protein extracts from the hippocampus of naïve P2Y<sub>1</sub> knockout mice revealed significantly lower GFAP and Iba1 protein levels in the hippocampus compared to wild-type mice (**Figure 4.4A, B**). There were also select changes to neurotransmitter systems. Expression of the kainate receptor GluR6/7 was higher in P2Y<sub>1</sub> knockout mice compared to wild-type levels (**Figure 4.4A, B**). No differences in the expression of GABA<sub>A</sub> or the P2X7 receptor were observed between wildtype and knockout mice (**Figure 4.4A, B**).



**Figure 4.4 – Naïve P2Y<sub>1</sub> knockout mice express less GFAP and Iba1, but shown an increase in the expression of the kainate receptor GluR6/7.**

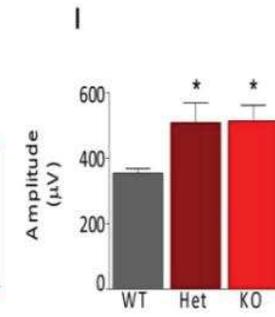
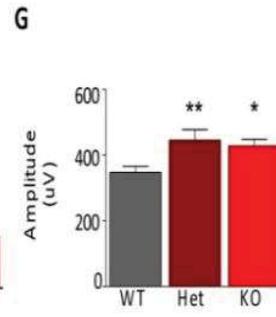
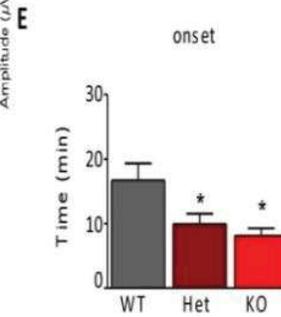
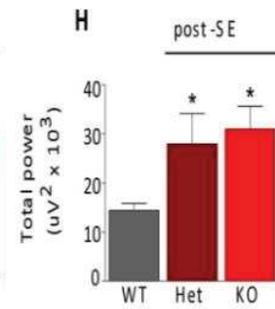
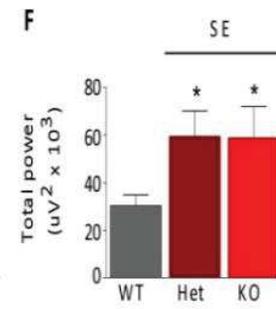
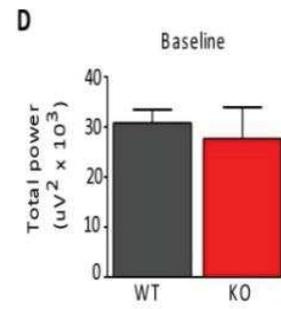
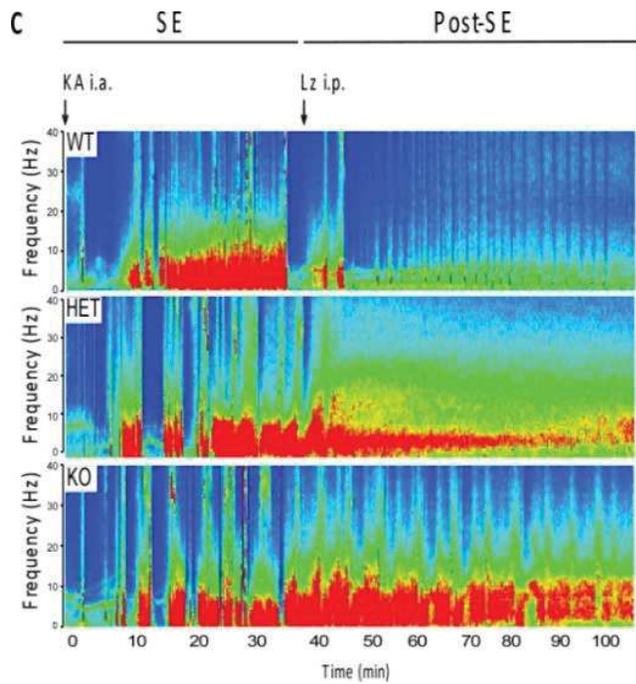
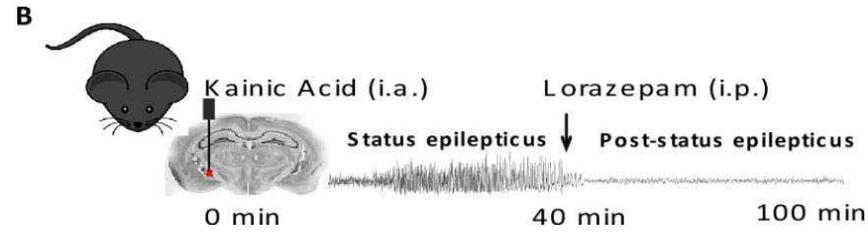
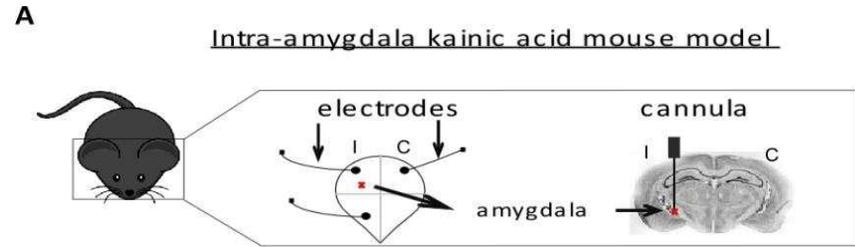
**(A)** Western blot (n= 1 per lane) and corresponding graphs **(B)** showing a decrease in the protein levels of GFAP and Iba1 in naïve P2Y<sub>1</sub> knockout mice in the hippocampus when compared to wild-type mice. On the other hand, GluR6/7 was increased in P2Y<sub>1</sub> knockout mice. No differences in the protein levels of the ionotropic P2X<sub>7</sub> receptor and GABA<sub>A</sub> receptor was observed (n= 6 WT and 6 P2Y<sub>1</sub> KO). β-actin is shown as guide to loading. **(C)** Presence of the *neo* gene in P2Y<sub>1</sub> knockout, which is replacing *P2ry1* gene in the P2Y<sub>1</sub> knockout mice (n=4 WT and 4 P2Y<sub>1</sub> KO). \*p<0.05.

#### 4.3.2.2 Increased seizure severity in P2Y<sub>1</sub> receptor knockout mice during status epilepticus

We first addressed the functional role of the P2Y<sub>1</sub> receptor during status epilepticus induced by intra-amygdala kainic acid in mice (Mouri et al., 2008) (**Figure 4.5A, B**).

Baseline EEG (1 hour) was first analyzed to determine whether loss of the P2Y<sub>1</sub> affects physiological activity. No differences in total power and amplitude were observed between both groups, suggesting similar brain activity between treatment groups (**Figure 4.5C, D**).

Next, we analyzed the EEG recordings made during and shortly after status epilepticus. P2Y<sub>1</sub> knockout and heterozygous mice displayed significantly higher total power and amplitude on EEG during status epilepticus compared to wild-type mice (**Figure 4.5C, F, G**). P2Y<sub>1</sub> knockout and heterozygous mice also had an earlier seizure onset when compared to wild-type mice (**Figure 4.5E**). During a 60 minutes recording period following lorazepam administration to reduce morbidity and mortality, P2Y<sub>1</sub> knockout and heterozygous mice continued to show higher EEG total power and amplitude when compared to wild-type mice (**Figure 4.5H, I**).



**Figure 4.5 - P2Y<sub>1</sub> knockout mice display increased seizure severity after status epilepticus.**

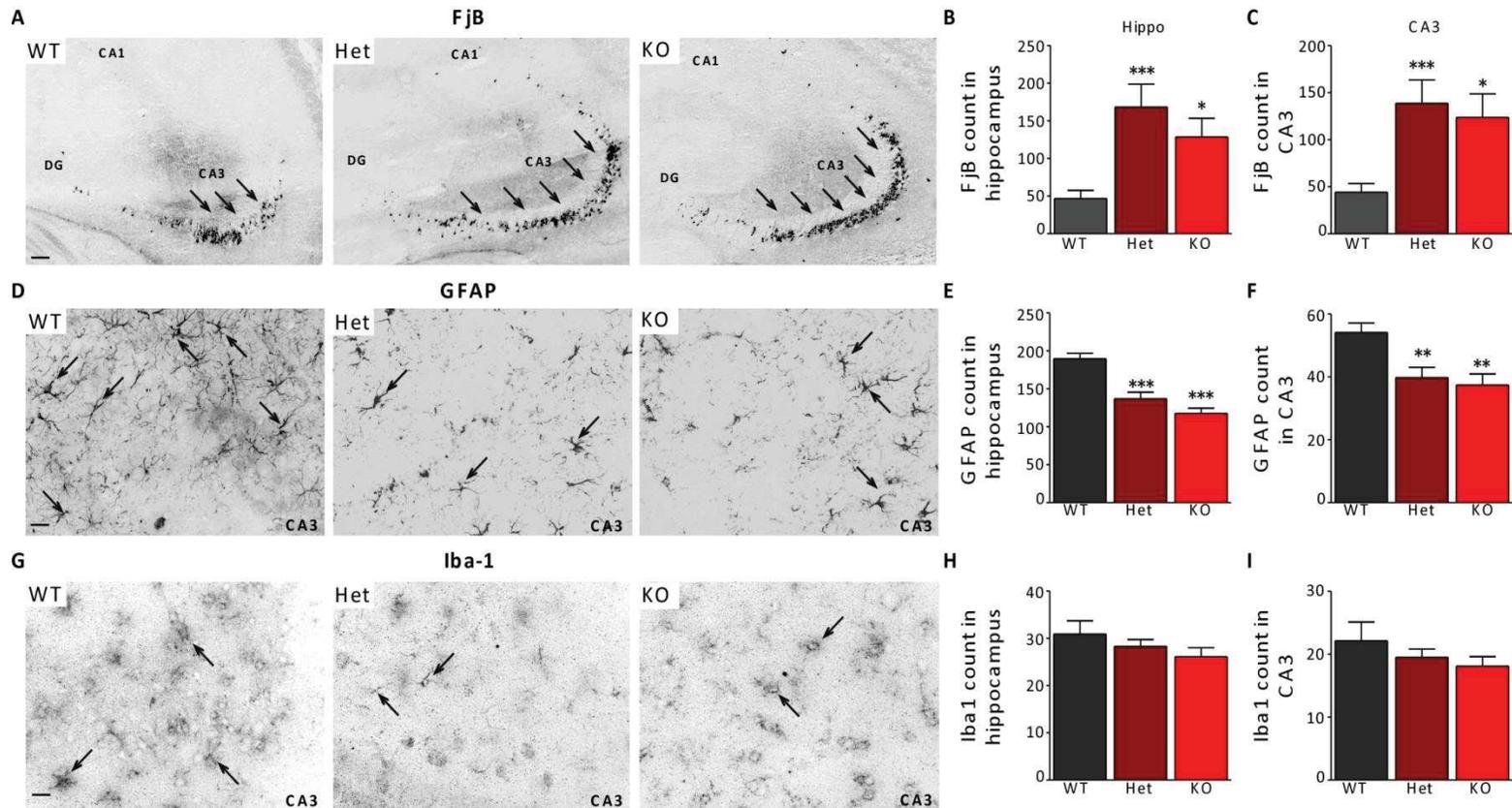
**(A)** Schematic diagram showing the placement of the electrodes on the mouse skull. In each hemisphere, one electrode was placed above the hippocampus. **(B)** Schematic diagram representing a coronal image of the mouse brain, showing the amygdala, region where kainic acid is injected. **(C)** Representative EEG recordings presented as heat maps of frequency and amplitude data during status epilepticus in P2Y<sub>1</sub> knockout mice and wild-type. **(D)** Graphic showing no difference in baseline conditions between wild-type and P2Y<sub>1</sub> knockout mice (n= 3WT and 4 P2Y<sub>1</sub> KO). **(F, G)** Graphics showing increased EEG total power and amplitude after status epilepticus and also **(H, I)** post-status epilepticus in P2Y<sub>1</sub> knockout mice (n= 14 WT, 13 P2Y<sub>1</sub> Het and 11 P2Y<sub>1</sub> KO). **(E)** Graphic showing a faster onset of seizures in P2Y<sub>1</sub> knockout mice when compared to wild-type mice (n= 14 WT, 13 P2Y<sub>1</sub> Het and 11 P2Y<sub>1</sub> KO). \*p< 0.05, \*\*p<0.01.

#### 4.3.2.3 Increased seizure-induced neuronal cell death in P2Y<sub>1</sub> knockout mice after status epilepticus

Next, we investigated brain damage outcomes in P2Y<sub>1</sub> knockout mice after status epilepticus. We hypothesized that the increased seizure severity would be associated with aggravated brain pathology.

As expected, P2Y<sub>1</sub> knockout and heterozygous mice displayed increased neuronal death in the hippocampus after status epilepticus, which was mainly found in the CA3 subfield (**Figure 4.6A-C**). Tissue sections from P2Y<sub>1</sub> knockout and heterozygous mice also displayed fewer astrocytes in the hippocampus when compared to wild-type mice (**Figure 4.6D-F**). Microglia counts were also somewhat lower although this did not reach statistical significance (**Figure 4.6 G-I**).

Thus, while seizure-induced cell death was increased in P2Y<sub>1</sub> knock-out and heterozygous mice, astrocytosis and microglia seemed to be suppressed.



**Figure 4.6 – Increased neuronal death and reduced glia in P2Y<sub>1</sub> knockout mice after status epilepticus.**

(A) Photomicrographs and (B, C) graphs showing an increased in FjB-positive cells in ipsilateral hippocampus, mainly in CA3 region at 24 hours after status epilepticus in P2Y<sub>1</sub> knockout mice and heterozygous when compared to wild-type. (D) Representative GFAP staining and (E, F) graphics of whole hippocampus and CA3 region from wild-type and P2Y<sub>1</sub> knockout and heterozygous mice. (G) Photomicrographs and (H, I) graphs showing no difference in the Iba-1 positive cells counted in the ipsilateral hippocampus at 24 hours after status epilepticus in the P2Y<sub>1</sub> knockout / heterozygous mice and wild-type mice (n= 14 WT, 13 P2Y<sub>1</sub> Het and 11 P2Y<sub>1</sub> KO). Scale bar: 100 μm (FjB) and 50 μm (GFAP, Iba1). \*p< 0.05, \*\*p<0.01, \*\*\*p<0.001.

#### 4.3.2.4 P2Y<sub>1</sub> activation prior to intra-amygdala kainic acid reduces seizure severity during status epilepticus

To complement and extend the genetic studies we used a pharmacological approach, taking advantage of the availability of a specific P2Y<sub>1</sub> receptor antagonist MRS2500 (von Kugelgen and Hoffmann, 2016) and specific P2Y<sub>1</sub> receptor agonist MRS2365 (von Kugelgen and Hoffmann, 2016). Status epilepticus was induced as described before, and MRS2500 or MRS2365 were delivered into the ventricle immediately prior to intra-amygdala kainic acid injection (**Figure 4.7A**).

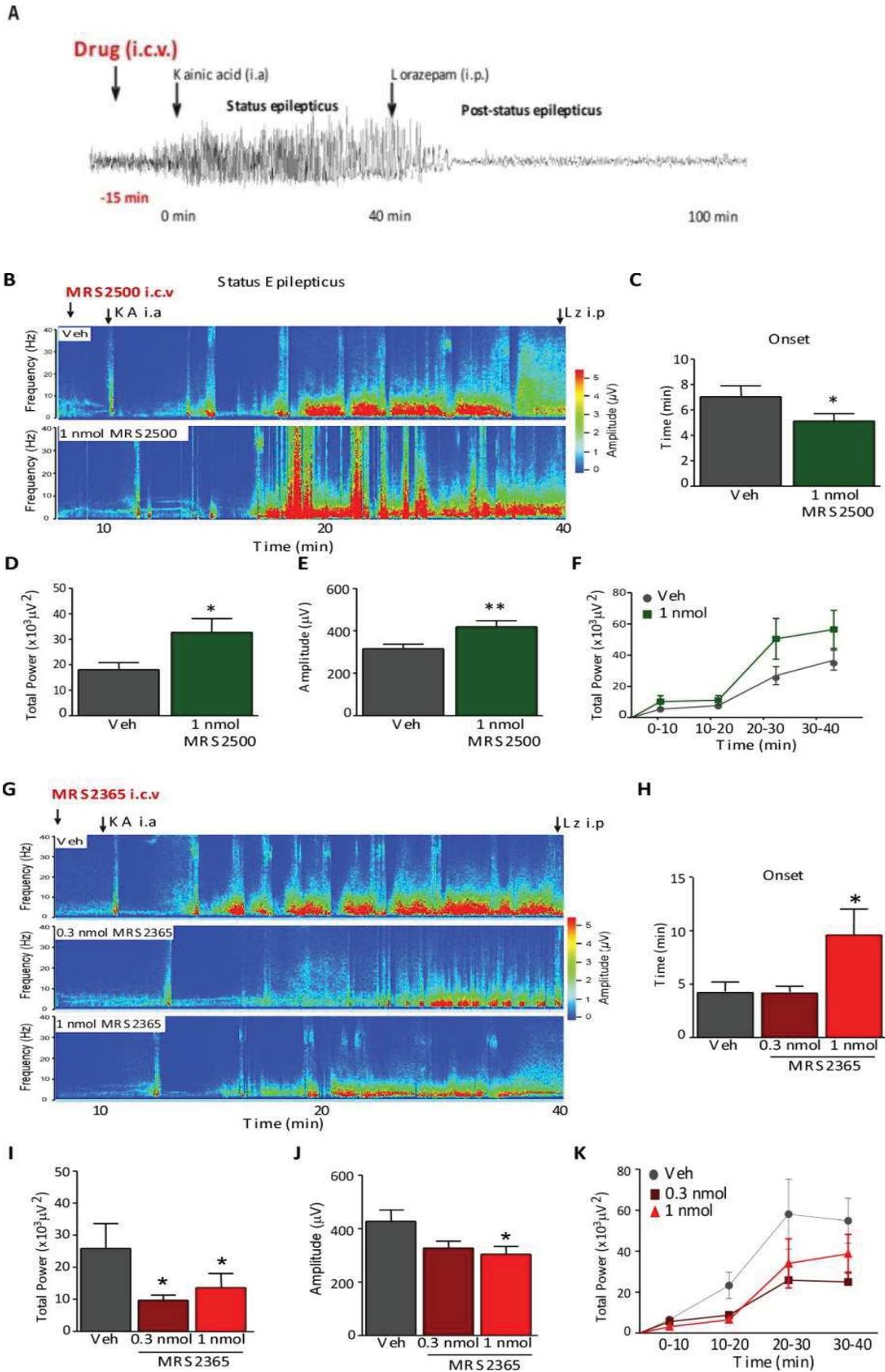
Consistent with P2Y<sub>1</sub>-deficiency resulting in a more severe seizure phenotype, mice pre-treated with the P2Y<sub>1</sub> antagonist MRS2500 (1nmol; dose were selected on the basis of the EC<sub>50</sub> of the compound) experienced increased seizure severity during status epilepticus (**Figure 4.7 B**). Seizures were more severe and of earlier onset when compared to wild-type mice (**Figure 4.7C-E**). Analysis of the total power in intervals of 10 minutes during status epilepticus revealed that the increase on seizure severity start 20 minutes following status epilepticus and progress over time (the result was not statistically significant using 2-way ANOVA analysis) (**Figure 4.7F**). The increase was apparent, however not significant, even 1 hour after lorazepam injection, suggesting that the effects are long-lasting (**Figure 4.8A-C**).

Next, we wanted to explore whether P2Y<sub>1</sub> activation protects the brain from seizure pathology. Here, we used the specific P2Y<sub>1</sub> agonist MRS2365, injected into the ventricle immediately prior to kainic acid as shown in the diagram (0.3 nmol and 1 nmol; again, doses were selected on the basis of the EC<sub>50</sub> of the compound) (**Figure 4.7A**).

Injection of mice with the P2Y<sub>1</sub> agonist MRS2365 before status epilepticus significantly reduced the recorded EEG total power and amplitude during status epilepticus when compared to vehicle-injected animals (**Figure 4.7H, I**). Pre-treatment with the P2Y<sub>1</sub> agonist also delayed seizure onset in the model by approximately 5 minutes (**Figure 4.7H**). Analysis of total power and amplitude in intervals of 10 minutes

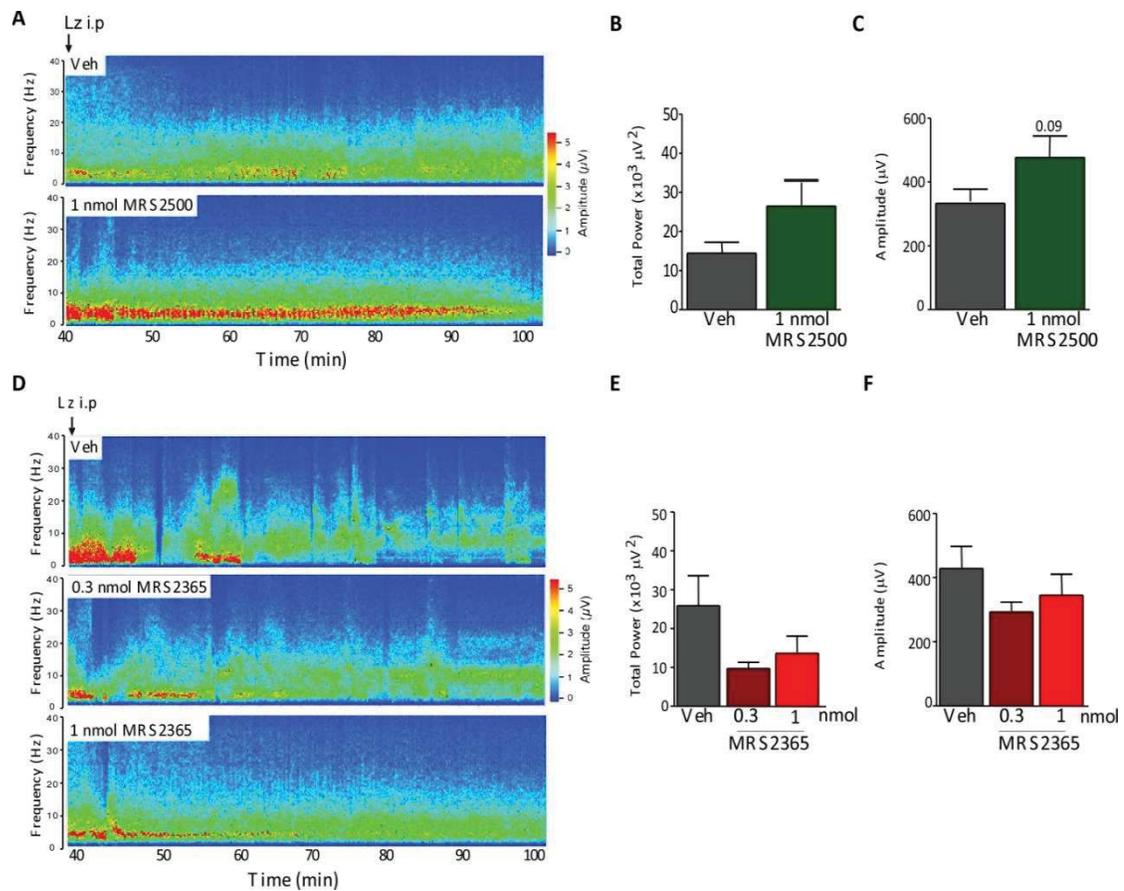
determined that the reduction in seizure severity was apparent within 10 minutes of kainic acid injection and remained over time (the result was not statistically significant using 2-way ANOVA analysis) (**Figure 4.7K**). To evaluate whether the effects of P2Y<sub>1</sub> agonist were long-lasting, we recorded seizures after lorazepam injection (at 40 minutes) for an additional 1 hour as before. EEG total power remained reduced, however not significant, in P2Y<sub>1</sub> agonist treated mice post-status epilepticus (**Figure 4.8D-F**).

These results suggest that P2Y<sub>1</sub> receptor activation may exert an anti-convulsive action when initiated prior to status epilepticus.



#### **Figure 4.7 - Pre-treatment with P2Y<sub>1</sub> targeting agonists and antagonists**

**(A)** Schematic diagram showing the experimental design of the treatment with P2Y<sub>1</sub> receptor ligands in the kainic acid mouse model. 15 minutes prior to kainic acid injection, mice were treated with the P2Y<sub>1</sub> agonist MRS2365 or antagonist MRS2500. 40 minutes after status epilepticus, mice were injected with the anticonvulsant lorazepam. **(B)** Representative EEG recordings presented as heat maps of frequency and amplitude data during status epilepticus in mice treated with P2Y<sub>1</sub> antagonist MRS2500 and vehicle. **(C)** Graphic showing a faster onset of seizure in mice treated with the P2Y<sub>1</sub> agonist MRS2500 **(D, E, F)** Graphics showing increased EEG total power and amplitude after status epilepticus in mice treated with P2Y<sub>1</sub> antagonist MRS2500 (n= 13 Vehicle (Veh), and 13 MRS2500). **(G)** Heat maps of frequency and amplitude data during status epilepticus in mice treated with P2Y<sub>1</sub> agonist MRS2365 and vehicle. **(H)** Graphic showing a significant reduction in the onset of seizures in mice treated with 1nmol of the P2Y<sub>1</sub> specific agonist MRS2365 **(I, J, K)** Graphics showing reduced EEG total power and amplitude after status epilepticus in mice treated with the P2Y<sub>1</sub> agonist MRS2365 when compared to vehicle-injected mice (n= 14 Vehicle (Veh), 9 0.3 nmol MRS2365 and 11 1 nmol MRS2365). \*p< 0.05, \*\*p<0.01.



**Figure 4.8 – P2Y<sub>1</sub> receptor activation decreases seizure severity after status epilepticus.**

(A) Representative EEG recordings presented as heat maps of frequency and amplitude data post-status epilepticus in mice treated with P2Y<sub>1</sub> antagonist MRS2500 and vehicle-treated mice. (B, C) Graphic showing a non-significant increase of total power and amplitude post-status epilepticus in mice pre-treated with P2Y<sub>1</sub> antagonist MRS2500 (n= 13 Vehicle (Veh) and 13 MRS2500). (D) Representative heat maps of frequency and amplitude data post-status epilepticus in mice treated with P2Y<sub>1</sub> agonist MRS2365 and vehicle-injected mice. (E, F) Graphics showing a non-significant decrease of total power and amplitude post-status epilepticus in mice pre-treated with the P2Y<sub>1</sub> agonist MRS2365 when compared to vehicle-treated mice (n= 14 Vehicle (Veh), 9 0.3 nmol MRS2365 and 11 1 nmol MRS2365).

#### 4.3.2.5 Effects of P2Y<sub>1</sub> ligands on hippocampal damage following status epilepticus

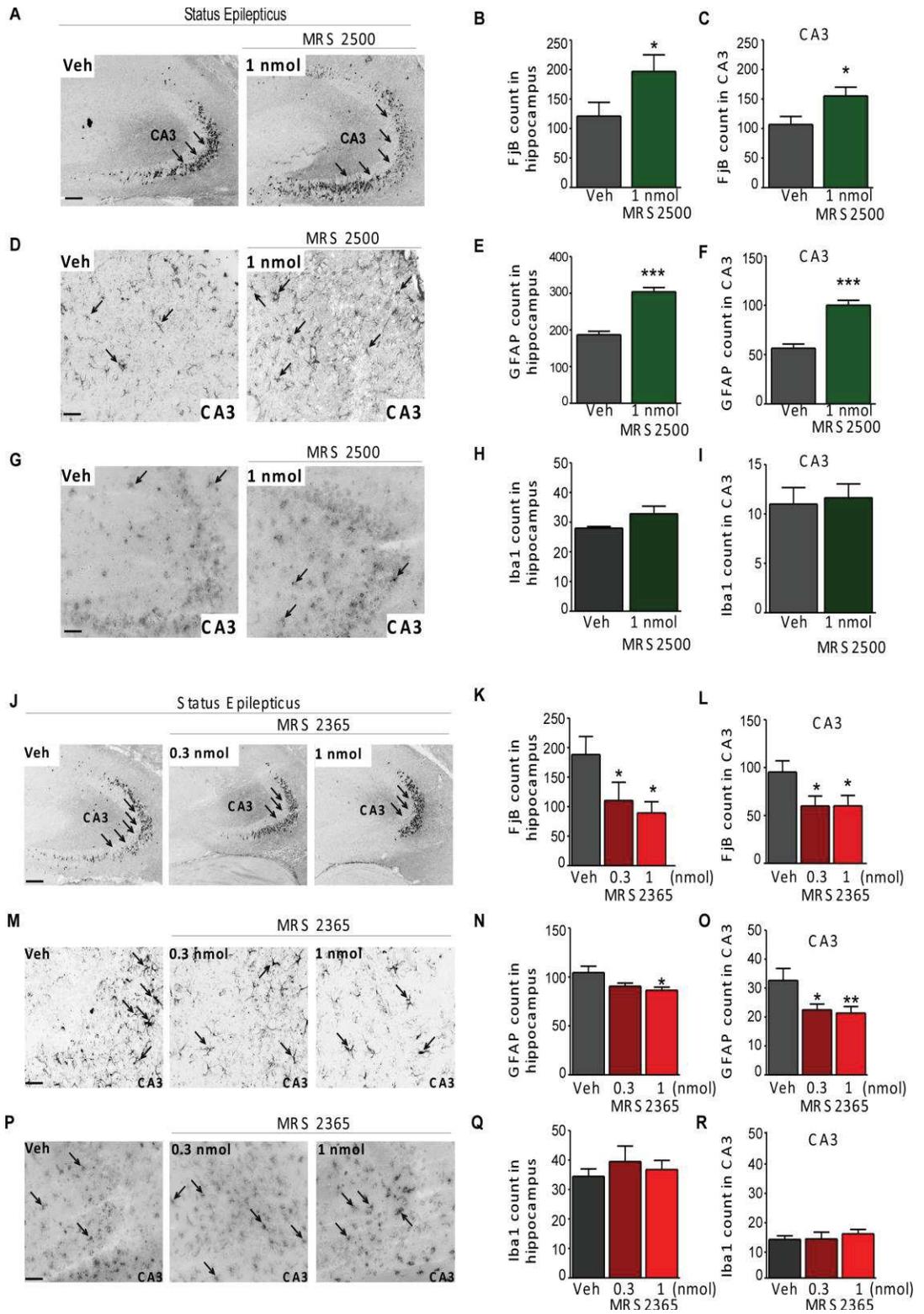
Next, we evaluated whether pre-treatment with either P2Y<sub>1</sub> agonist or antagonist had an effect on seizure-induced cell death.

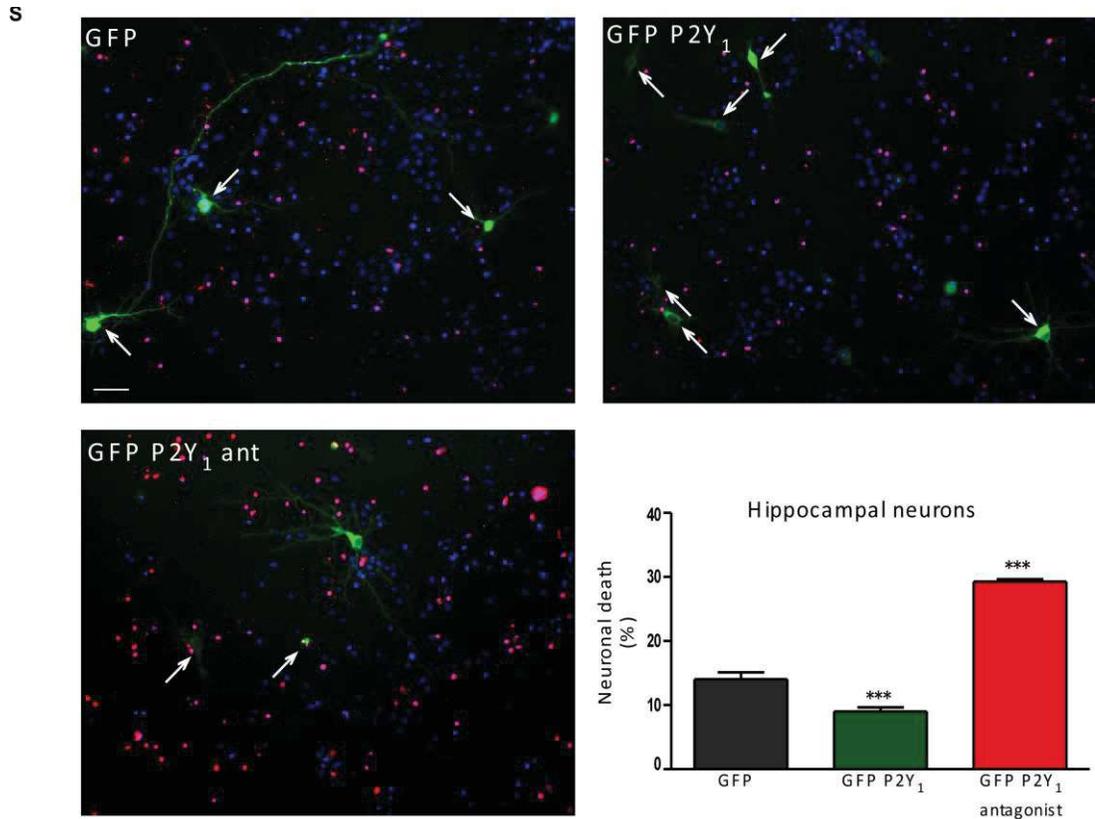
Mice treated with the P2Y<sub>1</sub> antagonist MRS2500 during status epilepticus showed a significant increase in FJB-positive cells in the CA3 (**Figure 4.9A-C**), while mice pre-treated with the P2Y<sub>1</sub> agonist MRS2365 displayed significant less FJB-positive cells in CA3 (**Figure 4.9J-L**), when compared to vehicle-injected mice.

Antagonist pre-treated mice displayed increased staining for the astrocyte marker GFAP compared to vehicle mice after status epilepticus (**Figure 4.9D-F**). Microglia staining was not different between antagonist-treated mice and vehicle-treated mice (**Figure 4.9G-I**). In contrast, astrocyte staining was reduced in the hippocampus of mice treated with the P2Y<sub>1</sub> agonist MRS2365 before status epilepticus particularly in the CA3 region (**Figure 4.9M-O**). Again, no differences were observed in numbers of microglia between mice injected with the P2Y<sub>1</sub> agonist MRS2365 and vehicle-injected mice (**Figure 4.9P-R**).

To support the evidence that P2Y<sub>1</sub> activation protects against seizure-induced cell death, we treated primary hippocampal neurons (transfected with green fluorescence protein (GFP)-P2Y<sub>1</sub>) with kainic acid. Cells expressing the receptor were identified by GFP-P2Y<sub>1</sub>. Kainic acid treatment resulted in death of a significant percentage of primary hippocampal neurons. Neuronal death was significantly lower in GFP-P2Y<sub>1</sub> hippocampal neurons treated with kainic acid (**Figure 4.9S**). GFP-P2Y<sub>1</sub> hippocampal neurons treated with the P2Y<sub>1</sub> receptor antagonist MRS2500 30 minutes prior to kainic acid treatment resulted in an increase in neuronal death, confirming this effect was specific to the P2Y<sub>1</sub> receptor (**Figure 4.9S**).

These results are in line with our *in vivo* experiment, suggesting that the activation of the P2Y<sub>1</sub> receptor has a neuroprotective effect.





**Figure 4.9 – Activation of the P2Y<sub>1</sub> receptor reduces cell death and astrogliosis after status epilepticus.**

(A) Photomicrographs and (B) graphs showing an increased in FjB-positive cells in the total hippocampus and (C) in CA3 24 hours after status epilepticus in mice treated with the P2Y<sub>1</sub> antagonist MRS2500. (D) Representative GFAP staining of hippocampus and (E) correspondent graph showing a significant increase of astrocytes in whole hippocampus and in the CA3 (F) in mice pre-treated with P2Y<sub>1</sub> antagonist MRS2500. (G) Representative Iba1 staining in hippocampus and (H, I) correspondent graph showing no difference in microglia cells counts in mice pre-treated with the P2Y<sub>1</sub> antagonist MRS2500 and vehicle-treated mice (n= 13 Vehicle (Veh) and 13 MRS2500). (J) Photomicrographs and (K) graphs showing a significant decrease in FjB-positive cells in the hippocampus and (L) in CA3 24 hours after status epilepticus in mice treated with P2Y<sub>1</sub> agonist MRS2365. (M) Representative GFAP staining of hippocampus and (N) corresponding graph showing a significant decrease in the number of astrocytes counted in whole hippocampus and CA3 (O) in mice pre-treated with P2Y<sub>1</sub> agonist MRS2365. (P) Representative Iba1 staining of hippocampus and corresponding graph (Q, R) showing no difference in microglia cells counted in the hippocampus of mice pre-treated with P2Y<sub>1</sub> agonist-MRS2365 and vehicle mice (n= 14 Vehicle (Veh), 9 for 0.3 nmol MRS2365 and 11 for 1 nmol MRS2365). Scale bar = 50 μm (Iba1 and GFAP) or 100 μm (FjB). (S) Representative photomicrographs and corresponding graph

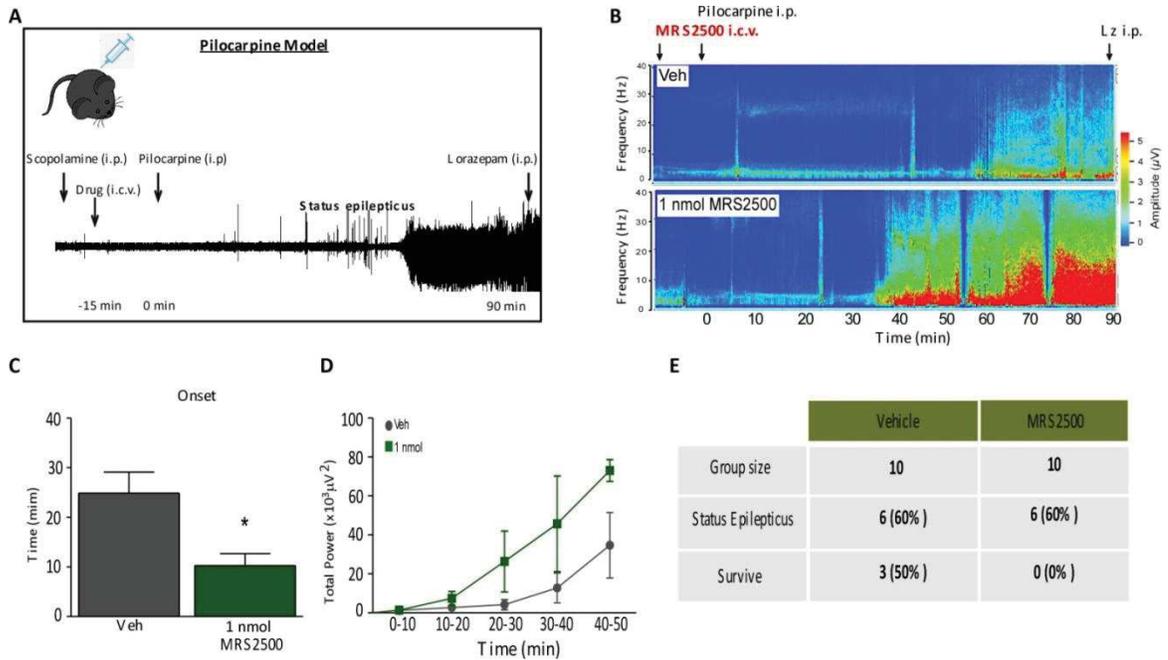
showing a significant reduction in the percentage of neuronal cell death in hippocampal neurons transfected with GFP-P2Y<sub>1</sub> plasmid after kainic acid treatment when compared with GFP control group. This protection was reversed in the presence of the P2Y<sub>1</sub> antagonist MRS2500 (n=6 GFP, 6 GFP-P2Y<sub>1</sub>R and 6 GFP-P2Y<sub>1</sub>R antagonist). Scale bar= 200 μm. \*p< 0.05, \*\*p<0.01, \*\*\*p<0.001.

#### 4.3.2.6 P2Y<sub>1</sub> antagonism reduces seizures in the pilocarpine model during status epilepticus

To assure that the functional response observed using the P2Y<sub>1</sub> receptor agonist and antagonist is a shared response to status epilepticus rather than model-specific, we used a second mouse model where status epilepticus is induced by a systemic injection of pilocarpine, a muscarinic receptor agonist (Curia et al., 2008) (**Figure 4.10A**). In these experiments, we used only the P2Y<sub>1</sub> antagonist MRS2500 (1 nmol), and as before, mice were treated prior to the induction of status epilepticus. EEG was recorded for a period of 90 minutes during status epilepticus (**Figure 4.10B**).

As observed in the kainic acid model, mice pre-treated with P2Y<sub>1</sub> antagonist MRS2500 showed an earlier seizure onset compared to vehicle-injected mice (**Figure 4.10C**). P2Y<sub>1</sub> antagonist-treated mice also showed a trend to increased total power during status epilepticus, starting at 20 minutes after status epilepticus (**Figure 4.10D**). Mortality was also different, with 50% of pilocarpine-treated mice surviving in the vehicle group compared to 0% in the P2Y<sub>1</sub> antagonist MRS2500 group (**4.10E**).

Thus, findings in the pilocarpine model corroborate the kainic acid mouse model, demonstrating a functional role for the P2Y<sub>1</sub> receptor during status epilepticus.



**Figure 4.10 - Effects of P2Y<sub>1</sub> receptor antagonist in seizures generation during status epilepticus in the pilocarpine model.**

(A) Schematic diagram showing the experimental design using P2Y<sub>1</sub> receptor antagonist MRS2500 in the pilocarpine mouse model. (B) Representative EEG recordings represented as heat maps of frequency and amplitude data in mice treated with P2Y<sub>1</sub> antagonist MRS2500 and vehicle. (C) Graphic showing an earlier seizure onset in mice treated with the P2Y<sub>1</sub> antagonist MRS2500. (D) Graph showing increased EEG total power in mice treated with P2Y<sub>1</sub> antagonist MRS2500 (n= 6 Vehicle (Veh) and 6 MRS2500). (E) Table showing the total number of mice used in the experiment, the number of mice that developed status epilepticus and the survival rate. \*p< 0.05.

### 4.3.3 Pharmacological targeting of the P2Y<sub>1</sub> receptor after induction of status epilepticus

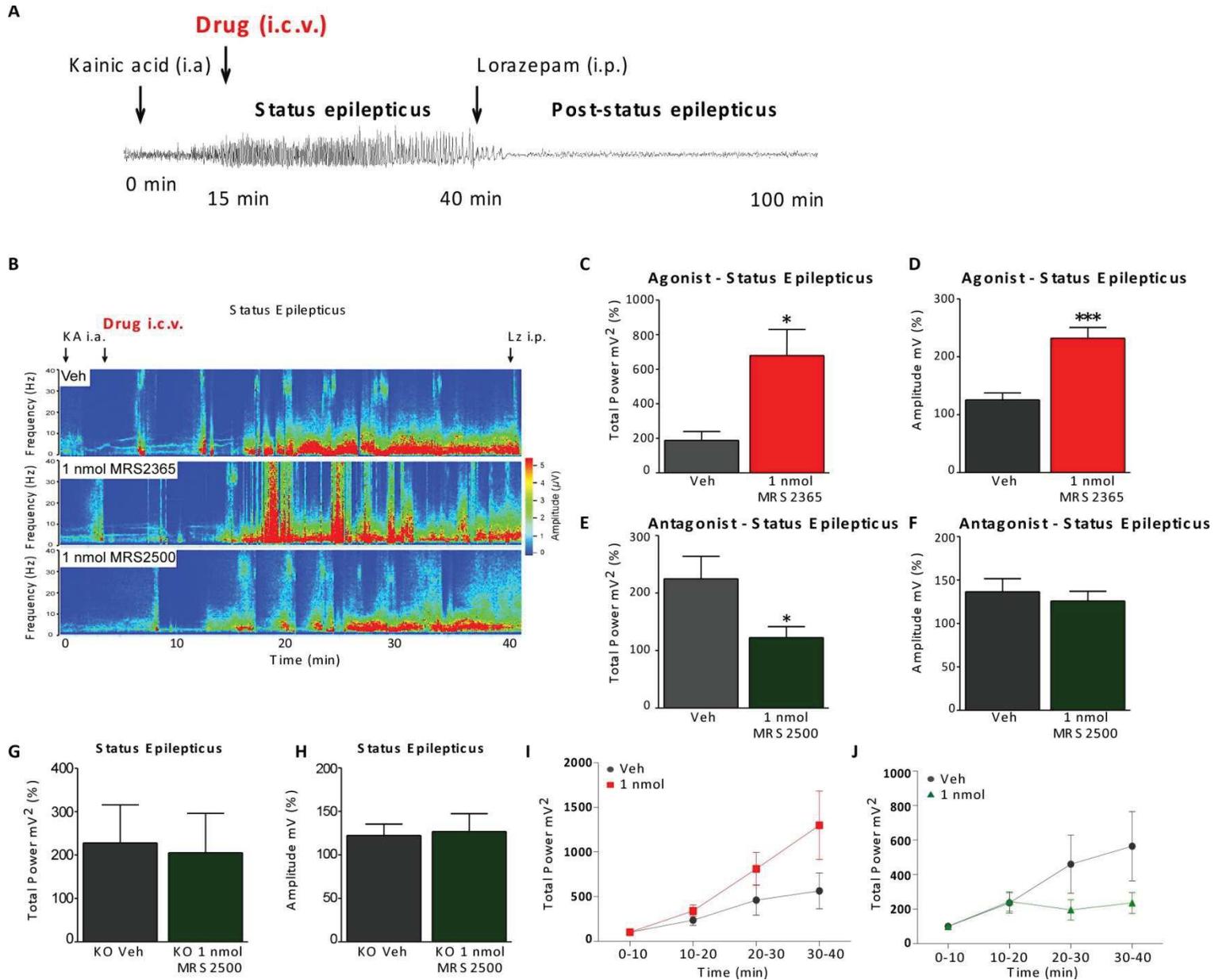
#### 4.3.3.1 Post-treatment with P2Y<sub>1</sub> receptor antagonist reduces seizure generation in the kainic acid mouse model

Operationally, clinicians will initiate aggressive anti-seizure therapy if seizures continue beyond 5 minutes. The above studies reveal P2Y<sub>1</sub> receptor ligands may have therapeutic potential for status epilepticus. However, the pre-treatment regime is not a clinically realistic scenario. Accordingly, we next performed experiments to test whether manipulating the P2Y<sub>1</sub> receptor after status epilepticus begins can alter seizure or pathologic outcomes. Thus, mice received i.c.v. injections of the same doses of either the P2Y<sub>1</sub> antagonist MRS2500 or agonist MRS2365 15 minutes following intra-amygdala kainic acid injection, once mice had experienced at least one seizure burst (**Figure 4.11A, B**).

Surprisingly and in contrast to what had been observed in the pre-treatment studies, injection of mice with the P2Y<sub>1</sub> agonist MRS2365 after kainic acid injection resulted in an exacerbation of seizure severity during status epilepticus (**Figure 4.11C, D**). This was apparent at 20 minutes post-kainate injection (**Figure 4.11I**).

Next, we investigated what happens when the P2Y<sub>1</sub> receptor is inhibited after status epilepticus is underway. Seizure severity was reduced in mice injected with the P2Y<sub>1</sub> antagonist MRS2500 15 minutes after kainic acid injection (**Figure 4.11E, F**). This effect appeared within a few minutes of injection (**Figure 4.11J**).

To support the specificity of the P2Y<sub>1</sub> receptor antagonist MRS2500, we injected P2Y<sub>1</sub> knockout mice with P2Y<sub>1</sub> antagonist MRS2500 15 minutes after intra-amygdala kainic acid injection and no effects on seizure severity in the P2Y<sub>1</sub> knockout mice was observed, confirming the specificity of the antagonist (**Figure 4.11G, H**).



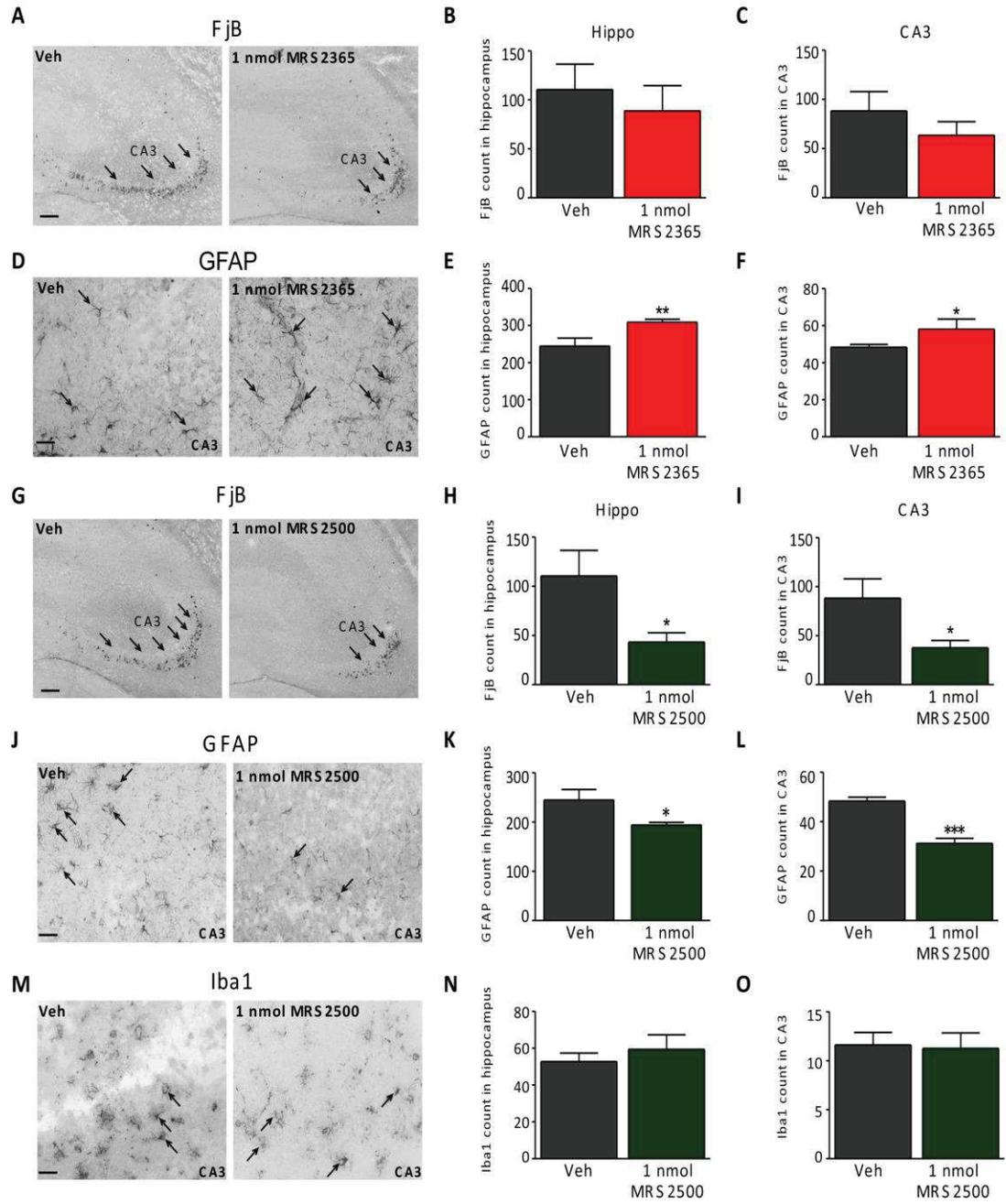
**Figure 4.11 – Inhibition of the P2Y<sub>1</sub> receptor reduces seizure severity in the kainic acid model.**

**(A)** Schematic diagram showing the experimental design of the treatment with P2Y<sub>1</sub> receptor ligands in the kainic acid mouse model. 15 minutes after kainic acid injection, mice were treated with P2Y<sub>1</sub> agonist (MRS2365) or antagonist (MRS2500). 40 minutes after status epilepticus, mice were injected with lorazepam. **(B)** Representative EEG recordings presented as heat maps of frequency and amplitude data during status epilepticus in mice treated with MRS2365, MRS2500 and vehicle. **(C, D, I)** Graphic showing an increase of seizure severity in mice treated with P2Y<sub>1</sub> agonist (n=7 Vehicle (Veh) and 9 MRS2365). **(E, F, J)** Graphics showing a significant reduction in the total power and amplitude in the EEG during 40 minutes of status epilepticus in mice treated with P2Y<sub>1</sub> antagonist MRS2500 when compared with vehicle-injected mice (n=7 Vehicle (Veh) and 8 MRS2500). **(G, H)** Graphs showing no differences in the total power and amplitude in P2Y<sub>1</sub>R KO mice treated with P2Y<sub>1</sub> receptor antagonist-MRS2500 and vehicle, confirming the specificity of the antagonist (n=4 Vehicle (Veh) and 4 MRS2500). \*p< 0.05.

#### **4.3.3.2 Post-treatment with P2Y<sub>1</sub> receptor antagonist reduces seizure-induced cell death and astrogliosis in the kainic acid model**

We next examined brain sections from mice from our post-treatment studies. Despite undergoing more severe seizures, FjB-positive cell counts were slightly lower in mice injected with the P2Y<sub>1</sub> agonist MRS2365 during status epilepticus when compared to vehicle-injected mice (**Figure 4.12A-C**). As expected, antagonist-treated mice had lower counts of FjB-positive cells in the hippocampus when compared to vehicle-injected mice. This was most evident in the CA3 subfield of the hippocampus (**Figure 4.12G-I**).

Finally, we analysed glial markers in tissue sections obtained from vehicle and P2Y<sub>1</sub> receptor antagonist/agonist-treated mice 24 hours after status epilepticus. Astrogliosis was significantly increased in the hippocampus of mice treated with the P2Y<sub>1</sub> receptor agonist MRS2365, and the most prominent increase was observed in the CA3 subfield (**Figure 4.12D-F**). On the other hand, astrogliosis was significantly reduced in the hippocampus of mice treated with the P2Y<sub>1</sub> receptor antagonist MRS2500 (**Figure 4.12J-L**). No differences were observed in microglia counts in the hippocampus of mice treated with the P2Y<sub>1</sub> antagonist (**Figure 4.12M-O**).



**Figure 4.12– Inhibition of the P2Y<sub>1</sub> receptor reduces seizures-induced neuronal death and astrogliosis.**

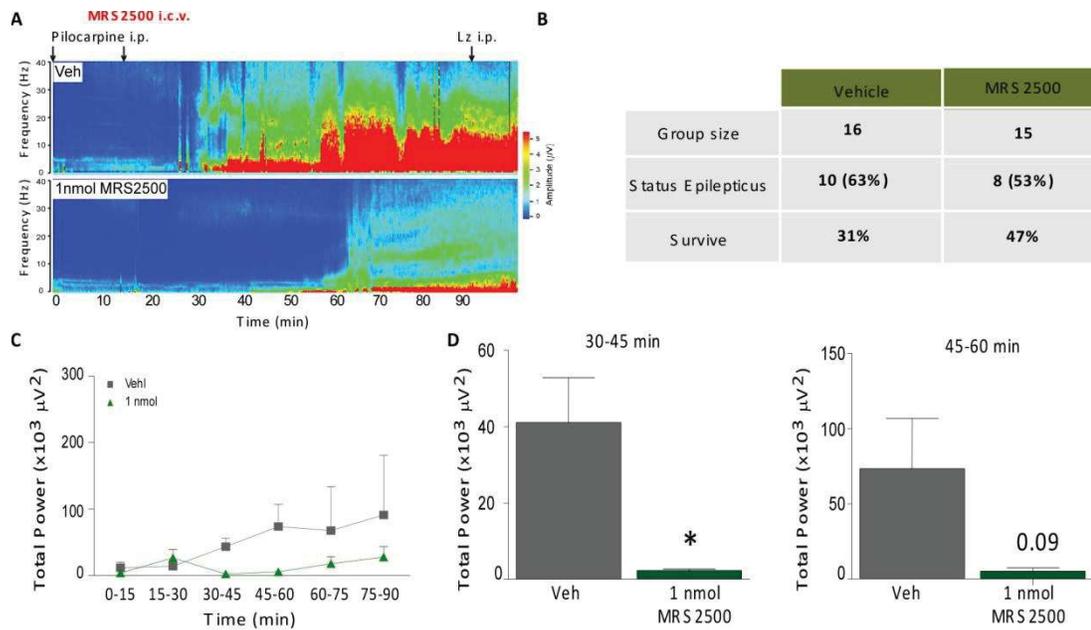
Representative images (A) and graph (B) showing a non-significant reduction in FjB-positive cells 24 hours after status epilepticus in mice treated with P2Y<sub>1</sub> receptor agonist MRS2365 in whole hippocampus and (C) in CA3. (D) Photomicrographs and (E) graphs showing an increase in GFAP-positive cells in the total hippocampus and (F) in CA3 24 hours after status epilepticus in mice treated with P2Y<sub>1</sub> receptor agonist MRS2365 (n=7 Vehicle (Veh) and 9 MRS2365). (G) Representative images and (H, I) corresponding graphs showing a significant decrease in FjB-positive cells 24 hours after status epilepticus in mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500. (J) Photomicrographs and (K, L) graph showing a reduction in the number of astrocytes counted in the hippocampus of mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500. (M) Representative images of Iba1 staining and (N, O) corresponding graphs showing no differences in the number of microglia cells in the hippocampus of mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500 when compared with vehicle-injected mice (n=7 Vehicle (Veh) and 8 MRS2500). Scale bar 100 μm in the FjB images and 50 μm in the GFAP and Iba1 images. \*p< 0.05, \*\*p<0.01, \*\*\*p<0.001.

#### **4.3.3.3 Post-treatment with P2Y<sub>1</sub> receptor antagonist reduces seizure severity during status epilepticus in the pilocarpine model**

To ensure that above findings were not unique to the kainic acid model, select experiments were repeated in the pilocarpine model.

Consistent with findings in the kainic acid model, injection of mice with the P2Y<sub>1</sub> receptor antagonist MRS2500 (1 nmol) during status epilepticus reduced EEG total power (**Figure 4.13A, C**), mainly at 30-45 minutes (**Figure 4.13C, D**). In line with P2Y<sub>1</sub> antagonism being protective when administered after the induction of status epilepticus, the survival rate in mice treated with the P2Y<sub>1</sub> receptor antagonist MRS2500 is higher (47%) compared to vehicle-injected mice (31%) (**Figure 4.13B**).

Thus, these results confirm that P2Y<sub>1</sub> antagonism when applied after the induction of status epilepticus acts as anticonvulsant.



**Figure 4.13 – P2Y<sub>1</sub> receptor antagonism reduces seizure severity in the pilocarpine model.**

(A) Representative EEG recordings presented as heat maps of frequency and amplitude data during status epilepticus in mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500 and vehicle. (B) Table showing the number of mice used in this experiment, the number of mice that developed status epilepticus and the survival rate in P2Y<sub>1</sub> receptor antagonist MRS2500 treated mice and vehicle-injected mice. (C, D) Graphic showing a decrease of seizure severity in mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500 compared to vehicle-treated mice (n=10 Vehicle (Veh) and 8 MRS2500). \*p< 0.05.

#### 4.3.4 P2Y<sub>1</sub>-driven microglia activation contributes to seizures during status epilepticus

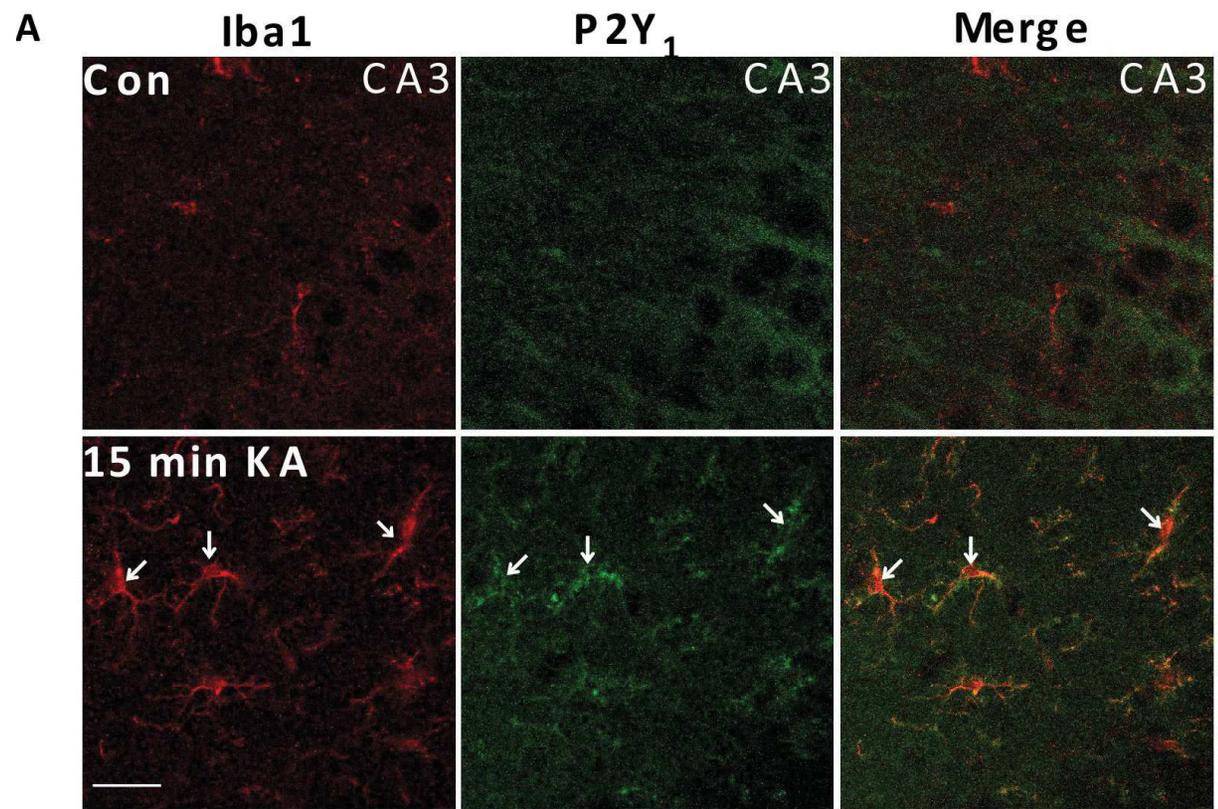
We next aimed to understand the mechanism by which modulating P2Y<sub>1</sub> receptor activity can produce such divergent response depending on when the receptor is targeted. One potential explanation is that as seizures begin there is a change in the cellular distribution of the receptor. This could then lead to different signaling pathways and responses than would occur under physiological conditions. At the beginning of this Chapter (4.3.1) we found that seizures caused the P2Y<sub>1</sub> receptor to appear more on the surface of microglia as well as on neurons. We therefore hypothesized that microglia may be activated by the P2Y<sub>1</sub> receptor in a manner similar to that reported for other P2 receptors such as P2X7. Indeed, the P2X7 emerges on microglia shortly after status epilepticus and is sufficient to drive microglia into a pro-inflammatory state (Monif et al., 2009). Targeting the P2X7 receptor after status epilepticus begins reduces seizures and neuroinflammation (Engel et al., 2012).

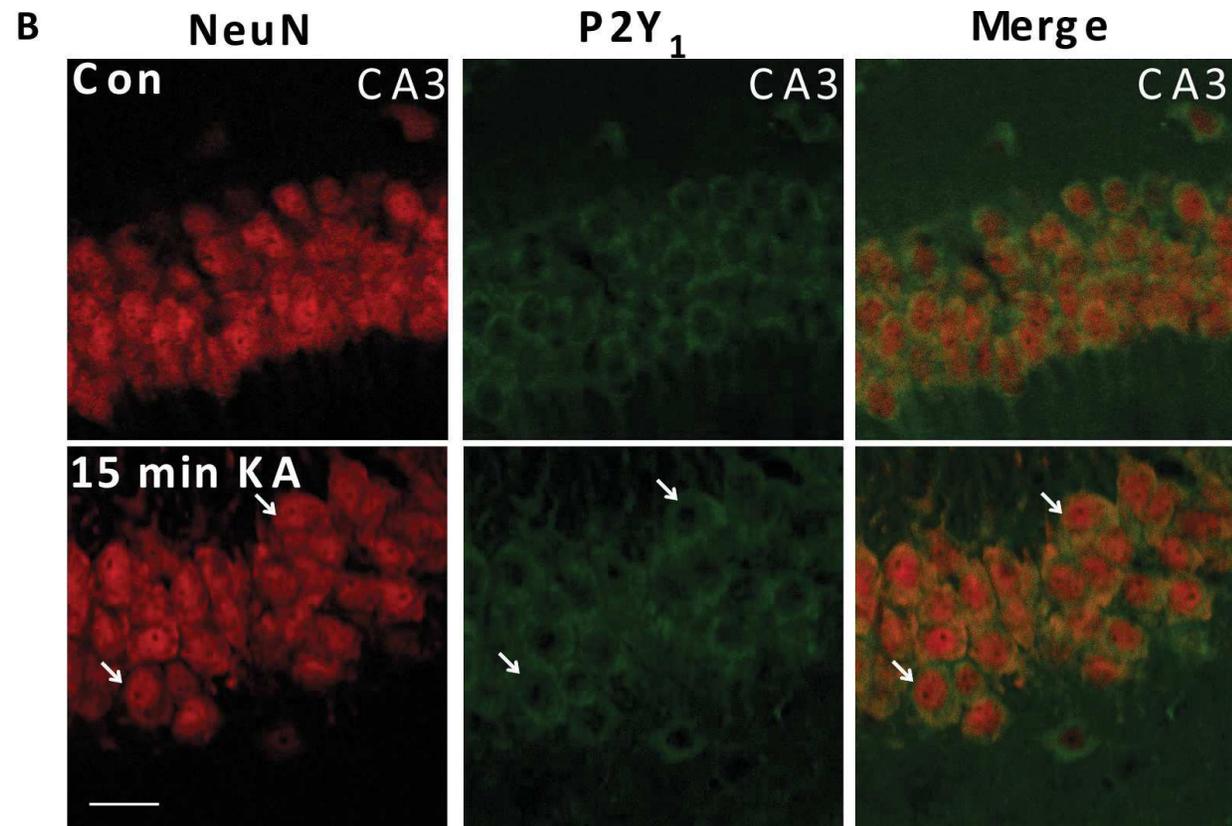
To test our hypothesis, we first performed immunostaining on brain tissue sections collected 15 minutes following kainic acid injection. We observed that P2Y<sub>1</sub> receptor expression was already increased at this time in microglia, but not yet in neurons (**Figure 4.14A-C**). These findings are consistent with status epilepticus driving an early appearance of P2Y<sub>1</sub> receptors in microglia that might influence their inflammatory state. To test whether microglial P2Y<sub>1</sub> receptor activity exerts a pro-inflammatory effect, we tested the effects of the broad spectrum anti-inflammatory drug minocycline in combination with manipulations of the P2Y<sub>1</sub> receptor. Minocycline has been suggested to be an inhibitor of microglia activation, blocking the proliferation of these cells (Hiragi et al., 2018). In order to confirm that minocycline is regulating microglia rather than astrocytes, mice were treated with minocycline and qPCR was performed to evaluate the levels of *Iba-1* and *GFAP* in the hippocampus. Our results confirmed that treating mice with minocycline significantly reduced hippocampal *Iba-1* levels (**Figure 4.15A**) but did not affect hippocampal *GFAP* levels (**Figure 4.15B**).

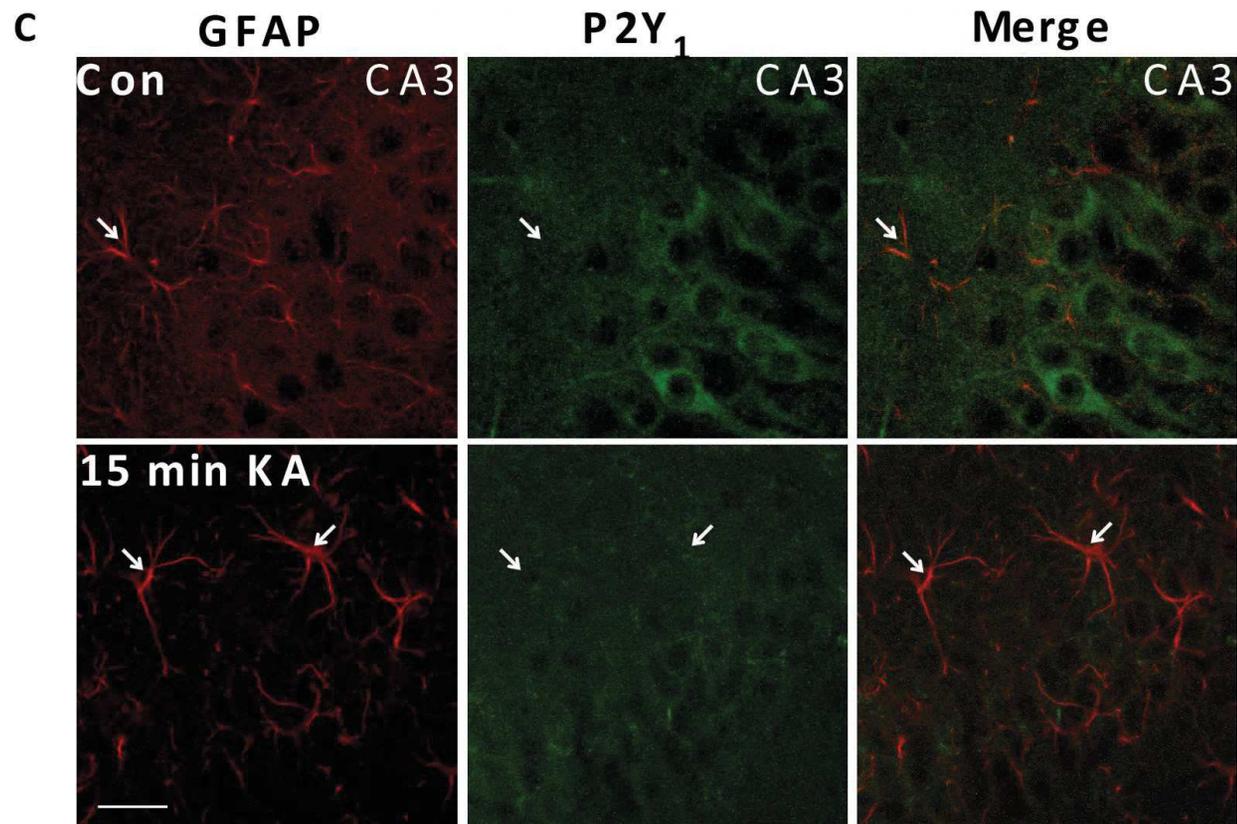
In our next experiment we measured seizure severity in mice given i.p. minocycline (30 mg/Kg) in combination with post-treatment with the P2Y<sub>1</sub> receptor antagonist MRS2500 (i.c.v., 1 nmol) or vehicle (**Figure 4.16A**). EEG recordings showed that co-delivery of minocycline with P2Y<sub>1</sub> antagonism resulted in increased seizure severity (**Figure 4.16B-D**).

To further explore the effects of the P2Y<sub>1</sub> antagonist on microglia activation, microglia cells were isolated from brain tissue and treated with glutamate (100 μM) or glutamate and P2Y<sub>1</sub> antagonist (10 μM). Our results show that the inhibition of the P2Y<sub>1</sub> receptor blocks the activation of microglia (**Figure 4.17A, B**) further supporting a role P2Y<sub>1</sub> in microglia regulation.

Thus, our results suggest that the effects of the P2Y<sub>1</sub> receptor on seizure severity are partly mediated via driving microglia activation.

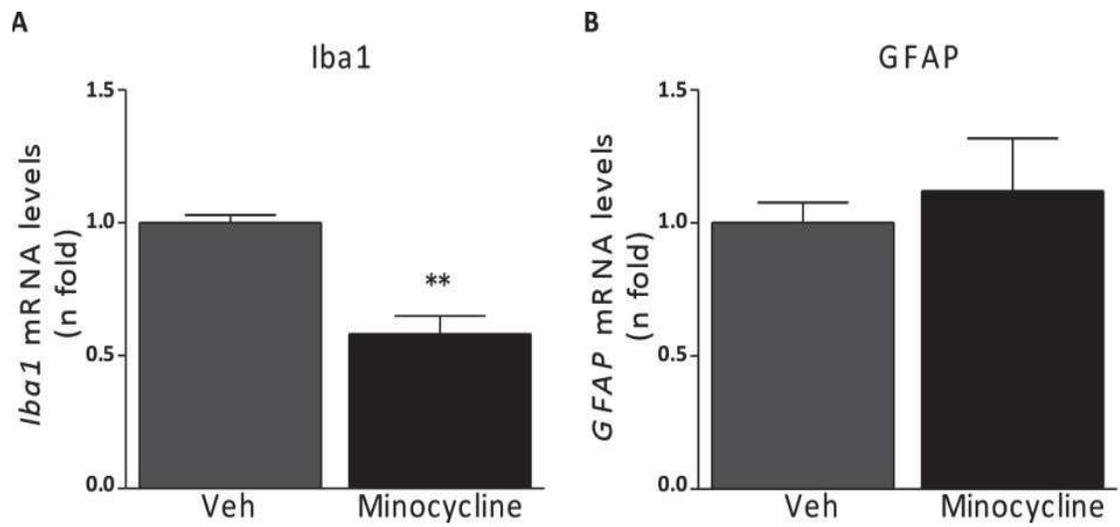






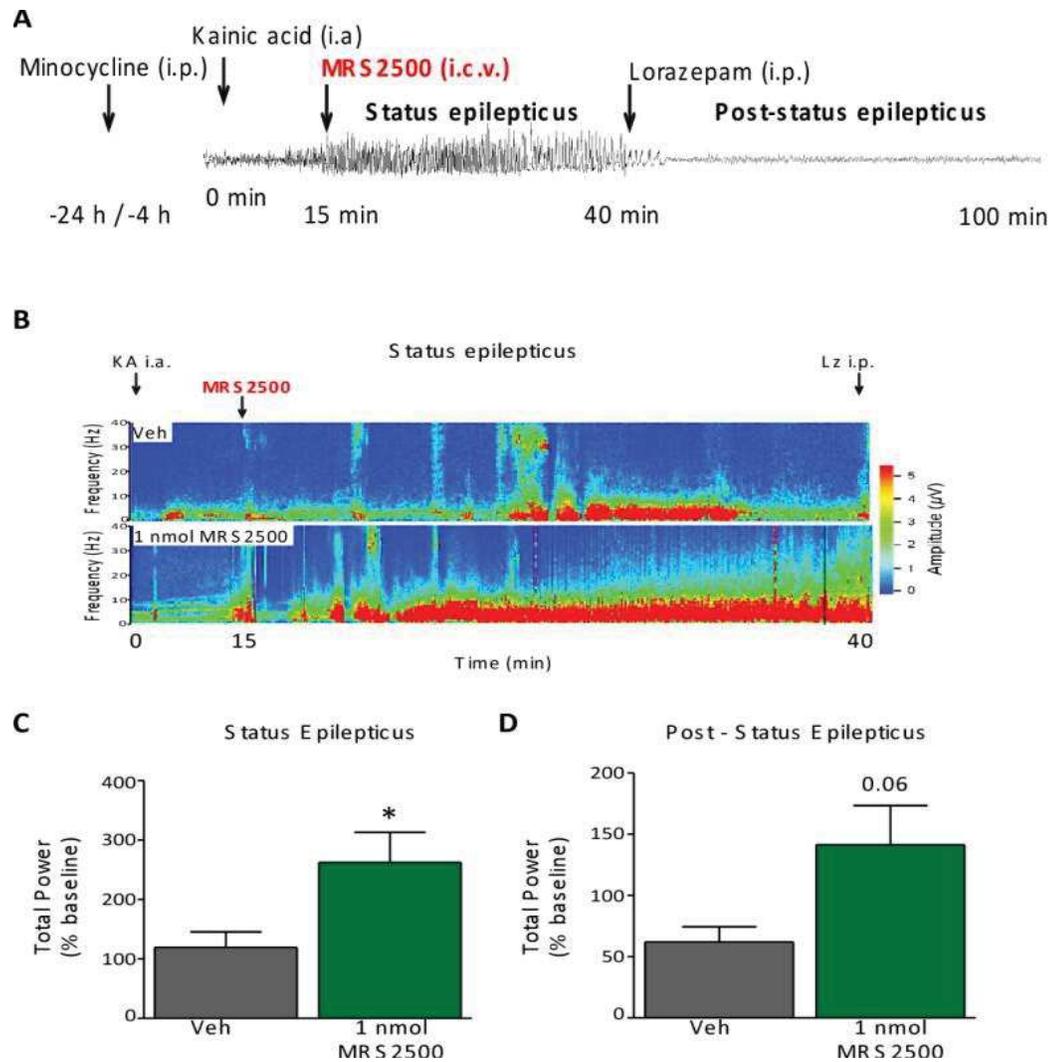
**Figure 4.14 – P2Y<sub>1</sub> receptor is expressed in microglia and neurons.**

**(A)** Photomicrograph showing co-localization of P2Y<sub>1</sub> (Green) with Iba1 (red), specific marker of microglia cells, 15 minutes after kainic acid injection in the CA3 region of the hippocampus. **(B)** Immunofluorescent staining revealing that P2Y<sub>1</sub> receptor (green) is expressed in neurons (red) in the hippocampus (CA3 region). **(C)** Immunofluorescent staining showing no co-localization of the P2Y<sub>1</sub> receptor (green) with astrocytes (red) in the hippocampal CA3 region. Scale bar= 50µm. Note that white arrows indicate the co-localization of the P2Y<sub>1</sub> receptor with microglia **(A)** and neurons **(B)** in the hippocampus.



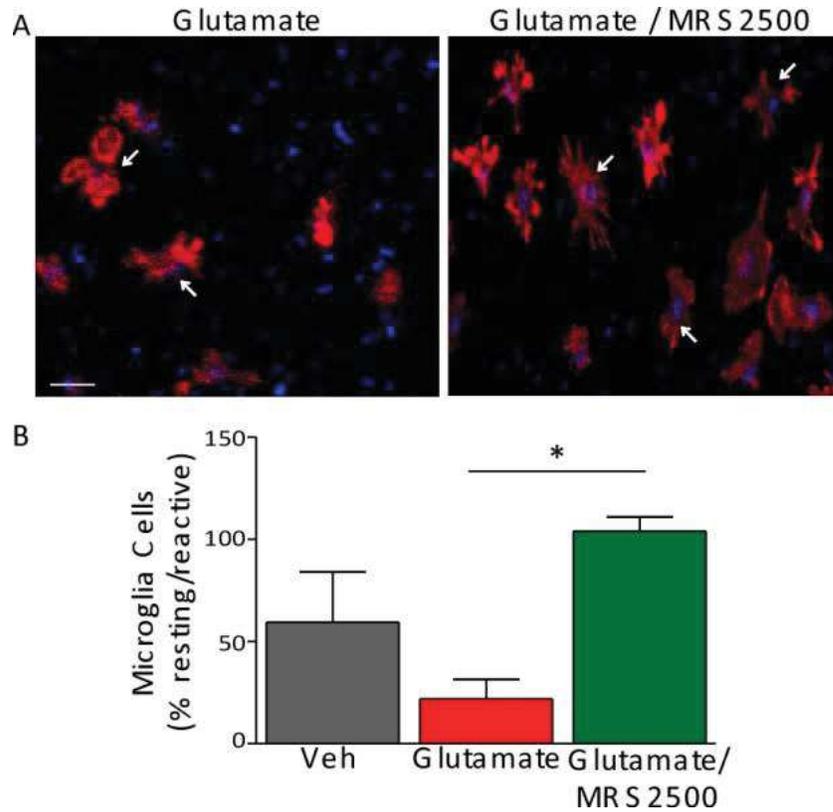
**Figure 4.15 – Minocycline inhibits microglia activation.**

**(A)** Graph showing a significant reduction of Iba-1 mRNA levels after treatment with minocycline. **(B)** Graph showing no differences in the mRNA levels of GFAP in the hippocampus after treatment with minocycline when compared with vehicle group (n= 4 vehicle (Veh) and 5 minocycline). \*\*p<0.01.



**Figure 4.16 – P2Y<sub>1</sub> receptor increases seizure severity through inflammation.**

**(A)** Schematic diagram showing the experimental design of the treatment with P2Y<sub>1</sub> receptor antagonist in kainic acid mouse model. Mice were treated with minocycline (30 mg/Kg, i.p.) 2 hours and 24 hours prior kainic acid-induced status epilepticus. 15 minutes after i.a. kainic acid injection, mice were treated with P2Y<sub>1</sub> receptor antagonist MRS2500, followed by i.p injection of lorazepam 40 minutes after status epilepticus. **(B)** Representative EEG recordings presented as heat maps of frequency and amplitude data during status epilepticus in mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500 and vehicle. **(C, D)** Graphic showing a significant increase of total power and amplitude in mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500 during status epilepticus (n= 4 vehicle (Veh) and 4 MRS2500). \*p< 0.05.



**Figure 4.17 – P2Y<sub>1</sub> receptor antagonism blocks activation of microglia cells *in vitro*.**

(A) Immunofluorescent staining showing the different morphology of microglia cells (red) in the presence of glutamate or glutamate / MRS2500 (n=3 different microglia culture/ group). Scale bar= 35  $\mu$ m. (B) Graph showing a significant increase in the percentage of the resting/reactive microglia cells treated with P2Y<sub>1</sub> receptor antagonist MRS2500 (n=3 different microglia cultures /group). \*p< 0.05.

#### 4.4 Discussion of the results

In Chapter 4, we demonstrate that P2Y<sub>1</sub> activation has a unique and remarkable dual effect on seizures and seizure-induced pathology according to the time-point at which the P2Y<sub>1</sub> receptor is stimulated. We showed that treatment with agonist of the P2Y<sub>1</sub> receptor prior to status epilepticus protects the brain from seizures and damage whereas a P2Y<sub>1</sub> antagonist aggravated seizures and brain damage. However, when the receptor was manipulated after the induction of status epilepticus, P2Y<sub>1</sub> agonists had the opposite effect, increasing seizure severity and neurodegeneration. P2Y<sub>1</sub> antagonists given during status epilepticus reduced seizures and protected the brain from damage. Finally, we showed that the pro-convulsive effects of P2Y<sub>1</sub> during status epilepticus seemed to be mediated via microglia activation. Thus, our results suggest that P2Y<sub>1</sub> protects the brain from damage when stimulated before status epilepticus induction, however when activated during status epilepticus, P2Y<sub>1</sub> seems to aggravate seizure pathology, which seems to be a cell-specific action.

A role for the P2Y<sub>1</sub> receptor has been described in an array of different brain diseases including acute and chronic diseases of the CNS such as TBI (Choo et al., 2013, Talley Watts et al., 2013), brain ischemia (Carmo et al., 2014) and, more recently, epilepsy (Simoes et al., 2018). However, there have been apparently conflicting findings in several studies. P2Y<sub>1</sub> receptor activation significantly reduced post-injury symptoms of mechanical trauma in mice, including edema, neuronal swelling and reactive gliosis (Talley Watts et al., 2013). In contrast, P2Y<sub>1</sub> receptor antagonism has been reported to improve cognitive deficits and reduce damage in other models of TBI (Choo et al., 2013, Carmo et al., 2014). These findings suggest the functional consequences of P2Y<sub>1</sub> receptor modulation may be highly context-specific. The mechanistic basis for this is unknown.

Mounting evidences suggests a functional role for different P2 receptors during status epilepticus including both ionotropic P2X receptors and metabotropic P2Y

receptors. Recently, however, P2Y receptors have also been linked to status epilepticus (Alves et al., 2017, Alves et al., 2018, Simoes et al., 2018).

In our first findings, we have shown that under physiological conditions the P2Y<sub>1</sub> receptor is expressed in neurons. This is in agreement with other previous studies (Moore et al., 2000). Although we did not focus on the specific expression patterns within neurons, previous work showed P2Y<sub>1</sub> is expressed both in the soma and mossy fiber terminals of the CA3 subfield of the hippocampus (Csolle et al., 2008). This would be consistent with a physiological function in reducing neurotransmitter release. We did not observe P2Y<sub>1</sub> under baseline conditions in either microglia or astrocytes in the mouse hippocampus. This is in contrast to other studies where functional P2Y<sub>1</sub> receptor has been suggested to be expressed in astroglia (Fischer et al., 2009a). The observed differences could be due to antibodies used and the detection rate in astrocytes and microglia might be below antibody threshold. Future studies using electrophysiology may shed a better light on the cell-specific expression of P2Y<sub>1</sub> receptor in these cell types.

In the present study we found that status epilepticus caused a strong upregulation of the P2Y<sub>1</sub> receptor in neurons but also in microglia. Again, this fits with previous studies showing microglia upregulate the P2Y<sub>1</sub> receptor following transforming growth factor- $\beta$  (TGF- $\beta$ ) addition (De Simone et al., 2010). As under physiological conditions, P2Y<sub>1</sub> was not expressed by astrocytes following status epilepticus. This contrasts with other reports that observed on astrocytes under different pathological conditions, including oxidative stress (Shinozaki et al., 2006, Fujita et al., 2009) and ischemia neuronal damage (Zheng et al., 2010). Where astrocyte expression has been reported, this has been linked with protective effects. For example, over-activation of P2Y<sub>1</sub> receptor was reported to prevent damage in astrocytes caused by oxidative stress (Shinozaki et al., 2006) or prevent neuronal damage through IL-6 release (Fujita et al., 2009). Also, treatment with P2Y<sub>1</sub> agonist 2-MeSADP reduced cytotoxic edema and ischemia lesions through enhanced astrocyte mitochondrial metabolism due to increased inositol trisphosphate-dependent Ca<sup>2+</sup>

release (Zheng et al., 2010). We do not know why P2Y<sub>1</sub> expression on astrocytes is not increased following status epilepticus. Again, this might be due to different antibodies used. In our studies we included specificity controls and demonstrated loss of the staining in P2Y<sub>1</sub> knock-out tissue as a negative control. P2Y<sub>1</sub> expression may also increase according to insult in a cell-specific manner.

Another finding was the subfield-specific regulation of the P2Y<sub>1</sub> receptor during status epilepticus. To the best of our knowledge no transcription factors which drive P2Y<sub>1</sub> receptor expression have been identified, however, because P2Y<sub>1</sub> is downregulated in the cell-death prone CA3 subfield of the hippocampus and upregulated in the from cell death protected hippocampal subfields CA1 and DG, it is tempting to speculate that these may be protective mechanisms. P2Y<sub>1</sub> may also be regulated via changes in its degradation. We have previously shown that status epilepticus leads to a subfield-specific inhibition of the ubiquitin-proteasome system (UPS), with proteasome inhibition being most evident in the CA1 and DG subfield of the hippocampus (Engel et al., 2017), brain region showing the strongest increase in P2Y<sub>1</sub> expression. Subfield-specific changes in seizure-induced changes in receptor internalization may also account for the subfield specific changes in P2Y<sub>1</sub> expression.

The main finding of the present Chapter was that manipulation of the P2Y<sub>1</sub> receptor causes bi-directional exacerbation of seizure severity according to when the manipulation was performed, which may have a direct impact on the activation of P2Y<sub>1</sub> receptor in specific cell types.

We found that activating the P2Y<sub>1</sub> receptor prior induction of status epilepticus reduced seizures and protected the hippocampus from seizure-induced pathology. This finding is consistent with a role for the P2Y<sub>1</sub> receptor in reducing excessive excitation in the brain. Notably, the P2Y<sub>1</sub> receptor is found presynaptically where it may function to attenuate neurotransmitter release, including release of glutamate (Mendoza-Fernandez et al., 2000, Rodrigues et al., 2005). Activation of P2Y<sub>1</sub> receptor inhibits long-term depression via reduction of Ca<sup>2+</sup>-influx through voltage sensitive

Ca<sup>2+</sup>-channels (Guzman et al., 2010). In line with P2Y<sub>1</sub> playing a role in synaptic transmission (Guzman and Gerevich, 2016), P2Y<sub>1</sub> was found to be increased in synaptoneuroosomes post-status epilepticus.

In addition to the pharmacologic approaches used to interrogate the function of P2Y<sub>1</sub>, we explored the effects of status epilepticus in mice deficient in the P2Y<sub>1</sub> receptor. A strong susceptibility to seizures was observed in the P2Y<sub>1</sub> knockout mice, which can be explained by the high levels of the kainate receptor GLUR6/7 present in the P2Y<sub>1</sub> knockout mice in naïve conditions. Increased levels of kainate receptors may contribute to the aggravated seizure phenotype during status epilepticus (Crepel and Mulle, 2015). In line with our study, another mouse model deficient in a different ADP-sensitive P2Y receptor, P2Y<sub>12</sub>, showed also an exacerbated seizure phenotype (Eyo et al., 2014).

Another important aspect of the experimental design in the present Chapter was to show that the core findings in the kainic acid model were reproduced in the pilocarpine model. This avoids a common concern that an observation is unique to a particular model. Since no single model represents all aspects of the condition it is important to replicate key findings. Here we show that the pre- and post-treatment consequences of P2Y modulation are similar in the pilocarpine model. Notably, other studies that investigated P2Y in epilepsy only looked at single models (Eyo et al., 2014, Simoes et al., 2018), therefore the present study represent an advance in the field.

Status epilepticus is known to elicit irreversible brain damage in both experimental models and humans (Betjemann and Lowenstein, 2015). The relationship between seizures and damage is not fully understood. Here we looked at damage in each study in which we modulated the P2Y<sub>1</sub> receptor. Overall, the damage tracked well with what happened to seizures – damage was less intense in mice with less seizures and more in those in which seizures were longer. There were some exceptions however. We found that despite altering seizures, activation of P2Y<sub>1</sub> receptor after status epilepticus induction did not cause more neuronal damage, suggesting that P2Y<sub>1</sub> has a neuroprotective effect on the brain.

The present study looked at glial responses as well as neuronal injury. Overall, we found that seizure intensity was also related with glial responses. Once again, mice that displayed intense seizures also displayed more gliosis and less gliosis was observed in mice that showed more seizures. This is consistent to a previous study in which reduction on seizures is correlated with the reduction on gliosis after status epilepticus (Engel et al., 2012).

Evidences from other studies showed that P2Y<sub>1</sub> receptor modulation can affects brain excitability. For example, selective blockade of spontaneous astroglial Ca<sup>2+</sup> elevations and the inhibition of P2Y<sub>1</sub> receptor attenuate the abnormal enhancement of synaptic strength in epileptic brain (Alvarez-Ferradas et al., 2015), which may contribute to reduce brain excitability.

A remarkable finding in the present study was that the effect of P2Y<sub>1</sub> receptor activation on seizure severity depended on when that manipulation took place relative to the insult. Activation of the P2Y<sub>1</sub> receptor after the induction of the status epilepticus potentiates the generation of seizures. We have shown that following status epilepticus, P2Y<sub>1</sub> is also expressed in microglia, suggesting a role of P2Y<sub>1</sub> on microglia activation and possibly driving inflammation. Our experiments using the microglia inhibitor minocycline confirmed this. In fact, during brain inflammation, microglia cells are the first responder cells, being activated in an early stage of status epilepticus (Davalos et al., 2005, Eyo et al., 2017). Microglia also responds rapidly to brain injury (Davalos et al., 2005) and acute neuronal hyperactivity during seizures through NMDA glutamate receptors (Eyo et al., 2014). It is well establish that microglia is activated in response to seizures, which cause morphological alterations in microglia (Eyo et al., 2017). Strong evidences suggest activation of microglia in the brain of patients suffering from recurrent seizure episodes and in animal models of epilepsy (Beach et al., 1995, Drage et al., 2002). Also, activation of purinergic receptors (expressed on microglia), including the P2Y<sub>1</sub> receptor, can mediate microglial migration towards neurons (Davalos et al., 2005, De Simone et al., 2010, Eyo et al., 2014). Additionally, another group has shown that microglia transform astrocytes into a

neuroprotective phenotype through down-regulation of the P2Y<sub>1</sub> receptor (Shinozaki et al., 2017). We cannot exclude however, P2Y<sub>1</sub> not having an effect on astrocytes in our model. Our P2Y<sub>1</sub> knock-out data, showing a decrease in astrocytosis with increased seizure severity suggest that astrocytes may not be important to seizure phenotype. However, to proof this would require the use of cell-specific P2Y<sub>1</sub> knock-out mice in the future.

Our study allowed us to speculate that P2Y<sub>1</sub> receptor function may be related with the cell types where the receptor is expressed at the time of the brain insult (status epilepticus). Our results proposed that activation of P2Y<sub>1</sub> receptor induce neuroprotection in the brain through activation of neuronal cells; and activation of the P2Y<sub>1</sub> receptor in microglia potentiate seizure phenotype, suggesting that glial cells activation leads to prolonged seizures. This is in line with other studies where P2Y<sub>1</sub> inhibition in glial cells seems to induce neuroprotection after brain injury (Choo et al., 2013) or focal cerebral stroke (Chin et al., 2013).

In conclusion, our study was the first study showing an important functional role for the P2Y<sub>1</sub> receptor on seizure generation and seizure-induced pathology in two models of status epilepticus (intra-amygdala kainic acid and pilocarpine models) with P2Y<sub>1</sub> antagonism during status epilepticus protecting against seizure pathology via decreasing microglia activation. Thus, our study is the first to demonstrate anticonvulsant properties of P2Y<sub>1</sub> antagonism providing a new anti-convulsive drug, with different mechanisms of action. Our results, however caution against P2Y<sub>1</sub> treatment, without an underlying microgliosis.

**Chapter 5 – Functional role of the P2Y<sub>1</sub> receptor during epilepsy development and epilepsy**

## 5.1 Introduction

### 5.1.1 New directions for the treatment of epilepsy

Despite available treatments, approximately 30% of patients suffering from epilepsy remain drug-refractory with TLE being the most common and drug-resistant form of epilepsy in adults (Zhao et al., 2014). While drugs targeting excitatory and inhibitory systems have successfully controlled seizures in almost 70% of patients (Bialer et al., 2013), in order to modify disease progression or offer efficacious drug treatment to currently pharmacoresistant epilepsy patients, alternative targets, however, with a novel mechanism of action must be identified.

There is an increasing interest in targeting gliosis and neuroinflammation as a possible approach to pharmacoresistant epilepsy and anti-epileptogenesis (Bialer and White, 2010, Vezzani et al., 2011, Devinsky et al., 2013, Marchi et al., 2014). Several experimental and clinical findings have demonstrated an important role of neuroinflammation in both ictal- and epileptogenesis (Vezzani et al., 2011, Vezzani et al., 2016). High levels of inflammatory mediators are present in the brains of both experimental rodent models of epilepsy and epilepsy patients (Aronica et al., 2017) and these processes have therefore received much attention in recent years. Selective blockade of the pro-inflammatory cytokine, IL-1 $\beta$ , has been shown to reduce seizures in *in vivo* models of epilepsy (Ravizza et al., 2008b, Vezzani et al., 2009), while in an epileptogenesis-resistant animal, the Amazon rodent, *Proechimys*, no acute brain inflammatory response was found following experimentally-induced status epilepticus (Scorza et al., 2017).

Emerging evidences showed that ATP is a key upstream trigger of neuroinflammation (Idzko et al., 2014). ATP has been shown to be released after excessive neuronal firing and during chronic inflammation, both processes taking place during epilepsy (Henshall and Engel, 2015). The causality between hyperexcitation, excitotoxicity and neuroinflammation is circular and intercellular signalling through

purines is an important mediator of these processes, making purinergic receptors an attractive treatment target.

Very little is known about the expressional and functional role of P2 receptors in epilepsy. Different studies have shown that the P2X7 receptor is overexpressed in epileptic rats (Rappold et al., 2006, Dona et al., 2009), and in resected neocortex from TLE patients (Jimenez-Pacheco et al., 2013). Recently our group showed an up-regulation of the ionotropic P2X7 receptor in experimental epilepsy and resected hippocampus from epilepsy patients (Jimenez-Pacheco et al., 2016). In the same study, we also showed that a specific P2X7 receptor antagonist JNJ-47965567 significantly reduced spontaneous seizures in epileptic mice, and this effect was continued even treatment was stopped suggesting possible disease-modifying effects in experimental epilepsy (Jimenez-Pacheco et al., 2016).

The functional role of P2Y receptor family in epilepsy remains, however, unclear and up to date no functional studies have been carried out in chronic epilepsy. There is however evidence suggesting a role for these receptors in epilepsy. Sukigara et al. (2014), showed an up-regulation of the P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors in astrocytes in patients with intractable epilepsy associated with focal cortical dysplasia (FCD) (Sukigara et al., 2014). In a chronic model of epilepsy, astrocytes from kindled rats show an enhanced spontaneous Ca<sup>2+</sup>- dependent signalling and astroglial hyperexcitability, which requires the activation of P2Y<sub>1</sub> receptor (Alvarez-Ferradas et al., 2015). P2Y<sub>1</sub> receptor has also been shown to be increased in neuronal progenitor cells after pilocarpine-induced status epilepticus (Rozmer et al., 2017).

In the Chapter 4, we showed a strong anti-convulsive effect of P2Y<sub>1</sub> receptor during status epilepticus. The inhibition of P2Y<sub>1</sub> receptor protects the brain from seizures and seizures-induce pathology. In our final Chapter, we investigated the role of P2Y<sub>1</sub> during epileptogenesis and in chronic epilepsy.

## 5.2 Chapter objectives

**Hypothesis:** P2Y<sub>1</sub> receptor inhibition prevents/ameliorates the development of epilepsy and provides anti-epileptogenic properties.

**Objectives:**

1. Determine the contribution of the P2Y<sub>1</sub> receptor to the epileptic phenotype.

1.1. Determine the contribution of the P2Y<sub>1</sub> receptor to epileptogenesis.

1.2. Establish the contribution of the P2Y<sub>1</sub> receptor to chronic epilepsy.

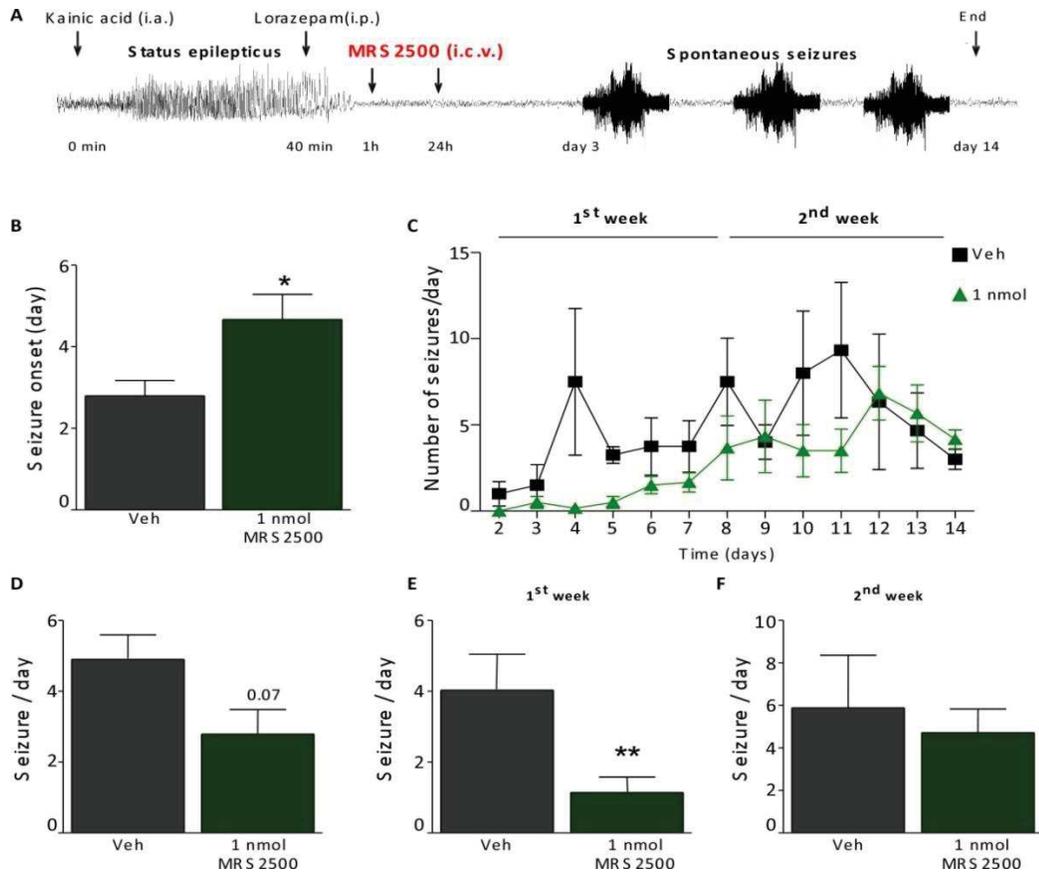
## 5.3 Results

### 5.3.1 Antagonism of the P2Y<sub>1</sub> receptor delays the development of epilepsy following status epilepticus

To investigate a potential anti-epileptogenic effect of P2Y<sub>1</sub> receptor antagonism, we used the intra-amygdala kainic acid mouse model. EEG telemetry units were implanted in a subcutaneous pocket in the back of the animals, as explained in Chapter 2, and continuous EEG recorded for 14 days after status epilepticus. Mice were treated with P2Y<sub>1</sub> antagonist MRS2500 (1 nmol) 1 hour and 24 hours after status epilepticus. Mice were then sacrificed 14 days later.

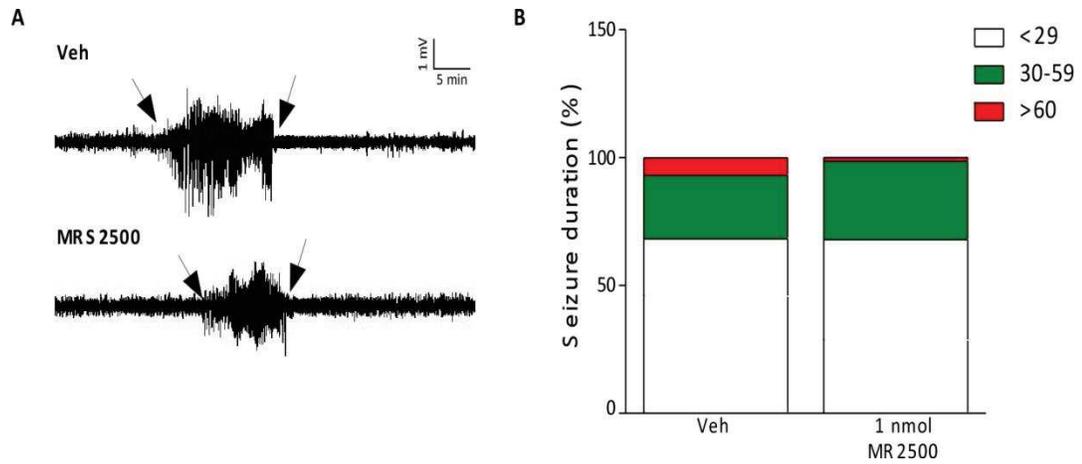
In the intra-amygdala kainate model, mice develop epilepsy after a short latent period of 3-4 days, experiencing 1-5 seizures per day thereafter (Mouri et al., 2008) (**Figure 5.1A**). Injection of the P2Y<sub>1</sub> antagonist MRS2500 shortly after status epilepticus resulted in a later onset of first spontaneous seizure (day 5) when compared with vehicle-injected mice (day 3) (**Figure 5.1B**). Thus, inhibition of the P2Y<sub>1</sub> receptor delays the emergence of epilepsy in this model. The P2Y<sub>1</sub> antagonist MRS2500 also slightly reduced the frequency of spontaneous seizures although this did not reach significance over the entire 14-day period (**Figure 5.3B, C**). However, post-hoc analysis found that seizure frequency was lower in P2Y<sub>1</sub> antagonist-treated mice if only the first week of recordings were considered, likely due to the effect of the delay to first seizure (**Figure 5.1C, E**). Over the second week seizure frequency in mice given the P2Y<sub>1</sub> antagonist MRS2500 gradually recovered toward the vehicle group (**Figure 5.1C, F**). Taken together, these findings suggest inhibition of the receptor can delay but not prevent epileptogenesis, perhaps via long-lasting anticonvulsant effects rather than anti-epileptogenic effect. A number of other EEG parameters were investigated. We found that high frequency high amplitude spiking (> 60 sec) was shorter in the P2Y<sub>1</sub> antagonist MRS2500 treated group compared to the vehicle-injected group (**Figure 5.2A, B**). These data suggest that early blockade of the P2Y<sub>1</sub> receptor after status epilepticus can produce a lasting disease-modifying action.

Next, to investigate whether P2Y<sub>1</sub> receptor antagonism is regulating inflammation during epileptogenesis, we analysed the mRNA levels of the microglia marker *Iba-1* and astrocyte marker *GFAP*. Mice were treated with P2Y<sub>1</sub> antagonist MRS2500 (1 nmol) 1 hour and 4 hours following status epilepticus and then killed 24 hours later. Our results showed that mice treated with P2Y<sub>1</sub> receptor antagonist MRS2500 showed a significant reduction of *Iba-1* mRNA levels at 24 hours (**Figure 5.3A**) and no differences were observed in the mRNA levels of *GFAP* (**Figure 5.3B**), suggesting that the effects of P2Y<sub>1</sub> receptor antagonism following status epilepticus are rather on microglia than on astrocytes.



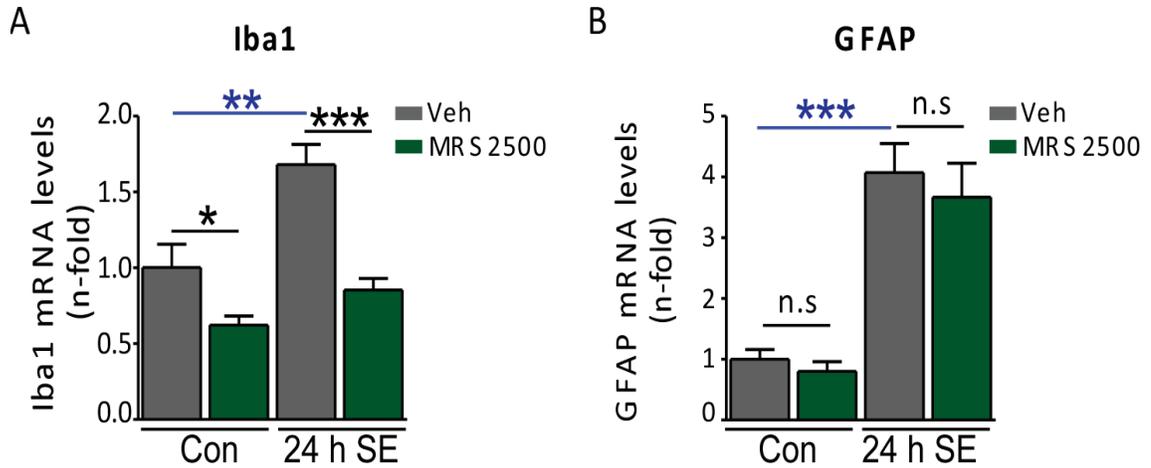
**Figure 5.1 – P2Y<sub>1</sub> receptor inhibition delays seizure generation in epileptogenesis process.**

**(A)** Schematic showing the intra-amygdala kainic acid model. Status epilepticus was induced by kainic acid injection. 40 minutes after status epilepticus mice received lorazepam to attenuate seizures. Then, 1 hour and 24 hours after status epilepticus, mice were treated with P2Y<sub>1</sub> antagonist MRS2500. EEG telemetry devices were recording for 14 days. **(B)** Graph showing the late onset of seizures in mice treated with P2Y<sub>1</sub> antagonist MRS2500 when compared with vehicle-injected mice. **(C, E)** Graph showing that mice treated with P2Y<sub>1</sub> antagonist MRS2500 displayed significantly less seizures per day than the vehicle-injected group, mainly in the first week. **(D, F)** Graph showing a non-significant reduction in the number of seizures per day in the treated group when compared with vehicle group. **(C, D)** Graph showing a reduction in the number of seizures in the group of mice treated with P2Y<sub>1</sub> antagonist MRS2500 during 14 days of experiment, comparing with vehicle-injected mice (n=4 vehicle (Veh) and 6 MRS2500). \*p<0.05, \*\*p<0.01.



**Figure 5.2 – P2Y<sub>1</sub> receptor antagonism after status epilepticus decreases the severity of spontaneous recurrent seizures.**

**(A)** Representative images showing examples of high frequency and high amplitude spiking on the EEG after drug treatment (see arrows). **(B)** Graph showing percentage of different seizure times after treatment with P2Y<sub>1</sub> antagonist MRS2500 or vehicle during 14 days of recording. Mice treated with P2Y<sub>1</sub> antagonist MRS2500 showed a reduced number in seizures longer than 60 seconds (n = 4 (vehicle (Veh)), 6 MRS2500).



**Figure 5.3 – P2Y<sub>1</sub> receptor antagonism decreases Iba-1 expression during epileptogenesis.**

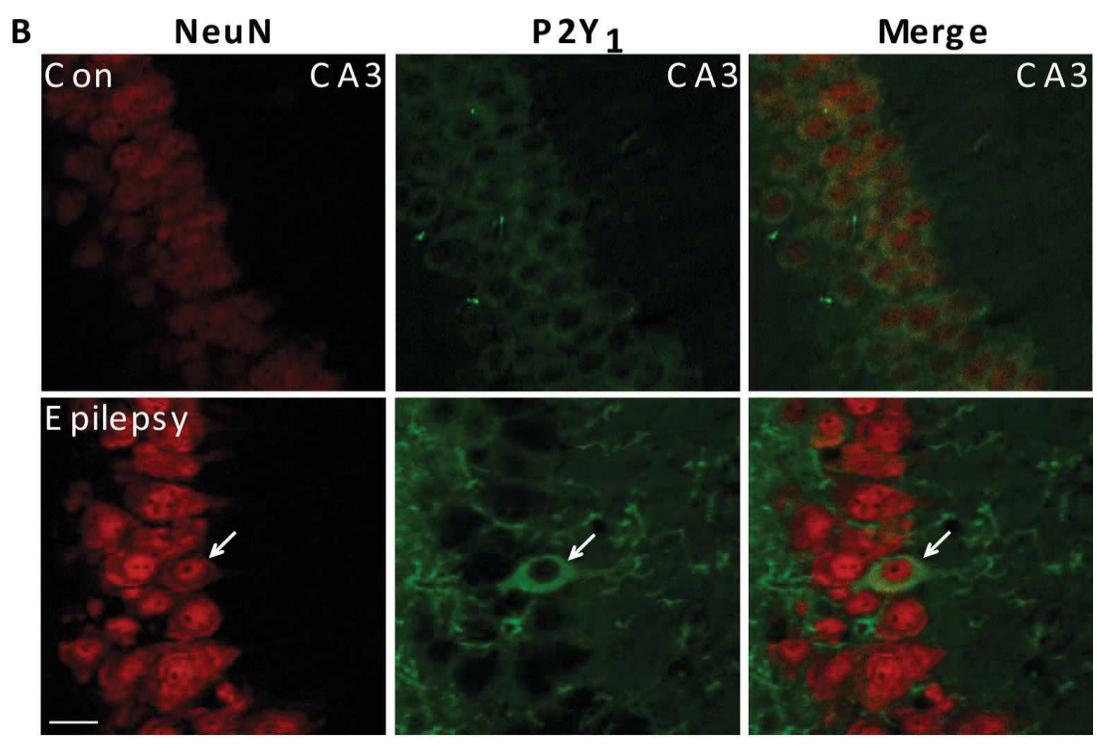
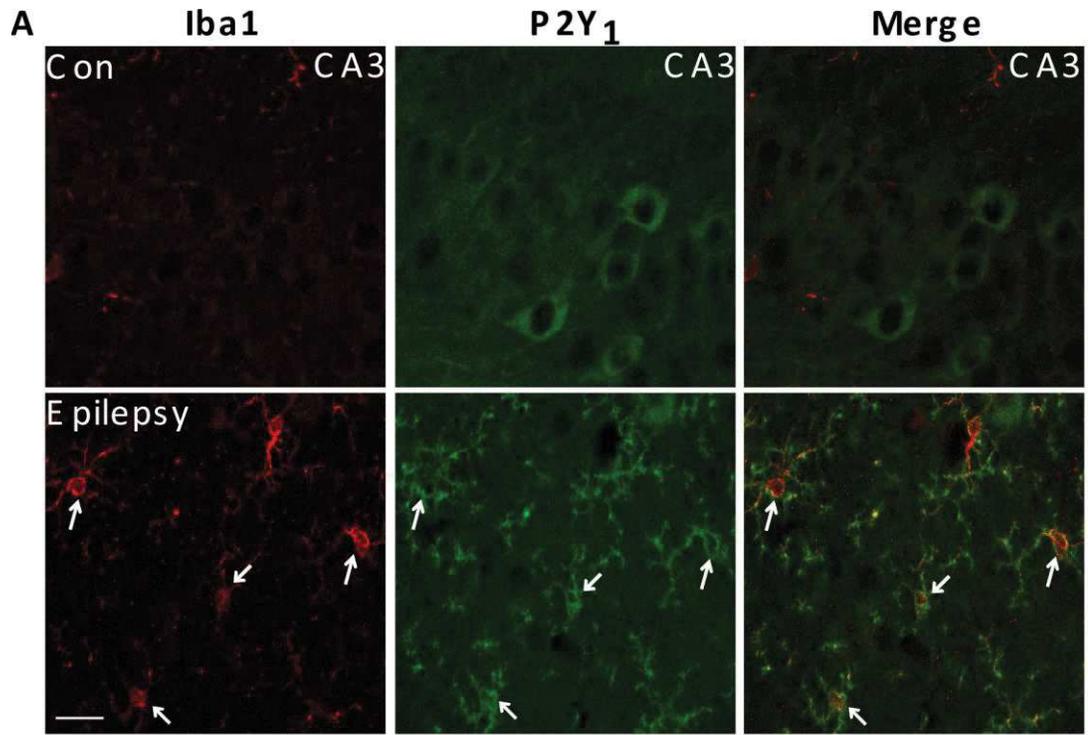
**(A)** Graph showing P2Y<sub>1</sub> antagonist MRS2500 decreased significantly the mRNA levels of Iba1 in control mice and 24 hours post-status epilepticus mice. **(B)** Graph showing no differences in the mRNA levels of GFAP between the control mice and 24 hours post-status epilepticus mice (n=4 vehicle (Veh) and 4 MRS2500). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

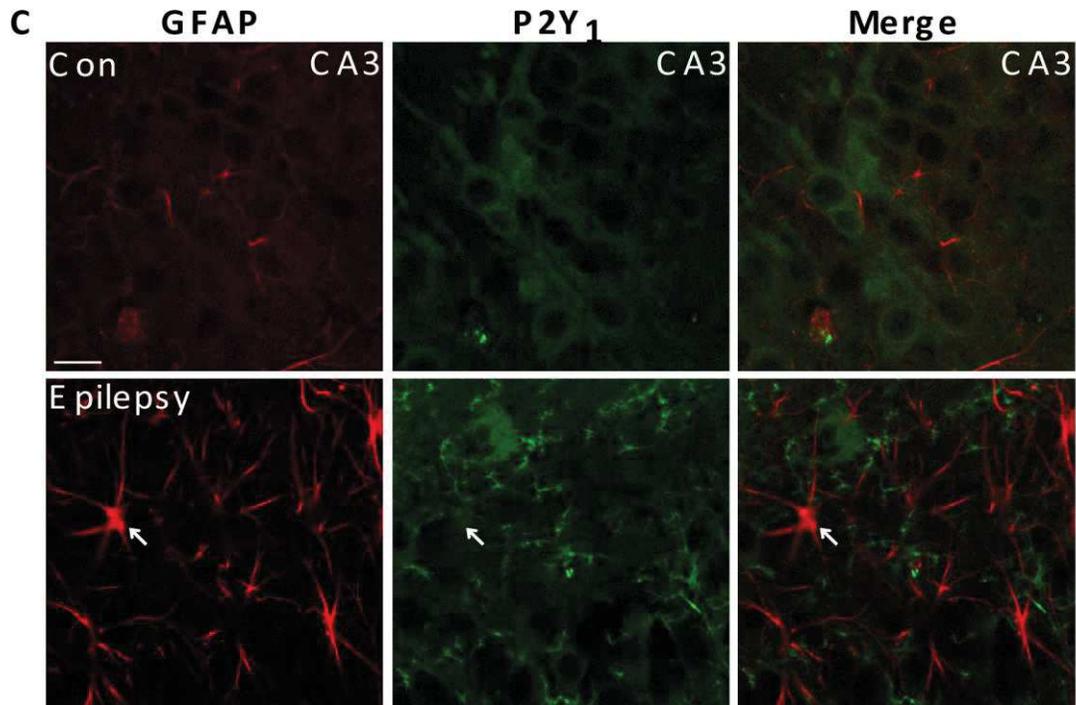
### 5.3.2 P2Y<sub>1</sub> receptor expression during chronic epilepsy

Next, we wanted to explore the cell-specific expression of the P2Y<sub>1</sub> receptor in mice that developed epilepsy. For these experiments mice were killed 14 days after status epilepticus and brain sections analysed by immunofluorescence using specific markers for microglia (Iba-1), neurons (NeuN) and astrocytes (GFAP).

Immunostaining results showed that the P2Y<sub>1</sub> receptor is expressed on microglia (**Figure 5.4A**) and neurons (**Figure 5.4B**), but not on astrocytes in tissue sections from epileptic mice (**Figure 5.4C**). We then also investigated whether P2Y<sub>1</sub> is enriched in synaptoneurosomes. Western blot revealed a significant increase of the P2Y<sub>1</sub> receptor in hippocampal synaptoneurosomes during epilepsy (**Figure 5.5A**).

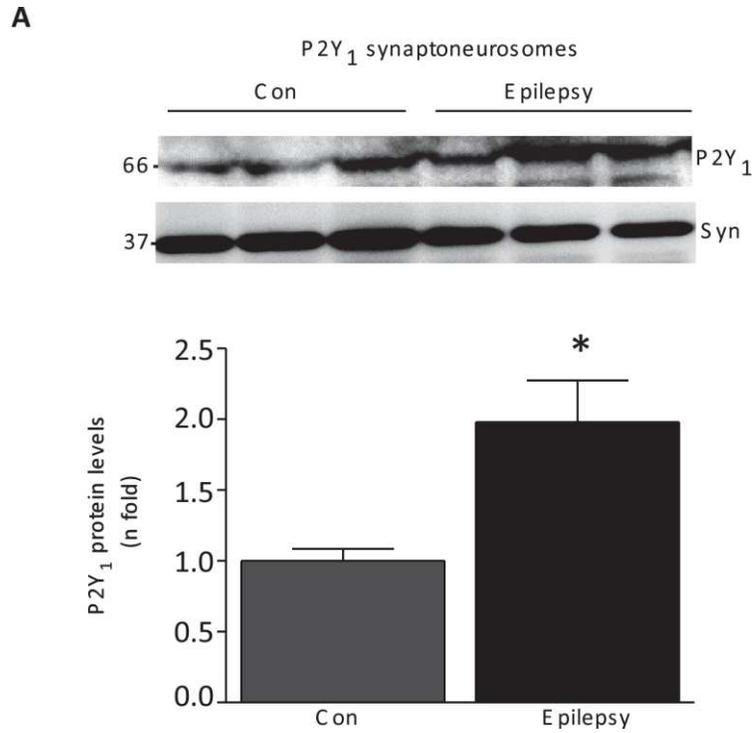
Thus, our results show, similar to what has been observed following status epilepticus, a mainly neuronal and microglial expression of the P2Y<sub>1</sub> receptor during chronic epilepsy.





**Figure 5.4 – P2Y<sub>1</sub> receptor is expressed in microglia and neurons during chronic epilepsy.**

(A) Photomicrograph showing the co-localization of the P2Y<sub>1</sub> receptor (Green) with Iba1 (red), specific marker of microglia cells, in chronic epilepsy in CA3 region of the hippocampus. (B) Immunofluorescent staining revealing that P2Y<sub>1</sub> receptor (green) is expressed in neurons (red) during chronic epilepsy in the CA3 hippocampal subfield. (C) Immunofluorescent images from CA3 region of the hippocampus showing no co-localization of the P2Y<sub>1</sub> receptor (green) with astrocytes (red). Scale bar= 50  $\mu$ m. Note that white arrows indicate the co-localization of the P2Y<sub>1</sub> receptor with microglia (A) and neurons (B) in the hippocampus.



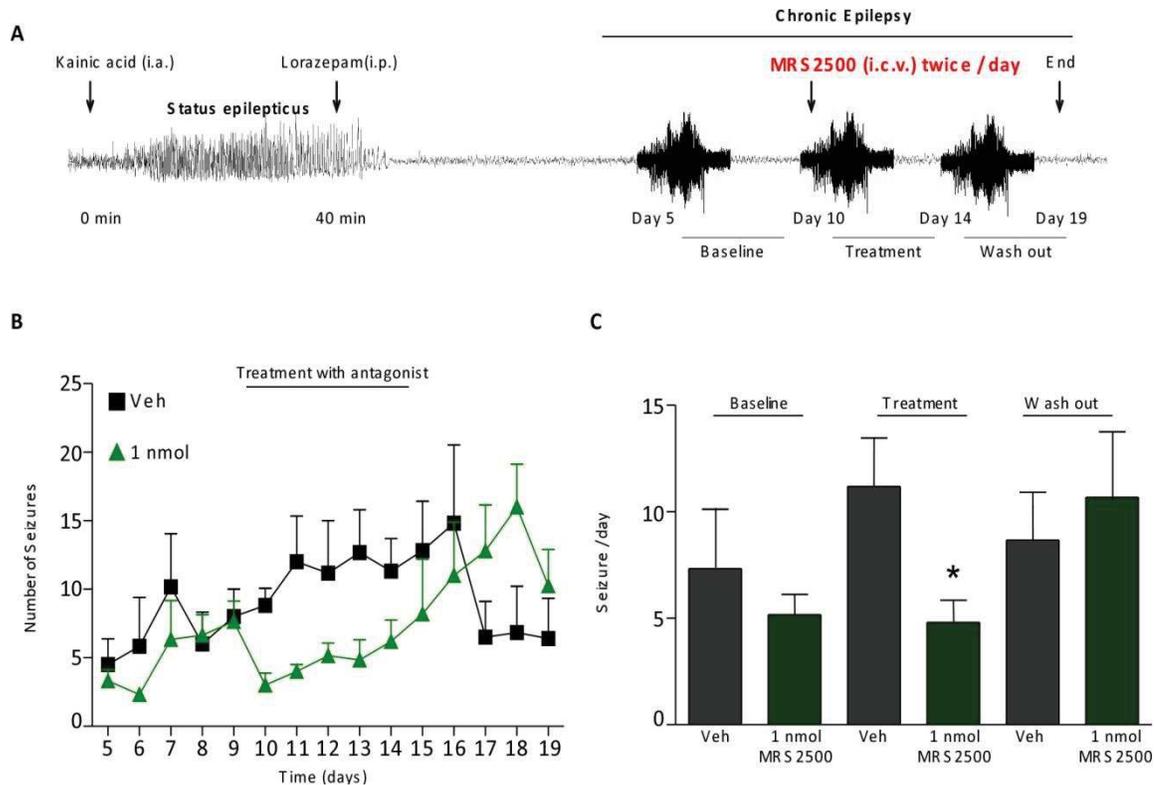
**Figure 5.5 – P2Y<sub>1</sub> receptor is increased in synaptoneurosomes during epilepsy.**

**(A)** Western blot (each lane is from n= 2 hippocampi) and representative graph showing a significant increase of P2Y<sub>1</sub> receptor protein levels in the synaptoneurosomes in the epileptic mice (n=4 control (Con) and 5 epilepsy). \*p<0.01.

### 5.3.3 Anti-convulsive effects of P2Y<sub>1</sub> receptor antagonism during chronic epilepsy

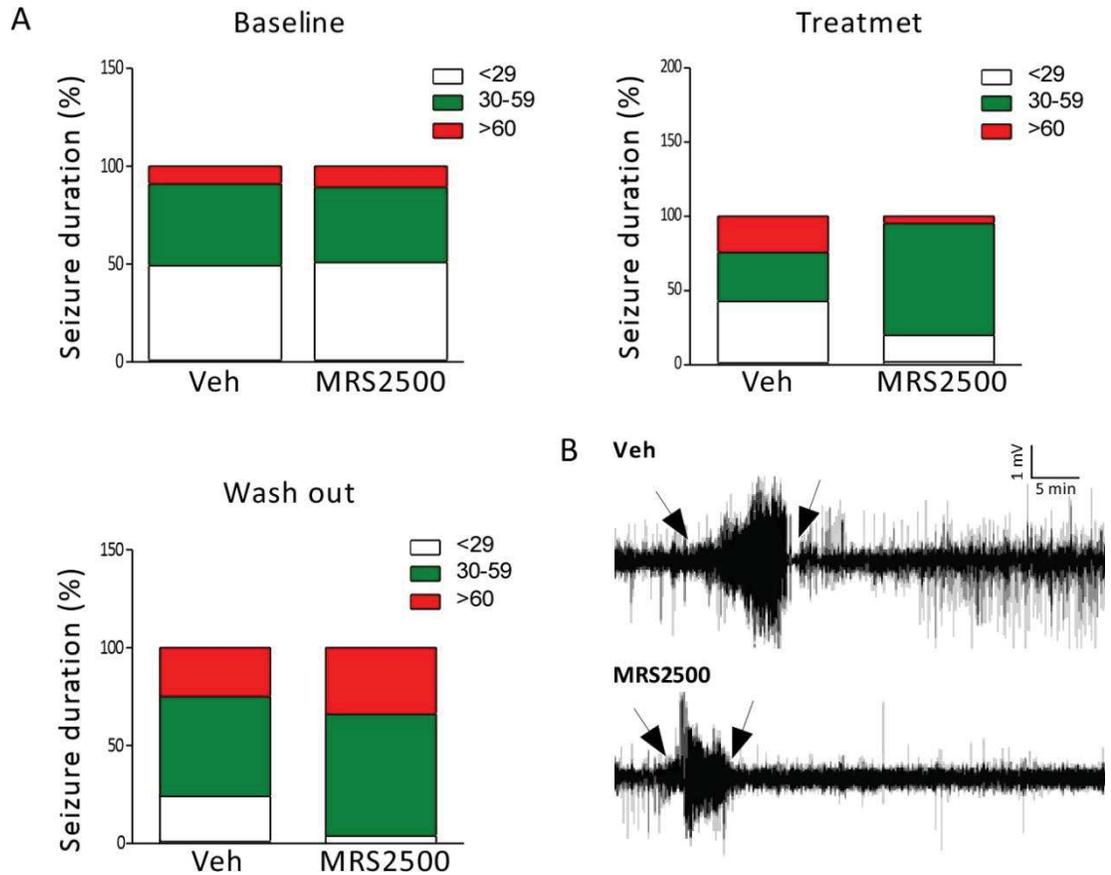
In our final experiments we wanted to explore whether P2Y<sub>1</sub> antagonism can also suppresses the occurrence of spontaneous seizures. As before, epilepsy was induced by using the intra-amygdala kainic acid model of status epilepticus. All mice used to perform this study had experienced at least one spontaneous seizure on day 3. From day 10 to day 14, mice were treated i.c.v. with the P2Y<sub>1</sub> antagonist MRS2500 (1 nmol) twice daily, followed by a 5 day wash out period (**Figure 5.6A**).

Our results showed a significant decrease in the number of seizure during the treatment phase in mice treated with P2Y<sub>1</sub> antagonist MRS2500 when compared with vehicle-injected mice (**Figure 5.6B, C**). Seizure suppressive effects provided by P2Y<sub>1</sub> antagonism were, however, absent during wash-out period, suggesting anticonvulsant effects rather than anti-epileptogenic effects (**Figure 5.6B, C**). In relation to seizure duration, during baseline no differences were observed between groups. During the treatment phase, mice injected with the P2Y<sub>1</sub> antagonist MRS2500, showed shorter seizures (>60 seconds) when compared to the vehicle-injected group. Again, effects on seizure duration were absent during wash-out period (**Figure 5.7A, B**), suggesting that inhibition of P2Y<sub>1</sub> receptor protects the brain from spontaneous seizures.



**Figure 5.6 – P2Y<sub>1</sub> receptor inhibitor decreases the number of seizures in epileptic mice.**

(A) Schematic showing the intra-amygdala kainic acid model. Kainic acid is used to induce status epilepticus followed by i.p. injection of lorazepam to attenuate seizures. Mice develop spontaneous seizures 3-4 days after status epilepticus and on day 10, mice were treated with 1 nmol of P2Y<sub>1</sub> antagonist MRS2500 (i.c.v.), twice a day during 5 days, followed by 5 days of wash out. EEG telemetry devices were recording for 19 days. (C, D) Graph showing that mice treated with P2Y<sub>1</sub> antagonist MRS2500 displayed significantly less seizures during treatment phase, when compared with vehicle-injected mice. This effect was lost during the wash out period, where mice treated with P2Y<sub>1</sub> antagonist MRS2500 presented more seizure per day than vehicle-injected group (n=6 vehicle (Veh) and 6 MRS2500). \*p<0.05.



**Figure 5.7 – P2Y<sub>1</sub> receptor inhibitor decrease the seizure severity in chronic epilepsy.**

**(A)** Graph showing percentage of different seizure times during baseline, treatment and wash out periods. In baseline period, no differences were observed in the percentage of seizures duration between both groups. During treatment phase, mice treated with P2Y<sub>1</sub> antagonist MRS2500 displayed less long seizures (60 seconds), when compared to vehicle-injected group. However, in the wash out period mice treated with P2Y<sub>1</sub> antagonist MRS2500 presented longer seizures (>60 seconds) compared with vehicle-group (n = 6 (vehicle (Veh)), 6 MRS2500). **(B)** Representative images showing examples of high frequency and high amplitude spiking on the EEG after drug treatment (see arrows).

## 5.4 Discussion

In this final Chapter we analysed the role of P2Y<sub>1</sub> in the development of epilepsy and in the maintenance of the epileptic state. One of the main findings of our last chapter is that P2Y<sub>1</sub> antagonism post-status epilepticus results in a temporary delay in onset of epilepsy and less severe spontaneous seizures. We also demonstrated that P2Y<sub>1</sub> antagonism can reduce spontaneous seizures in already-epileptic mice. However, this effect was lost during washout. Taken together, these studies suggest P2Y<sub>1</sub> drugs may have potential as disease-modifying treatments for status epilepticus and are a new class of treatment for epilepsy.

As reviewed recently by Engel et al (2016), the P2 receptor family has not only been implicated in status epilepticus but also in epilepsy (Engel et al., 2016). Again, similar to status epilepticus, most attention has been paid to the study of the expression and function of the P2X receptor family, particularly of the P2X7 subtype (Soni et al., 2015, Amhaoul et al., 2016, Jimenez-Pacheco et al., 2016, Beamer et al., 2017). In a recent study our laboratory has shown strong seizure suppressive and possible disease-modifying potential of P2X7 antagonism during epilepsy using the intra-amygdala kainic acid mouse model (Jimenez-Pacheco et al., 2016). Other studies have obtained similar results with P2X7 antagonism improving the epileptic phenotype. However, the P2Y receptor family has received much less attention. Up to now, only one study attempted to determine the functional role of P2Y<sub>1</sub> receptor in chronic epilepsy using a rapid kindling protocol to induce epilepsy in rats. Results from this study showed that the inhibition of P2Y<sub>1</sub> reduced Ca<sup>2+</sup> waves in hippocampal slices from epileptic mice (Alvarez-Ferradas et al., 2015).

Our first findings showed a strong increase of P2Y<sub>1</sub> in neurons and in microglia during epilepsy, similar to what we found following status epilepticus. Again, no expression of P2Y<sub>1</sub> was observed in astrocytes. These results suggest a similar regulation of the P2Y<sub>1</sub> receptor following acute seizures and during epilepsy. It is tempting to speculate that the up-regulation in microglia may contribute to the

maintenance of the inflammatory state since microglial P2Y<sub>1</sub> receptor activation has been proposed to contribute to microglia migration processes (De Simone et al., 2010). This is in line with several studies demonstrating an important role of microglia in epileptogenesis and epilepsy (Hiragi et al., 2018). For example, the number of microglia and the percentage of amoeboid microglia were increased in the hippocampus one week after status epilepticus induced by electric stimulation (Ali et al., 2015). In contrast to our study, P2Y<sub>1</sub> receptor was shown to be up-regulated in astrocytes in patients with intractable epilepsy (Sukigara et al., 2014). However, this study lacked basic controls to verify the specificity of the P2Y receptors antibodies.

Our second finding was a delayed onset of spontaneous seizures and a reduction in the duration of spontaneous seizures following the inhibition of the P2Y<sub>1</sub> receptor shortly after status epilepticus. Based on the results obtained during status epilepticus, we can speculate that the reduction on seizures was caused by a reduction on microglia activation through inhibition of the P2Y<sub>1</sub> receptor. Several studies have shown that blocking anti-inflammatory markers attenuate the number of spontaneous seizures (Vezzani et al., 2011). In fact, P2Y<sub>1</sub> receptor has been suggested to modulate inflammation in the brain (Guzman and Gerevich, 2016) and P2Y<sub>1</sub> receptor inhibition may reduce glial cell activation, which might reduce the Ca<sup>2+</sup> waves and excitability in the brain (Guzman and Gerevich, 2016).

In line with microglia and not astrocytes driving P2Y<sub>1</sub>-induced pathology, we observed a strong decrease in the microglia marker Iba-1 but not in the astrocyte marker GFAP during the latent period. Potentially, reduction of microglia activation in the latent period caused by inhibition of the P2Y<sub>1</sub> receptor may contribute to delay the occurrence of the first spontaneous seizure. The observed anti-epileptogenic effects, however, were decreasing over the second week suggesting that P2Y<sub>1</sub>-mediated effects on epileptogenesis are not long-lasting. Therefore, future studies should analyse how long-lasting the presence of P2Y<sub>1</sub> antagonists is in the brain and whether effects on seizure suppression are decreasing once P2Y<sub>1</sub> antagonist decreases in brain tissue. Nevertheless, our study is the first showing a functional role of any P2 receptor

on epileptogenesis, demonstrating that targeting this class of receptors may provide anti-epileptogenic effects via reducing inflammation.

The last finding in this study was a strong seizure-suppressive effect of P2Y<sub>1</sub> antagonism during chronic epilepsy with P2Y<sub>1</sub> inhibition reducing both the number of spontaneous seizures and seizure duration. Our study is the first study showing a strong anti-convulsive effect in chronic epilepsy targeting a subtype of the P2Y receptor family. The inhibition of the P2Y<sub>1</sub> receptor caused a reduction of 50% in the number of spontaneous seizures in epileptic mice. This result emphasises the importance of the P2Y<sub>1</sub> receptor in epilepsy and so far, no other functional studies have been carried out in chronic epilepsy, being our study the pioneer. We have not analysed the mechanism of how P2Y<sub>1</sub> antagonism reduces seizures during epilepsy, however the strong immunoreactivity of P2Y<sub>1</sub> on microglia suggest this via P2Y<sub>1</sub>-mediated activation of microglia. Data demonstrating a role for inflammation during epileptogenesis showed that the inhibition of upstream TLR signalling reduces spontaneous seizures and also reduces brain inflammation (Maroso et al., 2010, Maroso et al., 2011). Further, brain tissue from epileptic mice and epileptic patients present high levels of pro-inflammatory cytokines (Aronica et al., 2017) and blocking these cytokines have been implicated in the reduction of seizures during chronic epilepsy (Ravizza et al., 2008b). Following treatment, seizure numbers in P2Y<sub>1</sub> antagonist-treated mice, however, increased again to baseline levels. This suggests that effects provided by P2Y<sub>1</sub> antagonism are rather anti-convulsive than disease-modifying. Regardless of the lack of disease-modifying effects, this is the first study showing a seizure-suppressive effect resulting from the inhibition of the P2Y<sub>1</sub> receptor in drug-refractory chronic epilepsy. Therefore, P2Y<sub>1</sub> receptor antagonists may be a novel candidate to treat drugs-refractory epilepsy.

In conclusion, our study found potent seizure-suppressive effects of P2Y<sub>1</sub> antagonism during epileptogenesis and during chronic epilepsy suggesting that P2Y<sub>1</sub> receptor antagonist may be a novel candidate for the control of drug-refractory epilepsy.

## 6. Final discussion, conclusion and future work

## 6.1 Final discussion

The first objective of this thesis was to characterize the expression of the metabotropic P2Y receptor family following status epilepticus and during epilepsy. In our second and third objectives, we explored the functional role of the P2Y<sub>1</sub> receptor during status epilepticus, epileptogenesis and in the generation of spontaneous seizures during epilepsy and whether we are able to alter or suppress seizures using P2Y<sub>1</sub>-modifying drugs.

Up to date, TLE is the most common and drug-resistant form of epilepsy in adults. Despite the existence of numerous AEDs, 30% of patients with epilepsy remain pharmacoresistant (Bialer and White, 2010) and, even when effective, AEDs do not target the underlying disease pathology and come along with severe side-effects. Therefore, there is an urgent need to develop treatment strategies that impact upon both seizures and disease progression, preferably, with minimum adverse effects.

Treatments interfering with neuroinflammation have been suggested as promising therapeutic strategy in epilepsy, therefore, research to decipher the mechanism underlying inflammatory pathways have generated much interest. ATP has emerged as a potential contributor to prolonged seizures and is released following cell death, inflammation and during increased neuronal activity, contributing to the activation of inflammatory pathways (Rodrigues et al., 2015). Consequently, receptors responding to extracellular ATP or its breakdown products have been suggested as potential new treatment targets for epilepsy (Engel et al., 2016).

While much effort has been spent to characterize the expressional changes of the ionotropic P2X family members during epilepsy, this work is the first representing a complete characterization of the expression of the P2Y receptor family in experimental epilepsy and in brain tissue from TLE patients. The main findings include a substrate and down-stream-specific regulation of P2Y receptors following status epilepticus in the hippocampus (**Table 6.1**).

As discussed previously, the selective down and up-regulation during status epilepticus may represent protective mechanisms of the brain to protect itself from damage. In line with this, UTP protected the brain whereas ADP aggravated seizures and damage. However, whether this is a general defensive mechanism for the remaining members of the P2Y receptor family of the brain against seizures remains to be further explored. Nevertheless, our results strongly suggest a functional role of these receptors during status epilepticus. Further, a selective up-regulation of P2Y receptors in experimental and human epilepsy was also observed. This seems to suggest P2Y receptors driving inflammation (our results from Chapter 4 and 5 are in line with this) however, whether this is true for the remaining P2Y receptors remains to be explored in future studies. Thus, our studies have not only described the expression of a new class of receptors during epilepsy, but also provided novel possible target genes for the treatment of status epilepticus and drug-refractory epilepsy.

**Table 6.1-** Induction and expressional changes of the P2Y receptor family in the hippocampus following 1, 4, 8 and 24 hours following status epilepticus

Receptor	Agonist	Induction	G-coupling	Expression
P2Y <sub>1</sub>	ADP>ATP	decreased	Gq	increased
P2Y <sub>2</sub>	UTP/ATP	increased	Gq	increased
P2Y <sub>4</sub>	UTP>ATP	increased	Gq	increased
P2Y <sub>6</sub>	UDP>UTP>>ADP	increased	Gq	increased
P2Y <sub>12</sub>	ADP>ATP	decreased	Gi	decreased
P2Y <sub>13</sub>	ADP>>ATP	decreased	Gi	not changed
P2Y <sub>14</sub>	UDP-Glucose	decreased	Gi	not changed

The main objective in the Chapters 4 and 5 of this thesis was to explore the functional role of the P2Y<sub>1</sub> receptor during status epilepticus, epileptogenesis and during epilepsy. One of the major findings of the 4<sup>th</sup> Chapter was the dual effect of the P2Y<sub>1</sub> receptor on seizure generation and seizure severity (**Table 6.2**) according to time-

point of precipitating insult and cell type involved (neurons, physiological conditions) or during status epilepticus (microglia). Importantly, our findings are in line with studies using TBI mouse model (Choo et al., 2013). This not only strengthens our results, but also suggests that P2Y<sub>1</sub> activation may be protective without underlying inflammation, whereas once inflammation has started, P2Y<sub>1</sub> activation seems to aggravate pathology regardless of the model used.

A major weakness of previous studies was the use of only one epilepsy model (e.g. kainic acid, pilocarpine). In our study we have used two different models which showed the same response to P2Y<sub>1</sub>-interfering drugs. This is in contrast to, for example, the P2X<sub>7</sub> receptor, with P2X<sub>7</sub> antagonism provoking different responses according to model used (Amhaoul et al., 2016, Fischer et al., 2016, Jimenez-Pacheco et al., 2016, Rozmer et al., 2017). Whether, apart from reducing seizures in the brain, P2Y<sub>1</sub> signalling interference also alters seizure-induced cell death remains elusive. Our *in vitro* results and published data suggest so, however, with P2Y<sub>1</sub>-targeting drugs altering seizure severity we cannot exclude brain pathology being a mere consequence of a reduction/increase in seizure severity. Lastly, our results suggest P2Y<sub>1</sub> antagonism reducing seizures by preventing the activation of microglia. However, we cannot exclude other cell types being involved and future studies using, for example cell-specific P2Y<sub>1</sub> knock-out mice would have to be used in future studies.

Taken together, for the first time we have demonstrated a strong anti-convulsive effect caused by the inhibition of the P2Y<sub>1</sub> receptor following status epilepticus using two different mouse models of status epilepticus, suggesting P2Y<sub>1</sub> receptor antagonism as a new adjunctive treatment for refractory status epilepticus.

**Table 6.2** - Functional role of the P2Y<sub>1</sub> receptor following status epilepticus.

	P2Y <sub>1</sub> KO	Pre-Treatment		Post-Treatment		Post-treatment
		Antagonist MRS2500	Agonist MRS2365	Antagonist MRS2500	Agonist MRS2365	Minocycline / Antagonist MRS2500
Seizures	Increase	Increase	Decrease	Decrease	Increase	Increase
Cell death	Increase	Increase	Decrease	Decrease	Decrease (n.s)	-
Astrogliosis	Decrease	Increase	Decrease	Decrease	Increase	-

n.s. non-significant

One of the major priorities in the epilepsy field is to identify new drugs capable of modifying disease progression or offer efficacious seizure suppression in pharmaco-resistant epilepsy patients. Therefore, the last objective of this thesis was to investigate the role of the P2Y<sub>1</sub> receptor during epileptogenesis and epilepsy. Up to date, no functional studies in *in vivo* models of epilepsy with regards to the P2Y<sub>1</sub> receptor have been performed. However, our data from Chapter 3 and studies analysing P2Y<sub>1</sub> expression showed a significant increase of the P2Y<sub>1</sub> receptor in epileptic patients (Sukigara et al., 2014), suggesting a functional role during epilepsy. Our study demonstrated P2Y<sub>1</sub> delaying the onset of epilepsy, possibly by preventing microglia activation. This is the first study showing this effect on any P2 receptor so far and demonstrates that drugs targeting this class of receptors may have potential as a therapeutic strategy to prevent epilepsy development following brain injury. Finally, we also showed a strong seizure-suppressive potential of P2Y<sub>1</sub> antagonists when given to epileptic mice. Whether this was also mediated via reducing microglia activation we do not know. However, the strong upregulation of this receptor on microglia suggest this. While our results suggest anticonvulsive effects, this may be due to a too short treatment regime or a too low drugs dose. Our results showing long-lasting changes in seizure severity suggest at least some disease-modifying potential. Regardless of the mechanism and anticonvulsive vs disease modifying potential, our results demonstrate

P2Y<sub>1</sub> antagonism being effective during chronic epilepsy making this the only P2 receptor analysed to date, apart from the ionotropic P2X7 receptor (Beamer et al., 2017), to be functional during status epilepticus, epileptogenesis and epilepsy and drugs interfering with P2Y<sub>1</sub> signalling being protective. Our results are also further in line with neuroinflammation having a causal role during icto- and epileptogenesis and demonstrate that drugs interfering with inflammatory pathways may represent novel strategies to treat seizure-induced pathology and epilepsy.

In conclusion, our study has provided the first comprehensive characterization of the P2Y receptor family during status epilepticus and chronic epilepsy and identifies P2Y<sub>1</sub> receptor antagonism a novel therapy to treat status epilepticus and epilepsy.

## 6.2 Conclusions

During the present PhD thesis, we have characterized the expression and function of a new receptor family, metabotropic P2Y receptors, during status epilepticus and epilepsy. We have carried out a thorough characterization of the induction and expression changes of the P2Y receptor family in two different mouse models of epilepsy and in TLE patients. We have also demonstrated potent anticonvulsive and seizure-suppressive potential of antagonists against the P2Y<sub>1</sub> receptor in different seizure mouse models.

In conclusion, our results demonstrate a functional role of the P2Y receptor family during epilepsy and suggest that targeting of this receptor family may represent a new therapeutic strategy to treat status epilepticus and drug-refractory epilepsy.

### 6.3 Future work

To further progress P2Y<sub>1</sub> modulation towards a clinical application in epilepsy, the following future studies are proposed:

#### **1. Elucidate the protective mechanism of P2Y<sub>1</sub> during pre-treatment regime.**

When applied during status epilepticus, P2Y<sub>1</sub> antagonism protected against seizure-induced pathology via reducing microglia activation. However, we also must determine what makes P2Y<sub>1</sub> activation good during our pre-treatment regime. P2Y<sub>1</sub> seems to be highly expressed on neurons during physiological conditions. Therefore, a possible explanation may be P2Y<sub>1</sub>-induced alterations in neurotransmitter release. In line with this, P2Y<sub>1</sub> inhibits the release of glutamate in the hippocampus (Mendoza-Fernandez et al., 2000, Koizumi et al., 2003, Rodrigues et al., 2005), possibly through the inhibition of voltage-activated Ca<sup>2+</sup> channels (VACCs) (Gerevich et al., 2004). P2Y<sub>1</sub> is also involved in the inhibition of GABA transport (Jacob et al., 2014). P2Y<sub>1</sub> receptor function during status epilepticus may be dependent on the neurotransmitter release, but there is no studies performed up to now.

#### **2- Determine the cell-specific contribution of P2Y<sub>1</sub> to seizure-induced pathology.**

Based in our results, P2Y<sub>1</sub> receptor function on seizure generation seems to be dependent on the cell type where P2Y<sub>1</sub> is expressed. Up to now, no studies have been performed to investigate the functional role of the P2Y<sub>1</sub> receptor in specific cells during epilepsy. The use of a cell specific conditional knockout of P2Y<sub>1</sub> receptors (neurons, microglia and possibly astrocytes) will allow a better understanding of the role of the P2Y<sub>1</sub> receptor during status epilepticus and during epilepsy.

#### **3- Identify up-stream and down-stream P2Y<sub>1</sub> signalling during status epilepticus and during epilepsy.**

ADP, main P2Y<sub>1</sub>-activation substrate, may be the consequence of extracellular ATP break-down but may also be release in the brain (Rodrigues et al., 2015). In future studies we should address where and when ADP is available during seizures and epilepsy. Secondly, there is no information available about possible transcriptional factors of the P2Y<sub>1</sub> receptors. Identification of the mechanisms that control P2Y<sub>1</sub> receptor regulation may provide additional new targets for the treatment of epilepsy. Finally, we also must elucidate the consequences of interfering with P2Y<sub>1</sub> signalling during epilepsy. This is important to predict possible adverse side effects, and, on the other side, may also provide new target genes for epilepsy treatment.

## 7. References

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## 8. Appendices



# The Metabotropic Purinergic P2Y Receptor Family as Novel Drug Target in Epilepsy

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Epilepsy encompasses a heterogeneous group of neurological syndromes which are characterized by recurrent seizures affecting over 60 million people worldwide. Current anti-epileptic drugs (AEDs) are mainly designed to target ion channels and/or GABA or glutamate receptors. Despite recent advances in drug development, however, pharmacoresistance in epilepsy remains as high as 30%, suggesting the need for the development of new AEDs with a non-classical mechanism of action. Neuroinflammation is increasingly recognized as one of the key players in seizure generation and in the maintenance of the epileptic phenotype. Consequently, targeting signaling molecules involved in inflammatory processes may represent new avenues to improve treatment in epilepsy. Nucleotides such as adenosine-5'-triphosphate (ATP) and uridine-5'-triphosphate (UTP) are released in the brain into the extracellular space during pathological conditions such as increased neuronal firing or cell death. Once released, these nucleotides bind to and activate specific purinergic receptors termed P2 receptors where they mediate the release of gliotransmitters and drive neuronal hyperexcitation and neuroinflammatory processes. This includes the fast acting ionotropic P2X channels and slower-acting G-protein-coupled P2Y receptors. While the expression and function of P2X receptors has been well-established in experimental models of epilepsy, emerging evidence is now also suggesting a prominent role for the P2Y receptor subfamily in seizure generation and the maintenance of epilepsy. In this review we discuss data supporting a role for the P2Y receptor family in epilepsy and the most recent finding demonstrating their involvement during seizure-induced pathology and in epilepsy.

**Keywords:** epilepsy, status epilepticus, pharmacoresistance, purinergic signaling, metabotropic P2Y receptors

## INTRODUCTION

The primary treatment for epilepsy is the use of anti-epileptic drugs (AEDs). These drugs control seizures by shifting the balance of inhibitory and excitatory drive in the brain (Bialer et al., 2013). 30% of patients, however, are pharmacoresistant to all available AEDs and between 40 and 50% of patients on AEDs suffer adverse effects (Baker et al., 1997). Current major goals of epilepsy research are to develop treatment strategies that impact upon disease emergence and progression, show efficacy within the currently pharmacoresistant cohort and have a lower burden of adverse effects. To this end, the role of neuroinflammation in ictal- and epileptogenesis is

receiving growing attention (Terrone et al., 2017). Purinergic signaling provides a mechanism by which hyperexcitation can lead to an inflammatory response and whereby inflammation can lead to hyperexcited networks. As such, the targeting of purinergic signaling is a promising strategy for developing new treatment options (Engel et al., 2016). Purinergic signaling is mediated via two families of purinergic receptors: ionotropic P2X receptors and metabotropic P2Y receptors (Burnstock, 2007), both receptor subtypes responding to extracellular adenine or uridine nucleotides. While much of the focus of purinergic signaling in epilepsy has focussed on the P2X receptor family, the role of P2Y receptors in epilepsy has, to date, received much less attention (Engel et al., 2016; Rassendren and Audinat, 2016; Beamer et al., 2017). In this review, we summarize data from the emerging field and suggest directions in which P2Y research in epilepsy should develop.

## SEIZURES, STATUS EPILEPTICUS, AND EPILEPSY

Seizures are a transient symptom resulting from abnormally excessive or synchronous neuronal firing in the brain (Fisher et al., 2014). In general, seizures do not last longer than 1–2 min and are self-limiting (Jenssen et al., 2006). Prolonged or recurrent seizures without intervening recovery periods, however, are classified as status epilepticus, a medical emergency (Betjemann and Lowenstein, 2015). Beyond epilepsy, seizures can have many etiologies, including acute insults, such as fever, hypoxia, low blood sugar, brain tumors, lack of sleep, substance abuse, or traumatic brain injury (TBI). Seizures can be classified according to their etiology, semiology, and anatomical focus (Chang et al., 2017). The transition from seizures to status epilepticus is often due to a failure of endogenous anticonvulsant mechanisms, such as the internalization or desensitization of  $\gamma$ -aminobutyric acid (GABA)<sub>A</sub> receptors (Naylor and Wasterlain, 2005; Wasterlain et al., 2009; Betjemann and Lowenstein, 2015). Status epilepticus is the second most common neurological emergency behind stroke, with an annual incidence of 10–41 cases per 100,000 (Hesdorffer et al., 1998). It is associated with high mortality (up to 20%), morbidity and considerable costs to the health-care system (Betjemann and Lowenstein, 2015) and can cause severe damage to the brain, leading to serious neurological complications such as cognitive impairment (Kornigut et al., 2007), and the development of chronic epilepsy (Hesdorffer et al., 1998).

Where seizures are recurrent and spontaneous, epilepsy is diagnosed. According to the International League Against Epilepsy (ILAE), epilepsy is defined by any of the following conditions: “(1) at least two unprovoked (or reflex) seizures occurring > 24 h apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; and (3) diagnosis of an epilepsy syndrome” (Fisher et al., 2014). Epilepsy is one of the most common neurological disorders, globally. With an incidence of ~1%, epilepsy affects over 65 million people worldwide (Moshe

et al., 2015). This is associated with a global disease burden of 7M disability adjusted life years (DALYs) (Leonardi and Ustun, 2002) and with an estimated annual cost of over €20 billion in Europe alone according to the World Health Organization (2010). Beside the occurrence of spontaneous seizures, epilepsy is associated with an increased mortality and co-morbidities such as anxiety and depression, which severely impact quality of life (Moshe et al., 2015). Epilepsy affects people of all ages, but is most common in the young and, particularly, the elderly (Everitt and Sander, 1998). Epilepsy can either be innate or acquired, arise due to genetic mutations or via epigenetic mechanisms (Chen et al., 2017), structural or metabolic alterations (Reid and Staba, 2014), infection and immune dysregulation (Vezzani et al., 2011), some combination thereof or, as is often the case, be of unknown etiology (idiopathic epilepsy) (Pal et al., 2016). Common mutations underlying epilepsy include those affecting the function of ion channels, such as the Na<sup>+</sup> channel, Voltage-Gated Sodium Channel Alpha Subunit (SCN1A) (Kasperaviciute et al., 2013), reducing the action potential threshold in neurons. Structural causes often arise as a result of changes in neuronal network connectivity following an initial insult to the brain, such as head injury, stroke, or status epilepticus (Pitkanen et al., 2015). Epileptogenesis, the process of a normal brain becoming epileptic, is usually the result of a precipitating injury and characterized by an interplay of factors including ongoing cell death, inflammation and synaptic and axonal plasticity changes (Pitkanen et al., 2015). Temporal lobe epilepsy (TLE), the most prevalent form of acquired epilepsy, is characterized by hippocampal sclerosis, including neuronal loss, mossy fiber sprouting and the formation of aberrant neuronal networks which can form a unilateral seizure focus, typically in the CA3 region of the hippocampus (Rao et al., 2006), from which seizures often generalize. Possibly because of the importance of these network changes, TLE is associated with a particularly high prevalence of pharmacoresistance (Zhao et al., 2014).

## Current Treatments for Epilepsy and Status Epilepticus

Over 25 AEDs are currently used in the clinic (Bialer and White, 2010). Despite the relatively large range of options available, where the mechanisms of action are understood, they fit into three broad categories: increasing inhibitory transmission (e.g., the glutamate decarboxylase catalyst, Gabapentin), decreasing excitatory transmission [e.g., the non-competitive alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, Perampanel] and blockade of voltage-gated ion channels (e.g., Na<sup>+</sup> channel blocker, lamotrigine) (Bialer and White, 2010). In most cases, AEDs have multiple actions and are incompletely understood. For example, Topiramate exerts an inhibitory effect on Na<sup>+</sup> conductance, enhances GABA neurotransmission via unknown mechanisms, and antagonizes AMPA receptors (Shank et al., 2000). While there is a superficial diversity in mechanisms, all treatment options rely on the concept of redressing a balance between excitatory and inhibitory drive. This has proven largely successful in controlling seizures, but no treatments have been developed that act on the emergence or progression

of the epileptic condition. Further, approximately 30% of patients remain pharmacoresistant to all available AEDs; in most cases leaving surgery as their sole remaining option (Moshe et al., 2015). Choice of treatment strategy is based on seizure type, epilepsy syndrome, health problems, other medication used, lifestyle of the patients and considerations such as pregnancy (Moshe et al., 2015; Kinney and Morrow, 2016). AEDs are the frontline treatment for epilepsy. Although strides have been made in terms of safety, tolerability, and pharmacokinetics with the new generation of AEDs, such as felbamate, gabapentin, lamotrigine or oxcarbazepine, the number of patients resistant to all treatments has not moved from 30% for approximately 80 years (Bialer et al., 2013; Moshe et al., 2015) and the search for mechanisms which could disrupt the emergence or progression of the disease remains elusive. There is therefore an urgent need to identify new drug targets which can show efficacy in patients who are currently refractory to available treatment, and can demonstrate a disease modifying effect.

When treating status epilepticus, time is a key factor and terminating the seizure is the number one priority for preventing lasting damage. A protocol for treatment of status epilepticus has been developed whereby, a first line treatment is administered within 5–10 min of seizure onset, a second line treatment is administered within 20–40 min and a third line treatment around 60 min following seizure onset (Shorvon et al., 2008). The best first line treatment is with benzodiazepines, such as lorazepam, diazepam, or midazolam (Betjemann and Lowenstein, 2015). Evidence supporting the best treatment strategy for second and third line treatments is weaker, however current practice involves the use of AEDs such as fosphenytoin, valproic acid or levetiracetam (Glauser et al., 2016) and anesthetic drugs (Betjemann and Lowenstein, 2015). As with epilepsy, approximately 30% of status epilepticus patients are refractory to available drug treatment and these patients are particularly vulnerable to adverse clinical outcomes (Novy et al., 2010). In summary, the drug development challenges for epilepsy and status epilepticus are similar, with a need in both cases for drugs which show efficacy in currently pharmacoresistant patients, while reducing comorbidities and adverse drug effects. In the case of epilepsy, preventing the emergence or progression of the disorder is also an important goal.

## New Directions in Drug Development for Epilepsy

While drugs targeting excitatory and inhibitory drive have proven widely successful in controlling seizures (Bialer et al., 2013), it seems likely that in order to modify disease progression or offer efficacious drug treatment to currently pharmacoresistant epilepsy patients, alternative targets, with a novel mechanism of action, must be sought. Several experimental and clinical findings have demonstrated an important role for neuroinflammation in both ictal- and epileptogenesis (Vezzani et al., 2011, 2016). High levels of inflammatory mediators are present in the brains of both experimental rodent models of epilepsy and epilepsy patients (Aronica et al., 2017) and these processes have therefore received much attention in recent years. Selective

blockade of the pro-inflammatory cytokine, Interleukin-1 $\beta$  (IL-1 $\beta$ ), has been shown to reduce seizures in *in vivo* models of epilepsy (Ravizza et al., 2008; Vezzani et al., 2009), while in an epileptogenesis-resistant animal, the Amazon rodent, *Proechimys*, no acute brain inflammatory response was found following experimentally-induced status epilepticus (Scorza et al., 2017).

Following an insult, such as a seizure or period of status epilepticus, pro-inflammatory cytokines, such as IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-6 are released in the brain, primarily from astrocytes and microglia (Terrone et al., 2017). These pro-inflammatory cytokines exert a number of effects that contribute to a reduction in the seizure threshold and emergence of chronic epilepsy. Experimental evidence demonstrates that pro-inflammatory cytokines can have an effect on the firing properties of neurons directly, through the modulation of voltage-gated Na<sup>+</sup>, Ca<sup>2+</sup>, and K<sup>+</sup> ion channels (Viviani et al., 2007), facilitation of excitatory neurotransmission through both pre- and post-synaptic mechanisms, and disinhibition via antagonism of GABA<sub>A</sub> receptors (Garcia-Oscos et al., 2012). The effect of inflammation on seizures and epilepsy, however, is not limited to direct modulation of the excitatory/inhibitory balance. Gliosis, gliotransmission, increased permeability of the blood–brain barrier (BBB) and subsequent influx of peripheral cells and modulatory molecules, neuronal cell death and the aberrant reorganization of neuronal networks can all be consequences of a neuroinflammatory response (Vezzani et al., 2012). The causality between hyperexcitation, excitotoxicity, and neuroinflammation is circular and, as described below, intercellular signaling through purines is an important mediator of these processes, making purinergic receptors an attractive treatment target.

## PURINERGIC SIGNALING

It was not until 1972 that the role of adenosine-5'-triphosphate (ATP) as an intercellular molecule, was first described by Burnstock (1972). Today, it is well-recognized that a wide variety of nucleotides, including ATP, function as either sole or co-transmitter in both the peripheral and central nervous system (CNS). ATP can act as a fast, excitatory neurotransmitter or as a neuromodulator and is involved in a vast array of short- and long-term physiological and pathological processes including inflammation, cellular survival, proliferation, cellular differentiation, and synaptic plasticity (Burnstock et al., 2011; Khakh and North, 2012; Idzko et al., 2014). It has therefore been implicated in numerous different diseases of the CNS including epilepsy (Burnstock, 2017).

## Purine Release in the Brain

Purines and pyrimidines are a well-established source of energy in all living cells. These molecules, however, also play an important role in intercellular communications within the CNS (Lecca and Ceruti, 2008; Idzko et al., 2014). Adenine and uridine nucleotides are present in almost every synaptic and secretory vesicle where they are either present alone, functioning as a fast neurotransmitter or co-stored with classical neurotransmitters

(e.g., GABA or glutamate) (Abbracchio et al., 2009). Under physiological conditions, adenine and uridine nucleotides are usually present at micromolar concentrations in the extracellular space; however, under pathological conditions (e.g., inflammation, hyperexcitability, and cell death) extracellular nucleotide levels can reach the millimolar range (Dale and Frenguelli, 2009; Idzko et al., 2014; Rodrigues et al., 2015). ATP [and most likely uridine-5'-triphosphate (UTP)] can enter the extracellular space by crossing the compromised membranes of damaged and dying cells (Rodrigues et al., 2015). In addition, purines are actively released from different cell types including neurons, astrocytes, microglia, and endothelial cells to act as neuro- and glio-transmitters (Lecca and Ceruti, 2008; Rodrigues et al., 2015). Several mechanisms have been proposed to contribute to the release of nucleotides into the extracellular medium including cell damage, exocytosis of secretory granules, vesicular transport involving the vesicular nucleotide transporter (VNUT) and membrane channels such as ABC transporters, pannexins, connexins and via purinergic receptors themselves (Lecca and Ceruti, 2008; Rodrigues et al., 2015). Once released into the extracellular space, adenine and uridine nucleotides are rapidly metabolized by ectonucleotidases (e.g., ectonucleoside triphosphate diphosphohydrolases, ectonucleotide pyrophosphatase, alkaline phosphatases, ecto-5'-nucleotidase, and ecto-nucleoside diphosphokinase) into different breakdown products including adenosine-5'-diphosphate (ADP), adenosine, uridine-5'-diphosphate (UDP), and uridine. These metabolites, in turn, are important neurotransmitters/neuromodulators in their own right, with specific receptors for each expressed throughout the CNS (Zimmermann, 2006; Burnstock, 2007).

Direct evidence for ATP release during seizures is mixed. Large elevations in ATP on electrical stimulation of the cortex (Wu and Phillis, 1978) provided the first direct evidence that high levels of neuronal activity could induce the release of ATP. Subsequently, stimulation of the Schaffer collateral in hippocampal slices was demonstrated to induce ATP release in a  $Ca^{2+}$ -dependent, but glutamate receptor activation-independent manner (Wieraszko et al., 1989), suggesting the release of ATP was pre-synaptic. While ATP release was not detected following high frequency stimulation or electrically-induced epileptiform seizure like events in hippocampal slices (Lopatar et al., 2015), the induction of epileptiform activity in rat hippocampal slices with the use of the mGluR5-agonist, (S)-3,5-Dihydroxyphenylglycine induced the release of ATP through pannexin hemichannels (Lopatar et al., 2015). ATP release was also elevated in hippocampal slices in a high  $K^+$  model of seizures (Heinrich et al., 2012). Dona et al. (2016) used microdialysis and high-performance liquid chromatography in order to attempt to measure extracellular concentrations of ATP and its metabolites *in vivo* after pilocarpine-induced status epilepticus and following the onset of chronic epilepsy. They found no change in ATP concentrations for 4 h following status epilepticus, but a marked increase in ATP metabolites, including adenosine monophosphate (AMP) and ADP. Concentrations of ATP and all metabolites were reduced during chronic epilepsy, but ATP was elevated by 300% during spontaneous seizures. Because ectonucleotidases rapidly hydrolyze ATP in

the extracellular space and the concentration and activity of these enzymes are increased following seizures (Nicolaidis et al., 2005), it is difficult to measure changes in ATP release directly. Less interest has been shown in investigating UTP release following seizures, however, Koizumi et al. (2007) demonstrated that following kainic acid (KA)-induced-seizure-like events in hippocampal slices, extracellular concentrations of UTP were elevated approximately threefold (Koizumi et al., 2007).

Whereas the anticonvulsive properties of the nucleoside, adenosine, are well-documented (Boison, 2016), the possible contribution of extracellular nucleotides to seizure pathology is a relatively new research area (Engel et al., 2016). The discovery of increased extracellular levels of ATP in seizure-prone rats was one of the first studies to suggest a functional contribution of extracellular nucleotides to seizures (Wieraszko and Seyfried, 1989). Demonstrating a direct impact on seizures, another early study showed that the microinjection of ATP analogs into the prepiriform cortex led to the generation of motor seizures (Knutsen, 1997). More recent evidence implicating extracellular nucleotides in seizure generation stems from studies showing that the injection of ATP into the brain of mice led to the development of high spiking on the electroencephalogram (EEG) and exacerbated seizure severity during status epilepticus (Engel et al., 2012; Sebastian-Serrano et al., 2016). In contrast, treatment with UTP decreases the rate of neuronal firing in epileptic rats (Kovacs et al., 2013) and in mice subjected to status epilepticus (Alves et al., 2017). Further, UTP metabolites such as uridine reduce epileptic seizures in patients with epileptic encephalopathy (Koch et al., 2017).

## P2 Receptor Family

Once released, extracellular adenine and uridine nucleotides bind to and activate specific cell surface receptors termed P2 receptors which are ubiquitously expressed and functional on all cell types in the CNS (Burnstock, 2007). The P2 family of receptors include the ionotropic P2X channels and the metabotropic P2Y receptors. The fast acting P2X channels are a family of seven cation-permeable ionotropic receptor subunits (P2X1-7) which form both homo- and hetero-trimers, depolarizing the cell membrane upon activation (Khakh and North, 2006). All P2X receptors are activated by their main endogenous agonist, ATP, and are permeable to small cations including  $Na^+$ ,  $K^+$ , and  $Ca^{2+}$ . All P2X receptor subunits share a common topology with two transmembrane domains, a large extracellular loop and an intracellular amino and carboxyl terminus (Khakh and North, 2006; Burnstock, 2007). Much attention has been paid to the study of P2X receptors over the past decades, in particular in diseases of the CNS (Burnstock et al., 2011; Saez-Orellana et al., 2015). P2X receptor activation has been implicated in numerous pathological conditions including neurodegeneration, inflammation, ischemia, brain trauma, and hyperexcitability (Engel et al., 2016; Burnstock, 2017). Among the P2X receptor subtypes, the P2X7 receptor has attracted by far the most attention as a potential therapeutic target for brain diseases (Sperlagh and Illes, 2014; Rech et al., 2016).

While the P2X receptor family is made up of fast acting ligand-gated ion channels, the metabotropic P2Y receptor family

consists of eight G-protein coupled slower-acting receptors: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> (von Kugelgen, 2006; Burnstock, 2007). In contrast to P2X channels, P2Y receptors can be activated by more than one substrate including the adenine nucleotides ATP (P2Y<sub>2</sub> and P2Y<sub>11</sub>) and ADP (P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub>) and the uridine nucleotides UTP (P2Y<sub>2</sub> and P2Y<sub>4</sub>), UDP (P2Y<sub>6</sub> and P2Y<sub>14</sub>), and UDP-glucose (P2Y<sub>14</sub>). P2Y receptors contain the typical features of G-protein-coupled receptors which includes an extracellular amino terminus, intracellular carboxyl terminus and seven transmembrane-spanning motifs (Jacobson et al., 2015). P2Y receptors can be further subdivided into groups based on their coupling to specific G proteins. P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> receptors are coupled to G<sub>q</sub> proteins, which stimulate phospholipase C, ultimately resulting in the subsequent release of Ca<sup>2+</sup> from intracellular stores and activation of protein kinase C (PKC). Of these, P2Y<sub>11</sub> receptor can also couple to G<sub>s</sub>, stimulating adenylate cyclase and increasing the production of cyclic adenosine monophosphate (cAMP) (von Kugelgen, 2006). P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> are coupled to G<sub>i</sub> proteins, inhibiting adenylate cyclase and thereby decreasing cAMP production (von Kugelgen, 2006).

### Involvement of P2Y Receptor Signaling in Brain Inflammation and Excitability

P2Y receptors are involved in a myriad of different cellular functions and pathological processes pertinent to the process of epileptogenesis and epilepsy including neuroinflammation, neurodegeneration, synaptic reorganization, and changes in neurotransmitter release (von Kugelgen, 2006; Jacobson and Boeynaems, 2010; Pitkanen et al., 2015; Guzman and Gerevich, 2016) making them an attractive antiepileptic therapeutic target.

Inflammatory processes in the brain have received much attention over recent years and are thought to play a major role in seizure-induced pathology and the development of epilepsy (Vezzani et al., 2011). The principle ligands for P2Y receptors are the purine, ATP, the pyrimidine, UTP, and their metabolites, such as ADP and UDP (Burnstock, 2007). The role of each receptor in neuroinflammation is dictated by its affinity for different ligands and downstream targets. ATP is both released as a result of inflammation and promotes pro-inflammatory mechanisms. This circular causality can underpin a positive feedback loop whereby neuroinflammation becomes self-sustaining (Idzko et al., 2014). Less is known about the role of UTP in mediating neuroinflammation. The role of different P2Y receptors in mediating neuroinflammation and cell death seems to be divergent (Forster and Reiser, 2015), depending on downstream signaling pathways and mutually antagonistic actions, but is incompletely understood. The P2Y<sub>1</sub> receptor, activated by the ATP metabolite ADP, is expressed also on astrocytes and activated under conditions of oxidative stress, prompting the release of IL-6 (Fujita et al., 2009). IL-6 has been shown to play an anti-inflammatory role during 'classic signaling' involving the binding of IL-6 to the membrane-bound IL-6 receptor which induces the dimerization of the  $\beta$ -receptor glycoprotein 130 (gp130). In contrast however, IL-6 is also critical for pro-inflammatory signaling in a process termed

'trans-signaling,' whereby IL-6 stimulates distant cells which only express gp130 in the absence of the IL-6 receptor (Rothaug et al., 2016). A more recent study has shown that in a chronic model of epilepsy, astrocytes from kindled rats show enhanced Ca<sup>2+</sup>-dependent signaling and astroglial hyperexcitability, which requires the activation of the P2Y<sub>1</sub> receptor (Alvarez-Ferradas et al., 2015). P2Y<sub>1</sub> antagonism prevented cognitive deficits and neuronal damage in a model of ischemia in mice (Carmo et al., 2014). A recent study also showed improved histological and cognitive outcomes in a model of TBI in mice provided by P2Y<sub>1</sub> receptor antagonism (Choo et al., 2013). Activation of astrocytic P2Y<sub>2</sub> receptors promotes astrocyte activation and migration via an interaction with  $\alpha$ V-integrin (Wang et al., 2005). The P2Y<sub>2</sub> receptor has also been shown to play a protective role against chronic inflammation-induced neurodegeneration in a model of Alzheimer's disease (Kong et al., 2009). A role for the uridine-sensitive P2Y<sub>4</sub> receptor in mediating neuroinflammation has not been established (Beamer et al., 2016), with progress hamstrung by a lack of specific tools for targeting this receptor. The P2Y<sub>6</sub> receptor promotes the activation of microglia and the adoption of a phagocytic phenotype following activation by the UTP metabolite UDP (Koizumi et al., 2007). This is dependent on downstream signaling involving phospholipase C and PKC. Other studies have suggested a role for the P2Y<sub>12</sub> receptor in microglial activation (Ohsawa et al., 2010), showing that activation of integrin- $\beta$ 1 in microglia through P2Y<sub>12</sub> is involved in directional process extension by microglia in brain tissue. As discussed in more detail below, P2Y<sub>12</sub>-dependent process extension has been shown to be increased following status epilepticus in mice (Eyo et al., 2014).

The effects of P2Y signaling are not limited to inflammatory processes and cellular survival alone. P2Y signaling also impacts directly on neuronal excitability, synaptic strength, and synaptic plasticity (Guzman and Gerevich, 2016). Presynaptic P2Y receptors have been shown to affect the release of different neurotransmitters including glutamate, noradrenaline and GABA, most likely by reducing presynaptic Ca<sup>2+</sup> influx (Fischer et al., 2009). P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> inhibit the release of glutamate in the hippocampus (Mendoza-Fernandez et al., 2000; Koizumi et al., 2003; Rodrigues et al., 2005), possibly through the inhibition of voltage-activated Ca<sup>2+</sup> channels (VACCs) (Gerevich et al., 2004). Using the same mechanism, the release of noradrenaline in the hippocampus was also blocked via P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> activation (Csolle et al., 2008). Similarly, activation of P2Y<sub>4</sub> with UTP blocks the release of the inhibitory neurotransmitter GABA from cerebellar basket cells (Donato et al., 2008). P2Y receptors alter the expression/function of other membrane receptors and voltage-gated ion channels. P2Y<sub>1</sub> triggers the desensitization or internalization of the metabotropic glutamate receptor 1 (mGluR1) (Mundell et al., 2004) and inhibits N-methyl-D-aspartate (NMDA) receptor channels (Luthardt et al., 2003). P2Y<sub>1</sub> also increases the sensitivity of the GABA<sub>A</sub> receptor (Saitow et al., 2005) and inhibits P2X receptors (Gerevich et al., 2007). P2Y receptor activation can lead to the inhibition of VACCs (Diverse-Pierluissi et al., 1991) thereby potentially influencing neuronal excitability and synaptic

plasticity. P2Y receptors also block potassium channels [e.g., voltage-gated potassium channel subunit KvLQT2,3 (Filippov et al., 2006) or G protein-coupled inward rectifying channels 1, 2, and 4 (GIRK1,2,&4)] (Filippov et al., 2004), inhibiting membrane hyperpolarisation and thereby facilitating an increased frequency of neuronal firing (Brown and Passmore, 2009; Guzman and Gerevich, 2016). On a network level, P2Y<sub>1</sub> increases the firing of GABAergic inhibitory neurons either directly or via P2Y<sub>1</sub>-dependent activation of astrocytes in the hippocampus, eventually leading to an increase in inhibitory-postsynaptic currents (IPSCs) in pyramidal neurons (Bowser and Khakh, 2004). In a more recent study, Jacob et al. (2014) showed that astrocytic P2Y<sub>1</sub> activation increases extracellular concentrations of GABA by inhibiting Ca<sup>2+</sup> signaling dependent GABA transport (Jacob et al., 2014). In conclusion, while P2X receptors exert a mainly facilitatory effect on synaptic transmission (Khakh and North, 2012), the effects of P2Y receptors seem to be context-specific, either increasing or decreasing neuronal firing by altering excitatory and inhibitory neurotransmitter release or altering receptor function (e.g., NMDA and GABA<sub>A</sub>) and channel conductance (e.g., voltage-gated KCNQ2/3 potassium channel) (Guzman and Gerevich, 2016).

## PURINERGIC SIGNALING AS A NOVEL DRUG TARGET IN EPILEPSY

Mounting evidence has accumulated over the past decades demonstrating a causal role for purinergic signaling in numerous pathological conditions ranging from cancer (Di Virgilio, 2012), cardiovascular disease (Ralevic, 2015), blood cell diseases (McGovern and Mazzone, 2014) to diabetes (Fotino et al., 2015) and brain diseases (Puchalowicz et al., 2014). Among brain diseases, intervention in purinergic signaling has been postulated as a new therapeutic avenue for acute insults to the brain such as stroke (Kuan et al., 2015) and TBI (Kimbler et al., 2012) and for chronic brain diseases including neurodegenerative diseases (e.g., Huntington's, Alzheimer's, and Parkinson's disease) (Miras-Portugal et al., 2016), neuropsychiatric disorders (e.g., depression and schizophrenia) (Burnstock et al., 2011) and also epilepsy (Beamer et al., 2017). Emphasizing the potential for targeting purinergic signaling as a promising new therapeutic strategy, several compounds are already used in the clinic, including the P2Y<sub>2</sub> agonist Diquafosol for the treatment of dry eye (Lau et al., 2014) or Clopidogrel, a P2Y<sub>12</sub> antagonist used for the treatment of thrombosis (Sarafoff et al., 2012) while others have progressed into clinical trials such as antagonists of the ionotropic P2X<sub>3</sub> used against refractory chronic cough (Abdulqawi et al., 2015) and P2X<sub>7</sub> receptors used against rheumatoid arthritis (Keystone et al., 2012) and other inflammatory conditions (Rech et al., 2016).

To date, most of the studies performed to elucidate the changes in expression and functional contribution of purinergic P2 receptors to seizures and epilepsy have focused on the P2X receptor subtype, in particular the P2X<sub>7</sub> receptor (reviewed in Beamer et al., 2017), with relatively little attention paid to the P2Y receptor family. The lack of apparent interest was largely due to a lack of suitable tools (e.g., drugs to manipulate P2Y function)

and the strong focus on fast synaptic effects conferred by the ionotropic P2X receptors (Engel et al., 2016). Recent studies using experimental animal models of status epilepticus and epilepsy and analysis of patient brain tissue, however, suggest a prominent role for P2Y signaling during seizures and the development of epilepsy (Table 1). In the last section of this review we describe in detail the evidence linking a pathological activation of the metabotropic P2Y receptors to seizure generation and seizure-induced pathology and discuss the antiepileptic potential of drugs targeting P2Y signaling.

## P2Y Expression Following Status Epilepticus

One of the earliest studies analyzing P2Y expression changes following status epilepticus used the intraperitoneal KA-induced status epilepticus mouse model (Avignone et al., 2008). Here, the authors observed an increase in transcription of *P2ry6*, *P2ry12*, and *P2ry13* in the hippocampus. In another study using the intraperitoneal pilocarpine mouse model, Rozmer et al. (2016) show an increase in P2Y<sub>1</sub> activity in neuronal progenitor cells following status epilepticus. In a more recent study, our group published a comprehensive analysis of changes in transcription and expression across the entire P2Y family of receptors following status epilepticus using two different mouse models: the intraamygdala KA mouse model of status epilepticus (Mouri et al., 2008) and the intraperitoneal pilocarpine mouse model of status epilepticus (Alves et al., 2017). Both, intraamygdala KA and intraperitoneal pilocarpine-induced status epilepticus increased the transcription of the uridine-sensitive P2Y receptors *P2ry2*, *P2ry4*, and *P2ry6* in the hippocampus. At the same time, the transcription of the adenine-sensitive receptors *P2ry1*, *P2ry12*, and *P2ry13* was downregulated. At the protein level, hippocampal levels of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> were increased and P2Y<sub>12</sub> was decreased following status epilepticus. No immunohistochemistry was performed to identify cell types expressing the different P2Y receptors. Thus, these results show that changes in the transcription of P2Y receptors following status epilepticus closely correlate with the known profile of agonists (i.e., adenine-sensitive receptors are downregulated and uridine-sensitive receptors are upregulated) and, at the protein level, the G-protein coupling of the receptors with P2Y receptors coupled to G<sub>q</sub> being increased and P2Y receptors coupled to G<sub>i</sub> being downregulated or not changed (Alves et al., 2017).

## P2Y Expression During Chronic Epilepsy

Much less is known about the expression profile of P2Y receptors during epilepsy. To date, the only study carried out characterizing P2Y expression in experimental epilepsy was undertaken using the intraamygdala KA mouse model (Alves et al., 2017). In this model, mice become epileptic after a short latent period of 2–5 days (Mouri et al., 2008). Analysis of the hippocampus 14 days-post status epilepticus revealed increased *P2ry1*, *P2ry2*, and *P2ry6* transcription and increased P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>12</sub> protein levels. No changes were observed for the remaining receptors. Thus, P2Y upregulation seems to be the predominant response during experimental epilepsy, probably due to an increase in

**TABLE 1** | Summary of the main findings of P2Y receptor expression and function during status epilepticus and epilepsy in experimental models of epilepsy and patient brain.

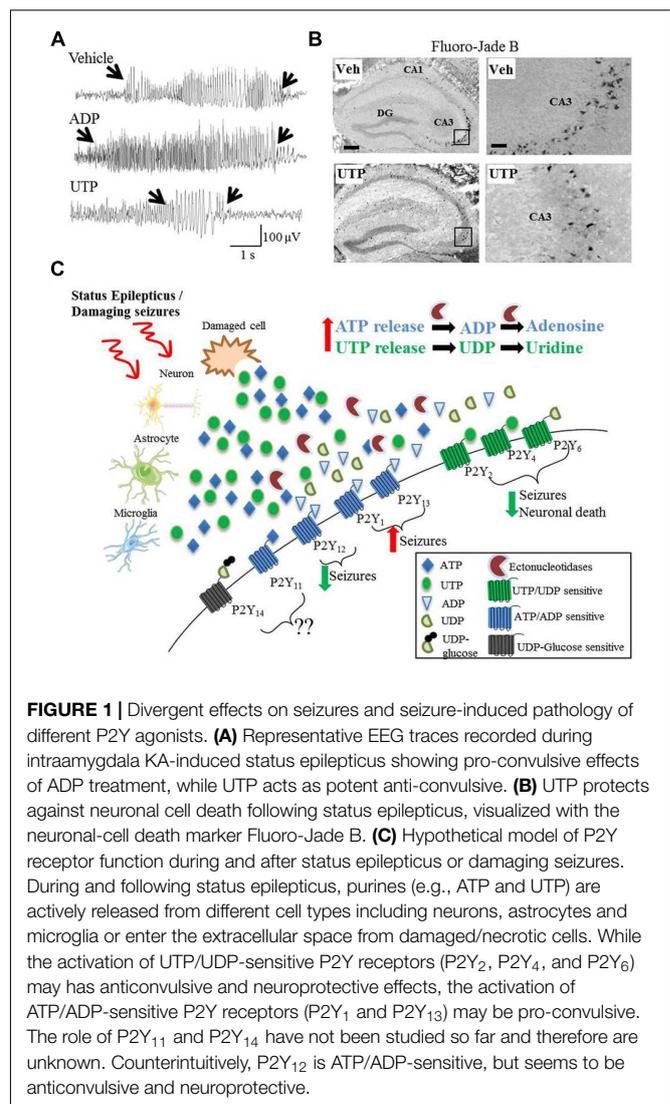
Disease process/stage	Epilepsy models/patients	Brain region	Techniques/drugs	Main results	Reference
Status epilepticus	i.p. KA-induced status epilepticus in mice	Hippocampus	GFP reporter mice; hippocampal slices; qPCR; UDP (broad-spectrum P2Y receptor agonist) and 2-MeSADP treatment (P2Y <sub>1</sub> , P2Y <sub>12</sub> , P2Y <sub>13</sub> agonist)	Upregulation of P2Y <sub>6</sub> , P2Y <sub>12</sub> transcripts after status epilepticus; transient downregulation of the P2Y <sub>13</sub> transcript 3 h following status epilepticus and increased P2Y <sub>13</sub> transcript 48 h post-status epilepticus; increased microglia currents after treatment with UDP and 2-MeSADP	Avignone et al., 2008
Status epilepticus	i.p. and i.c.v. KA-induced status epilepticus in mice	Hippocampus	P2Y <sub>12</sub> knock-out mice	Increased seizure phenotype; reduced hippocampal microglial processes	Eyo et al., 2014
Status epilepticus	i.p. KA-induced status epilepticus in mice	Hippocampus	GFP reporter mice; hippocampal slices and two photon microscopy; 2-MeSADP treatment (P2Y <sub>1</sub> , P2Y <sub>12</sub> , P2Y <sub>13</sub> agonist)	Increased velocity of microglia process extension toward a pipette containing 2-MeSADP following induction of status epilepticus	Avignone et al., 2015
Status epilepticus	i.p. pilocarpine-induced status epilepticus in mice	Hippocampus	IH	P2Y <sub>1</sub> activated in neuronal progenitor cells following status epilepticus	Rozmer et al., 2016
Status epilepticus and epilepsy	i.a. KA-induced epilepsy in mice; i.p. pilocarpine-induced status epilepticus in mice	Hippocampus (mice and patients)	WB; qPCR; i.c.v. treatment with ADP and UTP (broad-spectrum P2Y receptor agonists)	<b>Status epilepticus:</b> Increased P2Y <sub>2</sub> , P2Y <sub>4</sub> , and P2Y <sub>6</sub> and decreased P2Y <sub>1</sub> , P2Y <sub>12</sub> , P2Y <sub>13</sub> , and P2Y <sub>14</sub> transcript levels; increased P2Y <sub>1</sub> , P2Y <sub>2</sub> , P2Y <sub>4</sub> , and P2Y <sub>6</sub> and decreased P2Y <sub>12</sub> protein levels. ADP exacerbates seizure severity; UTP decreases seizure severity and neuronal death <b>Epilepsy:</b> Increased P2Y <sub>1</sub> , P2Y <sub>2</sub> , and P2Y <sub>6</sub> transcripts in mice; increased P2Y <sub>1</sub> , P2Y <sub>2</sub> , and P2Y <sub>12</sub> protein levels in mice. Increased P2Y <sub>1</sub> and P2Y <sub>2</sub> and decreased P2Y <sub>13</sub> protein levels in TLE patients	Alves et al., 2017
	TLE patient brain				
Epilepsy	Patients with intractable epilepsy associated with focal cortical dysplasia	Cortex	WB; IH	Increased P2Y <sub>1</sub> , P2Y <sub>2</sub> , and P2Y <sub>4</sub> expression in astrocytes	Sukigara et al., 2014
Epilepsy	Rapid kindling protocol in rats	Hippocampus	Hippocampal slices; treatment with P2Y <sub>1</sub> antagonist MRS2179	Enhanced spontaneous Ca <sup>2+</sup> -dependent signaling and astroglial hyperexcitability via P2Y <sub>1</sub> antagonism	Alvarez-Ferradas et al., 2015

GFP, green fluorescent protein; i.a., intraamygdala; i.c.v., intra-cerebro-ventricular; IH, immunohistochemistry; i.p., intraperitoneal; qPCR, quantitative polymerase chain reaction; TLE, temporal lobe epilepsy; WB, western blot.

inflammatory processes in the epileptic brain. In the same study, resected hippocampal samples from drug-refractory epilepsy patients were also analyzed. In these samples, as seen before in hippocampal samples from epileptic mice, the predominant response was an upregulation of P2Y receptors with P2Y<sub>1</sub> and P2Y<sub>2</sub> significantly upregulated. Of note, the only exception, and in contrast to findings from the mouse model of epilepsy, expression of the P2Y<sub>13</sub> receptor was found at lower levels in the epileptic brain compared to controls (Alves et al., 2017). In another previous study using brain tissue from patients suffering from intractable epilepsy associated with focal cortical dysplasia, Sukigara et al. (2014) showed increased levels of P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub>. Interestingly, the authors reported the main increase to be in astrocytes (Sukigara et al., 2014). Thus, P2Y receptor expression is altered during epilepsy, however, in contrast to status epilepticus, the main response was an upregulation of the P2Y receptor family.

## P2Y Function During Status Epilepticus

Despite the involvement of P2Y signaling in numerous pathological processes believed to play a key role during epilepsy, a possible involvement of the different P2Y receptor subtypes to seizure-induced pathology remains poorly explored and only three recent studies have suggested a functional contribution of P2Y receptors to seizures or seizure-induced pathology. The first study demonstrating a causal role for P2Y signaling during status epilepticus used mice deficient in P2Y<sub>12</sub> (Eyo et al., 2014). P2Y<sub>12</sub> is one of the most important therapeutic targets of the P2Y receptor family, with P2Y<sub>12</sub> agonists already routinely used in the clinic as an antithrombotic agent (Cattaneo, 2015). Eyo et al. (2014) report a P2Y<sub>12</sub>-dependent extension of microglial process toward neurons following KA-induced status epilepticus. Neuronal NMDA receptor activation led to an influx of Ca<sup>2+</sup>, stimulating ATP release, which subsequently activated microglial P2Y<sub>12</sub> receptors, which, in turn stimulated the extension of the processes. Interestingly, P2Y<sub>12</sub> knock-out mice, in which this process was inhibited, showed an increased seizure severity (Eyo et al., 2014). Thus, the authors concluded that microglial P2Y<sub>12</sub> receptors are necessary for microglia-neuron interaction during status epilepticus and that microglial process extension via P2Y<sub>12</sub> may serve an anti-ictal function. In a later study, Avignone et al. (2015) demonstrate that microglial processes extend toward a pipette containing methylthio-ADP, an agonist for P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> (and a weak agonist for P2Y<sub>11</sub>). The velocity of this chemotaxis was increased in activated microglia following status epilepticus. Because they also found an upregulation of P2Y<sub>12</sub> in activated microglia, the authors attributed this receptor as the likely mediator of this response (Avignone et al., 2015). More recently, our group has shown seizure altering properties of the broad-spectrum P2Y agonists ADP and UTP in the intraamygdala KA mouse model (Alves et al., 2017). Once status epilepticus was established, mice treated with ADP showed an increased seizure severity and mice treated with UTP showed a strong reduction in seizure severity and accompanying seizure-induced cell death (Alves et al., 2017). These results are in line with protective cellular mechanisms acting during status



epilepticus regarding the P2Y receptor family with adenine-sensitive receptors being generally downregulated during status epilepticus and uridine-sensitive receptors being upregulated (Figure 1).

In conclusion, while these results demonstrate a causal role for P2Y signaling during status epilepticus, we are still far from a clear and comprehensive picture of how individual P2Y receptors impact on seizure pathology.

## P2Y Function During Chronic Epilepsy

Although results from functional studies during status epilepticus and changes in expression of P2Y receptors during epilepsy strongly suggest a role for these receptors in epilepsy, to date, no studies have been performed to determine the functional contribution of P2Y receptors to epileptogenesis or the epileptic phenotype. Possible reasons are the lack of centrally available P2Y-targeting drugs and the lack of mouse models with conditional deletion of P2Y receptors, both essential for the study of the involvement of P2Y receptors during epilepsy.

## CONCLUSION AND FUTURE PERSPECTIVES

What remains to be done to establish P2Y receptors as potential drug target for epilepsy in the future? Despite the exciting emerging data revealing P2Y signaling in the brain, we are only at the beginning of understanding the potential role in seizure generation and during epileptogenesis. Recent studies have shown distinct changes in expression of the P2Y receptor family following status epilepticus and during seizures and a functional contribution has been postulated using broad-spectrum P2Y agonists (ADP and UTP) (Alves et al., 2017) and P2Y<sub>12</sub> knock-out mice (Eyo et al., 2014), there are many key issues, however, which will have to be resolved before considering P2Y receptors as valid drug target.

(i) Studies have demonstrated altered P2Y receptor expression following status epilepticus and during epilepsy (Alves et al., 2017). To get a better picture about the potential role of P2Y signaling during seizure-related pathologies, however, we must determine what cell types (e.g., neurons vs. glia; inhibitory vs. excitatory neurons) express the receptor and their sub-cellular localization (e.g., somatic vs. synaptic). (ii) Treatment of mice during status epilepticus with P2Y broad-spectrum agonists suggest a role of these receptors in seizure generation and seizure-induced pathology (Alves et al., 2017), however, we still do not know the role of individual P2Y receptors during seizures, with the only exception being the P2Y<sub>12</sub> receptor (Eyo et al., 2014). P2Y receptor-specific, centrally available drugs or P2Y knock-out mice, if possible cell-specific, must be used to determine the possible impact of the different P2Y receptors on seizures and epilepsy. (iii) P2Y receptors have been shown to be involved in numerous pathological processes in the brain (Beamer et al., 2016), however, signaling downstream of P2Y during seizures and epilepsy remains elusive, with the only exception being P2Y<sub>12</sub> functioning on microglia (Eyo et al., 2014). P2Y receptors have been shown to alter both excitatory (e.g., glutamate) and inhibitory neurotransmitter release in the brain (Garcia-Oscos et al., 2012), therefore, future studies must determine whether P2Y signaling impacts on the release of neurotransmitters and what neurotransmitters are altered during seizures. Do seizure-induced changes in P2Y function impact on the function of other cell membrane channels/receptors (e.g., potassium channels, calcium channels, NMDA receptors, GABA receptors) thereby altering neuronal excitability? (iv) Different P2Y receptors respond to different agonists (e.g., UTP, UDP, ATP, and ADP) (von Kugelgen, 2006), however, we still do not know at what concentrations these nucleotides are available during seizures/epilepsy and when, where and from which cell types these nucleotides are released or what mechanisms (e.g., ectonucleotidases) are responsible for extracellular nucleotide

concentration changes. (v) To date, studies have solely used the KA and pilocarpine mouse model of status epilepticus to analyze P2Y signaling during seizures (Avignone et al., 2008, 2015; Eyo et al., 2014; Alves et al., 2017). These mouse models rely, however, on chemically-induced seizures and only recapitulate certain aspects of the disease (Reddy and Kuruba, 2013). Results must therefore be confirmed in other models of acute seizures and chronic epilepsy. (vi) To date, we do not know what drives P2Y receptor expression during seizures. The clear expression pattern according to P2Y receptor agonists during status epilepticus, however, points toward common pathways. The identification of what drives P2Y expression during and following seizures may also therefore provide much needed new target genes for seizure control. (vii) While changes in P2Y receptor expression and, to an extent, function, have been analyzed in hippocampal tissue, extrahippocampal brain areas, in particular the cortex, may also contribute to the epilepsy phenotype (Thompson and Duncan, 2005; Helmstaedter, 2007). Status epilepticus is associated with significant extrahippocampal injury, including in the cortex (Fujikawa et al., 2000) and cortical thinning has also been reported in patients with pharmacoresistant TLE (Bernhardt et al., 2010). Consequently, the P2Y expression profile must also be analyzed in non-hippocampal brain regions. (viii) Data obtained by using the broad-spectrum agonists ADP and UTP with ADP exacerbating and UTP decreasing seizure pathology (Alves et al., 2017), suggest that a mix of antagonist (e.g., adenosine-specific receptors) and agonists (e.g., uridine-specific receptors) may provide better protection than single receptor targeting.

In conclusion, P2Y signaling is altered during and after status epilepticus and during epilepsy. Functional studies demonstrate an involvement of P2Y receptors in seizure pathology. Despite promising results, however, we are only at the beginning of understanding the role of P2Y signaling during seizures to ultimately establish P2Y targeting as possible therapeutic avenue in epilepsy.

## AUTHOR CONTRIBUTIONS

MA wrote the manuscript and designed the Figure and Table. EB edited the manuscript. TE wrote and edited the manuscript.

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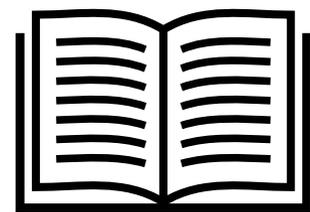
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Expression and function of the metabotropic purinergic P2Y receptor family in experimental seizure models and patients with drug-refractory epilepsy

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## SUMMARY

**Objective:** ATP is released into the extracellular space during pathologic processes including increased neuronal firing. Once released, ATP acts on P2 receptors including ionotropic P2X and metabotropic P2Y receptors, resulting in changes to glial function and neuronal network excitability. Evidence suggests an involvement of P2Y receptors in the pathogenesis of epilepsy, but there has been no systematic effort to characterize the expression and function of the P2Y receptor family during seizures and in experimental and human epilepsy.

**Methods:** Status epilepticus was induced using either intra-amygdala kainic acid or pilocarpine to characterize the acute- and long-term changes in hippocampal P2Y expression. P2Y expression was also investigated in brain tissue from patients with temporal lobe epilepsy. Finally, we analyzed the effects of two specific P2Y agonists, ADP and UTP, on seizure severity and seizure-induced cell death.

**Results:** Both intra-amygdala kainic acid and pilocarpine-induced status epilepticus increased the transcription of the uracil-sensitive P2Y receptors P2ry<sub>2</sub>, P2ry<sub>4</sub>, and P2ry<sub>6</sub> and decreased the transcription of the adenine-sensitive P2Y receptors P2ry<sub>1</sub>, P2ry<sub>12</sub>, P2ry<sub>13</sub>. Protein levels of P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub> were increased after status epilepticus, whereas P2Y<sub>12</sub> expression was decreased. In the chronic phase, P2ry<sub>1</sub>, P2ry<sub>2</sub>, and P2ry<sub>6</sub> transcription and P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>12</sub> protein levels were increased with no changes for the other P2Y receptors. In hippocampal samples from patients with temporal lobe epilepsy, P2Y<sub>1</sub> and P2Y<sub>2</sub> protein expression was increased, whereas P2Y<sub>13</sub> levels were lower. Demonstrating a functional contribution of P2Y receptors to seizures, central injection of ADP exacerbated seizure severity, whereas treatment with UTP decreased seizure severity during status epilepticus in mice.

**Significance:** The present study is the first to establish the specific hippocampal expression profile and function of the P2Y receptor family after experimental status epilepticus and in human temporal lobe epilepsy and offers potential new targets for seizure control and disease modification.

**KEY WORDS:** Purinergic signaling, P2Y receptors, Status epilepticus, Temporal lobe epilepsy.



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## KEY POINTS

- Status epilepticus increases uracil-sensitive P2Y receptor transcription and decreases transcription of adenine-sensitive P2Y receptors
- Status epilepticus increases the expression of P2Y receptors coupled to Gq proteins and decreases Gi-coupled P2Y receptor levels
- P2Y receptor upregulation is the predominant response during experimental epilepsy and in epilepsy patient brain
- The P2Y receptor agonist ADP increases seizure severity and the P2Y receptor agonist UTP decreases seizure severity during status epilepticus

Pharmacoresistance in epilepsy remains as high as 30%,<sup>1</sup> probably in part due to the relatively narrow spectrum of mechanisms targeted by available antiepileptic drugs (AEDs) (e.g. Na<sup>+</sup>, Ca<sup>2+</sup>, or K<sup>+</sup> channels and  $\gamma$ -aminobutyric acid [GABA] or glutamate receptors).<sup>1</sup> An increasing recognition of the importance of neuroinflammation in epileptogenesis has spurred interest in targeting inflammatory pathways for developing AEDs with a nonclassical mechanism of action.<sup>2</sup>

Known primarily for its role as the main molecular unit of currency for intracellular energy transfer, adenosine 5'-triphosphate (ATP) also functions as an intercellular signaling molecule.<sup>3,4</sup> Present under normal physiologic conditions at micromolar extracellular concentrations in the brain, ATP mediates signaling between neurons and glia via the activation of purinergic P2 receptors, including fast-acting P2X cationic channels and slower-acting, metabotropic, G protein-coupled P2Y receptors (P2YRs).<sup>4</sup>

In response to insults, such as neuronal hyperexcitation, inflammation, and cell death, ATP is released at millimolar concentrations, where it mediates the release of gliotransmitters, and influences synaptic structure and neuroinflammatory cascades.<sup>3</sup> Extracellular ATP is rapidly metabolized by ectonucleotidases into different breakdown products including adenosine 5'-diphosphate (ADP) and adenosine, which themselves function as signaling molecules.<sup>5</sup> Although the P2XR family seems to be exclusively activated by ATP, P2YRs can be activated by extracellular adenine and/or uracil nucleotides,<sup>6</sup> including ATP metabolites and uridine 5'-triphosphate (UTP), which are also released in the brain.<sup>7</sup> To date, eight mammalian P2YRs have been cloned and functionally characterized including P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub>.<sup>6</sup> P2YRs are expressed ubiquitously throughout the brain and are functional on all cell types present in the central nervous system including neurons, astrocytes, microglia, and oligodendrocytes.<sup>4</sup> The different P2YR family members can be

grouped according to their downstream targets: P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> are coupled to Gq proteins stimulating phospholipase C, resulting in the subsequent release of Ca<sup>2+</sup> and activation of protein kinase C (PKC). However, P2Y<sub>11</sub> can also couple to Gs, thereby stimulating adenylate cyclase and increasing production of cyclic adenosine monophosphate (cAMP).<sup>6</sup> P2Y<sub>12-14</sub> are coupled to Gi proteins, inhibiting adenylate cyclase and decreasing cAMP production.<sup>6</sup> P2Y signaling is involved in numerous physiologic processes, including neurotransmission, neurogenesis, and glial cell communication,<sup>6</sup> and causal effects of P2Y signaling on disease pathology have been reported for both acute insults to the brain, such as stroke and traumatic brain injury,<sup>8</sup> and chronic brain diseases such as Alzheimer's disease.<sup>9</sup>

A contribution of P2YRs to seizure pathology has recently been suggested.<sup>10</sup> Early studies showed changes in the induction of a subset of P2YRs after kainic acid (KA)-induced status epilepticus.<sup>11</sup> More recently, P2Y<sub>1</sub> has been shown to be activated in neuronal progenitor cells after pilocarpine-induced status epilepticus.<sup>12</sup> Furthermore, P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> protein levels are upregulated in astrocytes in brain tissue from patients with cortical dysplasia.<sup>13</sup> P2Y<sub>12</sub> appears to promote the formation of microglial extension after seizures, and P2Y<sub>12</sub> deficiency results in an aggravated seizure phenotype during status epilepticus.<sup>14</sup> To date, however, no systematic attempt has been undertaken to determine the expression profile of the complete P2YR family after status epilepticus and chronic experimental and human epilepsy. Here we used two different mouse models of status epilepticus and brain tissue from patients with temporal lobe epilepsy (TLE) to examine the expression responses of the P2YR family. We also investigated the in vivo effects of P2YR agonists ADP and UTP on acute seizures and hippocampal pathology.

## METHODS

All reagents and antibodies were purchased from Sigma-Aldrich (Dublin, Ireland) if not stated otherwise.

### Mouse seizure models

All experiments with animals were performed in accordance with the principles of the European Communities Council Directive (86/609/EEC); procedures were approved by the Research Ethics Committee of the RCSI (REC 205) and performed under license from the Department of Health and Children, Ireland. Status epilepticus was induced in male C57BL/6 mice (Biomedical Research Facility, RCSI, Dublin, Ireland).<sup>15</sup> A midline scalp incision was performed in completely anesthetized mice to expose the skull, analgesic treatment was applied (buprenorphine), and a guide cannula (anteroposterior

[AP] = -0.94 mm, lateral [L] = -2.85 mm) and electrodes for EEG (electroencephalography; Bilaney Consultants Ltd, Sevenoaks, United Kingdom) recording were fixed in place. EEG was recorded using an Xltek EEG system (Optima Medical Ltd, Guildford, UK). To trigger status epilepticus, KA was administered (0.3  $\mu$ g KA in 0.2  $\mu$ l phosphate-buffered saline [PBS]) into the basolateral amygdala nucleus. Vehicle-injected control animals received 0.2  $\mu$ l of PBS. After 40 min, lorazepam (6 mg/kg) was administered intraperitoneally to stop seizures and reduce morbidity and mortality. All mice receiving intra-amygdala KA develop epilepsy after a short latency period (3–5 days) with 2–5 seizures per day.<sup>16</sup> In a subgroup of mice, status epilepticus was induced with a subcutaneous injection of pilocarpine 30 min after the injection of methyl-scopolamine (1 mg/kg), and EEG was recorded for the following 90 min.<sup>15</sup> Mice received lorazepam (6 mg/kg) 90 min after pilocarpine injection. At the time of death, deeply anesthetized mice were perfused with saline and killed at different time-points after status epilepticus.

### Drug treatment

Mice were assigned randomly to receive either vehicle or drug 10 min after intra-amygdala KA injection, a time-point at which each intra-amygdala KA-injected mouse has experienced at least one electrographically recorded seizure burst. Mice were injected with a 2  $\mu$ l, intracerebroventricular (i.c.v.), infusion of 9 nmol of ADP or UTP resulting in approximately 300  $\mu$ M ADP or UTP in the ventricle (ventricular volume was calculated as 30  $\mu$ l). In the vehicle group, animals were injected with 2  $\mu$ l of vehicle (sterile H<sub>2</sub>O).

### EEG analysis

EEG recordings were analyzed by uploading EEG into LabChart7 software (ADInstruments) to calculate amplitude and total seizure power of the EEG signal.<sup>15</sup> The duration of high-frequency (>5 Hz) and high-amplitude (>2 times baseline) polyspike discharges of  $\geq$ 5 s duration, synonymous with injury-causing electrographic activity,<sup>17</sup> was also counted manually by a reviewer who was blinded to treatment.

### Human samples

Studies involving human tissue were approved by the ethics committee of Beaumont Hospital, Dublin (05/18) (Medical Research), and written informed consent was obtained from all patients. TLE patients (n = 6) were referred for surgical resection of the temporal lobe for the treatment of drug-refractory TLE. After temporal lobe resection, hippocampal tissue was frozen in liquid nitrogen and stored at -80°C. Hippocampal sclerosis and other pathologic changes were assessed by a pathologist. Control (autopsy) temporal hippocampal tissue

(n = 6) was obtained from the NIH Brain NeuroBio-Bank (Baltimore, MD, U.S.A.). Control and patient pathology and clinical data are presented in Figure S4 and Table 1.

### Autopsy control experiment

Hippocampi were extracted from adult mice (C57Bl/6) after deep pentobarbital anesthesia and decapitation and either frozen immediately (control) or extracted and frozen 4 or 8 h after being left at room temperature.<sup>15</sup>

### RNA extraction and quantitative polymerase chain reaction

The extraction of messenger RNA (mRNA) was performed using the Trizol method (QIAzol Lysis Reagent, Qiagen, Hilden, Germany).<sup>18</sup> Only samples with an absorbance ratio at 260/280 between 1.8 and 2.2 were considered acceptable; 500 ng of total mRNA was used to produce complementary DNA (cDNA). Primers were designed using Primer3, and data were analyzed and normalized to the expression of  $\beta$ -actin. Primers: *P2ry1* (Forward (F): gtagtagtagtacgccagggtc, Reverse (R): aagtagtctggctgtccca; *P2ry2* (F: ggccttgaaacctttaccct, R: actgtcatgcattccaggga); *P2ry4* (F: agctgtcttgatccagtct, R: gcagtagtccccttctctgt); *P2ry6* (F: aagcctgtgactcttgggac, R: caccctgggaagccatg); *P2ry12* (F: atcacagagggttgggaa, R: atgggaagagaacctg ggtg); *P2ry13* (F: cagctccgaggattgtgt, R: agaacatcagggac cagacg); *P2ry14* (F: aacaactccaccaccacaga, R: tcttgagctg ggcacataa); *c-Fos* (F: ggaattaacctggtgtctgga, R: cattcagaccac ctcgacaa); *Arc* (F: agcagcagacctgacatcct, R: gtgatgcccttcca gacat) and  $\beta$ -actin (F: gggtgtgatggtgggaatgg, R: ggttgccctta gggttcagg).

### Western blotting

Hippocampal tissue was homogenized in lysis buffer, and 30  $\mu$ g of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)<sup>15</sup> and probed with antibodies against P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> (Alomone Labs, Hadassah Ein Kerem, Jerusalem, Israel), c-Fos (Santa Cruz, Heidelberg, Germany), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA, U.S.A.), glial fibrillary acidic protein (GFAP), and  $\beta$ -actin. Protein bands were visualized using a Fujifilm LAS-4000 system (Fujifilm, Tokyo, Japan) with chemiluminescence (Pierre Biotechnology, Rockford, IL, U.S.A.), which was followed by analysis using Alpha-EaseFC4.0 software.

### Histopathology

Neuronal death was assessed using Fluoro-Jade B (FjB) staining.<sup>15</sup> Sections were post-fixed, rehydrated, and transferred to a 0.006% potassium permanganate solution followed by incubation with 0.001% FjB (Chemicon Europe Ltd, Chandlers Ford, United Kingdom) and mounted in DPX mountant. Cell counts were the average of two

adjacent sections determined with a 40× lens by an observer blinded to treatment.

### Data analysis

Data are presented as means ± standard error of the mean (SEM). To determine statistical differences between groups, analysis of variance (ANOVA) parametric statistics with post hoc Fisher's protected least significant difference test, and for two-group comparison Student's *t*-test (STATVIEW software, SAS Institute, Cary, NC, U.S.A.) was used. Significance was accepted at  $p < 0.05$ .

## RESULTS

### Status epilepticus evoked by intra-amygdala kainic acid leads to select changes to P2YR expression

To determine the induction and expression profile of the P2YR family after status epilepticus, we used the well-characterized mouse model of intra-amygdala KA-induced status epilepticus<sup>16</sup> (Fig. 1A). Increased hippocampal transcript levels of the activity-regulated gene *Arc* confirmed recruitment of the ipsilateral hippocampus (Fig. 1B). In this model, KA-induced neurodegeneration is most prominent in the CA3 subfield. Diffuse cell death is also evident, however, within the ipsilateral dentate gyrus (DG) and CA1 region (Fig. 1C).<sup>16</sup>

P2YR expression was analyzed in the ipsilateral hippocampus at different time-points following intra-amygdala KA (Fig. 1D,E). Transcript levels of *P2ry<sub>2</sub>*, *P2ry<sub>4</sub>*, and *P2ry<sub>6</sub>* were all increased following status epilepticus, whereas mRNA levels were lower after status epilepticus for *P2ry<sub>1</sub>*, *P2ry<sub>12</sub>*, *P2ry<sub>13</sub>*, and *P2ry<sub>14</sub>* (Fig. 1D,F). Of interest, transcript downregulation was the predominant response among the P2YRs known to be sensitive to ATP/ADP (P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub>), whereas transcript upregulation was the predominant response among P2YRs sensitive to UTP/UDP (P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>), except for P2Y<sub>14</sub>, which is activated by UDP-glucose (Fig. 1D,F).<sup>6</sup> *P2ry<sub>11</sub>* transcript and expression levels were not analyzed due to the lack of a *P2ry<sub>11</sub>* gene orthologue in the murine genome.<sup>19</sup>

Western blotting revealed a strong increase of P2Y<sub>1</sub> and P2Y<sub>4</sub> protein levels following status epilepticus. A modest increase in protein levels could be observed for P2Y<sub>2</sub> and a nonsignificant increase for P2Y<sub>6</sub> (Fig. 1E). In contrast, P2Y<sub>12</sub> expression decreased and no changes in expression could be observed for P2Y<sub>13</sub> and P2Y<sub>14</sub> (Fig. 1E). Therefore, P2YR expression changes correlate with changes in P2YR induction following status epilepticus, with the only exception being P2Y<sub>1</sub>. Antibody specificity for P2Y<sub>1</sub> was confirmed by pre-absorption tests (Fig. S1). These results further suggest that status epilepticus leads to select changes in the expression of the P2YR family, with P2YRs coupled to Gq being increased (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, and P2Y<sub>6</sub>) and P2YRs

coupled to Gi being downregulated or not changed (P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>) (Fig. 1F).

### Seizure-induced changes in P2YR transcription are not model-specific

To ensure that the observed changes in P2Y induction are a shared response to status epilepticus rather than model-specific, we used a second commonly used mouse model, where status epilepticus is induced by a systemic injection of the muscarinic receptor agonist pilocarpine<sup>15</sup> (Fig. 2A). Increased *Arc* mRNA levels confirmed recruitment of the hippocampus during pilocarpine-induced status epilepticus (Fig. 2B). Seizure-induced cell death was restricted mainly to the CA1 subfield of the hippocampus (Fig. 2C).<sup>15</sup> As seen before for the intra-amygdala KA mouse model, *P2ry<sub>1</sub>* and *P2ry<sub>14</sub>* transcript levels were decreased in the pilocarpine model, with *P2ry<sub>12</sub>* and *P2ry<sub>13</sub>* showing a nonsignificant minor tendency toward a reduction in mRNA transcription (Fig. 2D). Further in line with results from the intra-amygdala KA mouse model, *P2ry<sub>2</sub>*, *P2ry<sub>4</sub>*, and *P2ry<sub>6</sub>* mRNA levels were increased (Fig. 2D). These data suggest that bi-directional P2YR changes are a common response to experimental status epilepticus in mice and are not unique to the intra-amygdala KA model.

### Opposing effects of the P2YR agonists ADP and UTP on seizure severity during status epilepticus

P2YR expression changes observed following status epilepticus would suggest there may be agonist-specific responses of P2YRs during seizures. Therefore, we injected the two well-known P2Y agonists ADP and UTP<sup>4</sup> into the ventricle of mice 10 min after the induction of status epilepticus by intra-amygdala KA. Injection of mice with 9 nmol ADP resulted in an increase in total EEG power and amplitude during a 30-min recording period until the time of anti-convulsant injection (Total power: 26,230 ± 7,259 μV<sup>2</sup> [Veh] vs. 67,628 ± 18,614 μV<sup>2</sup> [ADP];  $p = 0.01$ ; Amplitude: 428 ± 44 μV [Veh] vs. 645 ± 91 μV [ADP];  $p = 0.01$ ; Fig. 3B,C). This increase was most evident 20 min following treatment with ADP (Total power: 50,245 ± 8,960 μV<sup>2</sup> [Veh] vs. 106,953 ± 28,133 μV<sup>2</sup> [ADP];  $p = 0.01$ ; Amplitude: 540 ± 48 μV [Veh] vs. 904 ± 148 μV [ADP];  $p = 0.006$ ). In contrast, injection of UTP into mice during status epilepticus decreased seizure severity (Fig. 3B,C). The effect was only significant, however, during the initial 10 min post-UTP injection (Total power: 29,794 ± 4,573 μV<sup>2</sup> [Veh] vs. 9,278 ± 1,152 μV<sup>2</sup> [UTP];  $p = 0.03$ ; Amplitude: 360 ± 23 μV [Veh] vs. 254 ± 14 μV [UTP];  $p = 0.006$ ) (Fig. 3D,E). No difference in EEG total power and amplitude was observed during the 10 min post-KA injection before drug treatment (baseline) (Fig. 3D,E).

EEG recordings were also analyzed for an additional 60 min following lorazepam. Although no significant

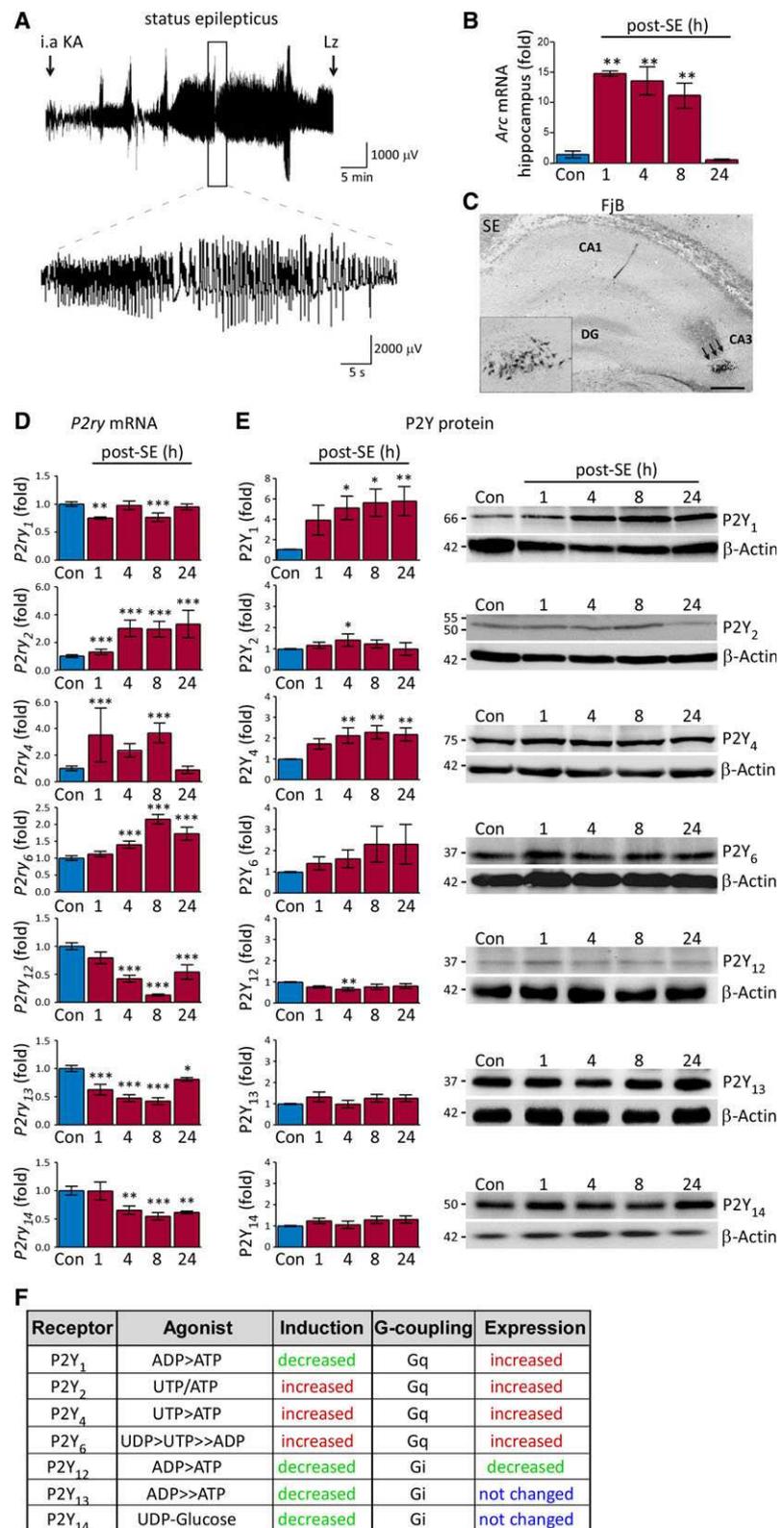
**Figure 1.**

P2YR induction and expression in the hippocampus following intra-amygdala KA-induced status epilepticus. **(A)** Representative EEG traces showing high amplitude and high frequency spiking following intra-amygdala KA injection. Status epilepticus develops shortly after intra-amygdala KA injection and mice are treated with intraperitoneal lorazepam (Lz) 40 min later. **(B)** Increased Arc mRNA transcription in the ipsilateral hippocampus after intra-amygdala KA induced status epilepticus ( $n = 8$  [control (Con)] and 4 [post-status epilepticus]). **(C)** Representative image ( $5\times$  lens) of FjB staining showing seizure-induced cell death mainly localized to the ipsilateral CA3 subfield of the hippocampus 24 h following intra-amygdala KA injection (arrows and insert). (Note, image invert reveals these as black-stained cells.) The ipsilateral CA1 and DG hippocampal subfields are mainly spared from damage. CA, cornu ammonis; DG, dentate gyrus; Scale bar = 500  $\mu\text{m}$ . **(D)** Graphs showing changes in P2ry mRNA levels post-status epilepticus (SE) in the ipsilateral hippocampus ( $n = 12-14$  [control (Con)] and 6-8 [post-status epilepticus]). **(E)** Graphs and representative Western blots showing expression changes of P2YR family after intra-amygdala KA-induced status epilepticus in the ipsilateral hippocampus ( $n = 8$ /group).  $\beta$ -Actin is shown as guide to loading. **(F)** Table summarizing P2YR induction and expression following intra-amygdala status epilepticus.

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*

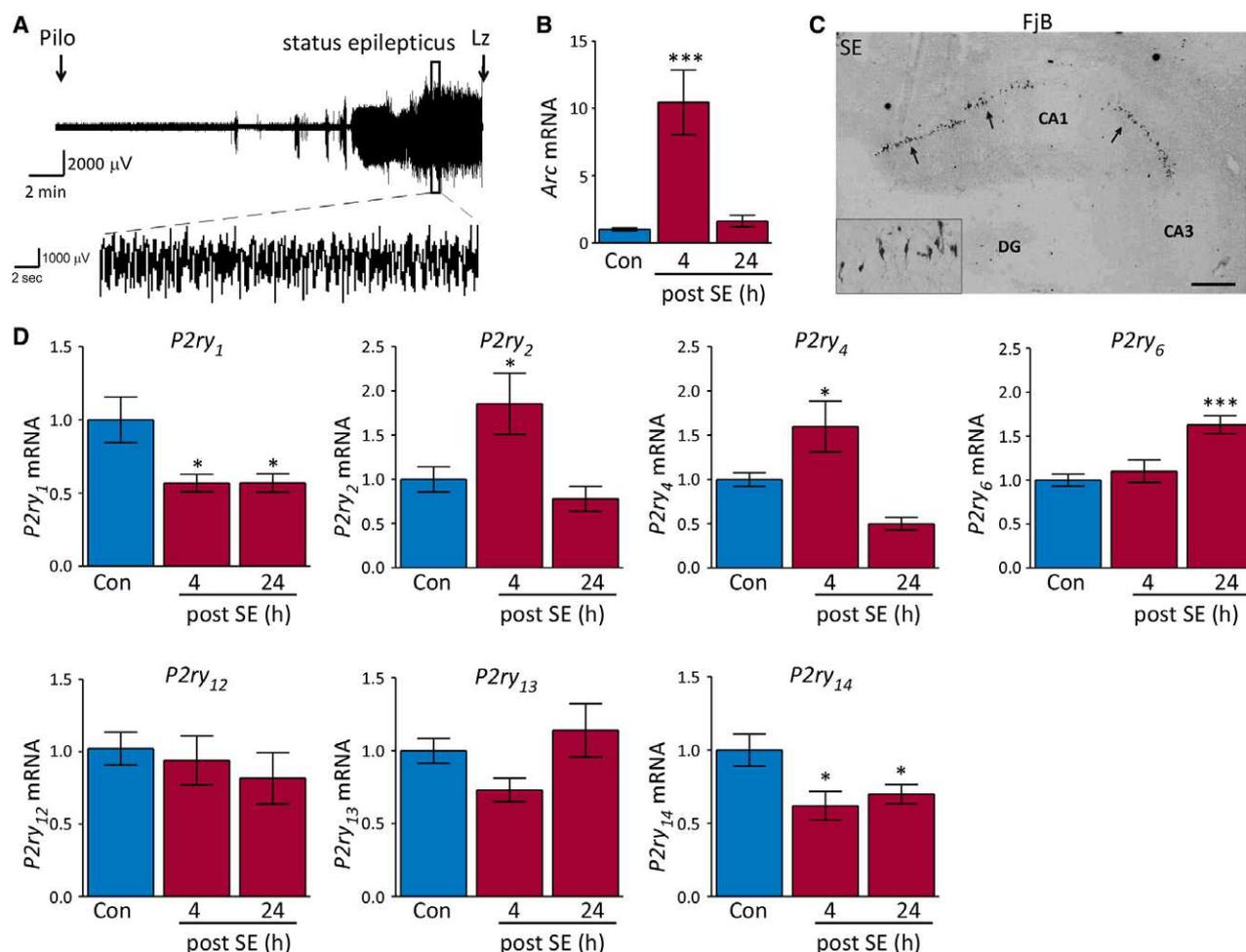
\* $p < 0.001$ .

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differences between treatment groups were observed, the same trend continued with ADP increasing EEG power (Total power:  $21,745 \pm 4,539 \mu\text{V}^2$  [Veh] vs.  $42,181 \pm 11,786 \mu\text{V}^2$  [ADP];  $p = 0.06$ ; Amplitude:  $438 \pm 34 \mu\text{V}$

[Veh] vs.  $588 \pm 93 \mu\text{V}$  [ADP];  $p = 0.08$ ) and UTP showing a tendency toward lower EEG power (Total power:  $14,727 \pm 1,713 \mu\text{V}^2$  [UTP];  $p = 0.52$ ; Amplitude:  $395 \pm 27 \mu\text{V}$  [UTP];  $p = 0.61$ ) (Fig. S2).



**Figure 2.**

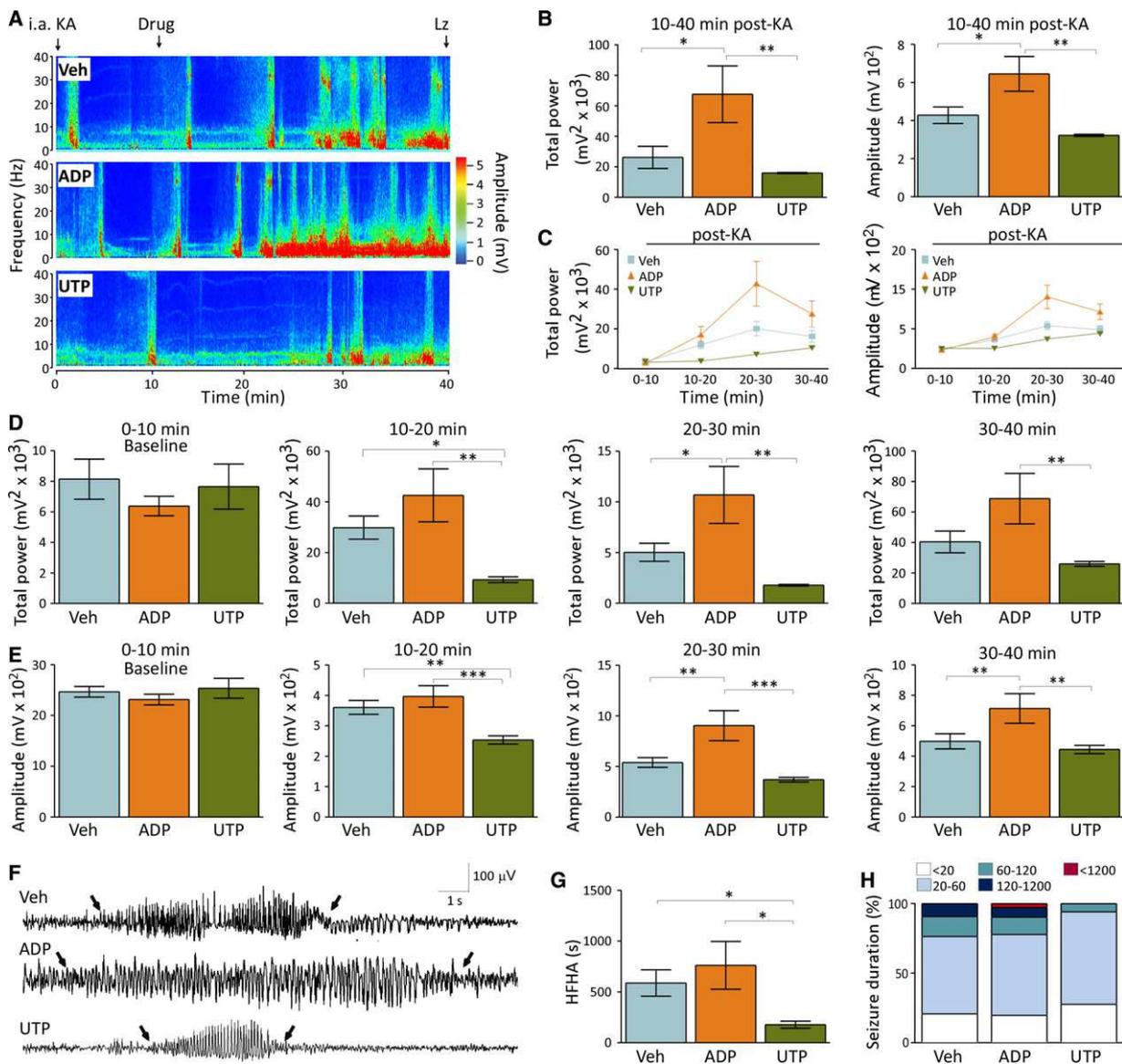
Hippocampal *P2ry* transcript levels after pilocarpine-induced status epilepticus. **(A)** Representative EEG traces showing high amplitude and high frequency spiking after systemic pilocarpine (Pilo) injection. Lorazepam (Lz) was administered 90 min following pilocarpine injection. **(B)** Graph showing increased *Arc* mRNA levels after pilocarpine injection in the hippocampus ( $n = 8$  [control (Con)] and 4 [post–status epilepticus (SE)]). **(C)** Representative image ( $5\times$  lens) showing cell death mainly localized to the CA1 subfield of the hippocampus 24 h following pilocarpine-induced status epilepticus as shown by FjB-positive cells (arrows and insert). Scale bar  $500\ \mu\text{m}$ . **(D)** Graphs showing *P2ry* mRNA changes in the hippocampus following pilocarpine-induced status epilepticus ( $n = 5\text{--}6/\text{group}$ ). \* $p < 0.05$ , \*\* $p < 0.001$ .

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Previous work in the intra-amygdala KA model of status epilepticus has shown that HFHA paroxysmal discharges correlate with seizure-induced brain pathology.<sup>17</sup> high frequency high amplitude (HFHA) analysis revealed that ADP-injected mice showed a trend toward longer durations of HFHA spiking, whereas UTP-treated mice showed a reduction in HFHA spiking ( $588 \pm 130\ \text{s}$  [Veh] vs.  $761 \pm 235\ \text{s}$  [ADP;  $p = 0.40$ ] vs.  $178 \pm 35\ \text{s}$  [UTP;  $p = 0.04$ ] (Fig. 3F,G). Of interest, only ADP-treated mice showed continuous HFHA spiking for  $>20$  min. In contrast, HFHA burst time remained  $<60$  s in UTP-treated mice (Fig. 3H), suggesting that ADP promoted and UTP attenuated status epilepticus.

### UTP treatment reduces status epilepticus-induced brain pathology

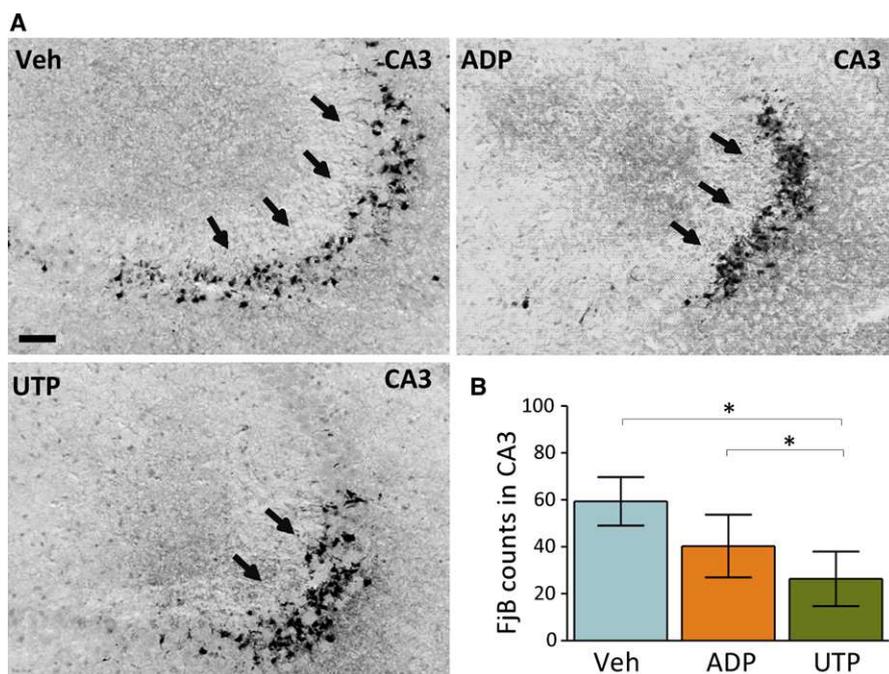
We next assessed hippocampal pathology in mice treated with P2YR agonists during status epilepticus. As reported before, vehicle-injected mice subjected to status epilepticus showed the typical CA3 lesion, as evidenced by FjB-positive cells (Fig. 4A,B).<sup>16</sup> Mice treated with ADP during status epilepticus showed a nonsignificant reduction in FjB-positive cells in the CA3 subfield, whereas mice injected with UTP displayed significantly fewer FjB-positive neurons in the CA3 subfield when compared to vehicle-injected mice ( $59 \pm 10$  [Veh] vs.  $40 \pm 13$  [ADP,  $p = 0.40$ ] vs.  $26 \pm 12$  [UTP,  $p = 0.04$ ] (Fig. 4A,B).



**Figure 3.**

P2Y agonist treatment alters seizure severity during status epilepticus. **(A)** Representative heatmaps depicting EEG frequency and amplitude of mice treated with i.c.v. vehicle, ADP (300  $\mu\text{M}$ ) or UTP (300  $\mu\text{M}$ ), during a 40-min recording period, starting at the time-point of intra-amygdala KA injection until the administration of lorazepam (Lz). Drug (Vehicle, ADP or UTP) was injected 10 min following intra-amygdala KA. **(B)** Graphs showing increased total power and amplitude after the injection of ADP in mice subjected to intra-amygdala KA during a 30-min recording period post-drug injection. No significant changes in total power and amplitude could be observed in the UTP-treated group when compared to vehicle-injected mice ( $n = 10$  [vehicle (Veh)] and 7 [ADP and UTP]). **(C)** Graphs showing an increase in seizure severity (total power and amplitude) in mice treated with ADP and a decrease in seizure severity (total power and amplitude) in mice when treated with UTP and compared to vehicle-injected animals. EEG was analyzed in 10 min segments ( $n = 10$  [vehicle (Veh)], 8 [10–20 min and 20–30 min (ADP and UTP)] and 7 [30–40 min (ADP and UTP)]). EEG total power **(D)** and amplitude **(E)** of 10 min segments analyzed separately starting at injection of intra-amygdala KA until treatment with lorazepam. Note, increased seizure severity after ADP treatment and decreased seizure severity after treatment with UTP when compared to control ( $n = 10$  [vehicle (Veh)], 8 [10–20 min and 20–30 min (ADP and UTP)], and 7 [30–40 min (ADP and UTP)]). **(F)** Representative images showing examples of high frequency and high amplitude spiking on the EEG during the 30-min recording period after drug treatment (see arrows). **(G)** Graph showing a decrease in high frequency and high amplitude spiking (HFHA) in mice treated with UTP ( $n = 10$  [vehicle (Veh)] and 7 [ADP and UTP]). **(H)** Graph showing percentage of different seizure times during the 30-min recording period from drug injection until the administration of lorazepam. Note, only ADP-treated mice develop seizures lasting longer than 1200 s (20 min). UTP-treated mice do not develop seizures lasting longer than 60 s ( $n = 10$  [vehicle (Veh)], 8 [10–20 min and 20–30 min (ADP and UTP)], and 7 [30–40 min (ADP and UTP)]). \* $p < 0.05$ , \*\* $p < 0.01$ .

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**Figure 4.**

UTP treatment protects the brain from seizure-induced damage. Representative images (A) and graph (B) showing fewer FjB-positive cells 24 h post-status epilepticus in mice treated with UTP (n = 8 [Veh], 6 [ADP], and 6 [UTP]). Scale bar = 100  $\mu$ m; \*p < 0.05. Epilepsia © ILAE

### P2YR expression in experimental epilepsy

To investigate whether P2YR expression is also altered in epilepsy, we analyzed hippocampal samples from epileptic mice 14 days post-status epilepticus induced by intra-amygdala KA.<sup>16,20</sup> Tissue sections showed a visible lesion within the ipsilateral CA3 subfield of the hippocampus involving neuron loss and astrogliosis (Fig. 5A), similar to neuropathologic changes found in human TLE.<sup>21</sup> Increased hippocampal levels of the neuronal activity-regulated gene *c-Fos* further confirmed involvement of the hippocampus during chronic epilepsy (Fig. 5B). In addition, as expected, GFAP levels were increased in the hippocampus of epileptic mice (Fig. 5B).

Analysis of P2YR expression determined increased *P2ry1*, *P2ry2*, and *P2ry6* transcript levels in the hippocampus of epileptic mice. *P2ry4* transcription showed a non-significant tendency to be increased, and no changes were observed for *P2ry12*, *P2ry13*, and *P2ry14* transcripts (Fig. 5C). Thus the main transcriptional response of the P2YR family in this model of experimental epilepsy is increased expression.

Western blotting using ipsilateral hippocampi from epileptic mice revealed an increase in P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>12</sub> protein levels (Fig. 5D). In line with a general increase in *P2ry* transcription observed during epilepsy, P2YR expression levels were also mainly increased.

### P2YR expression in human temporal lobe epilepsy

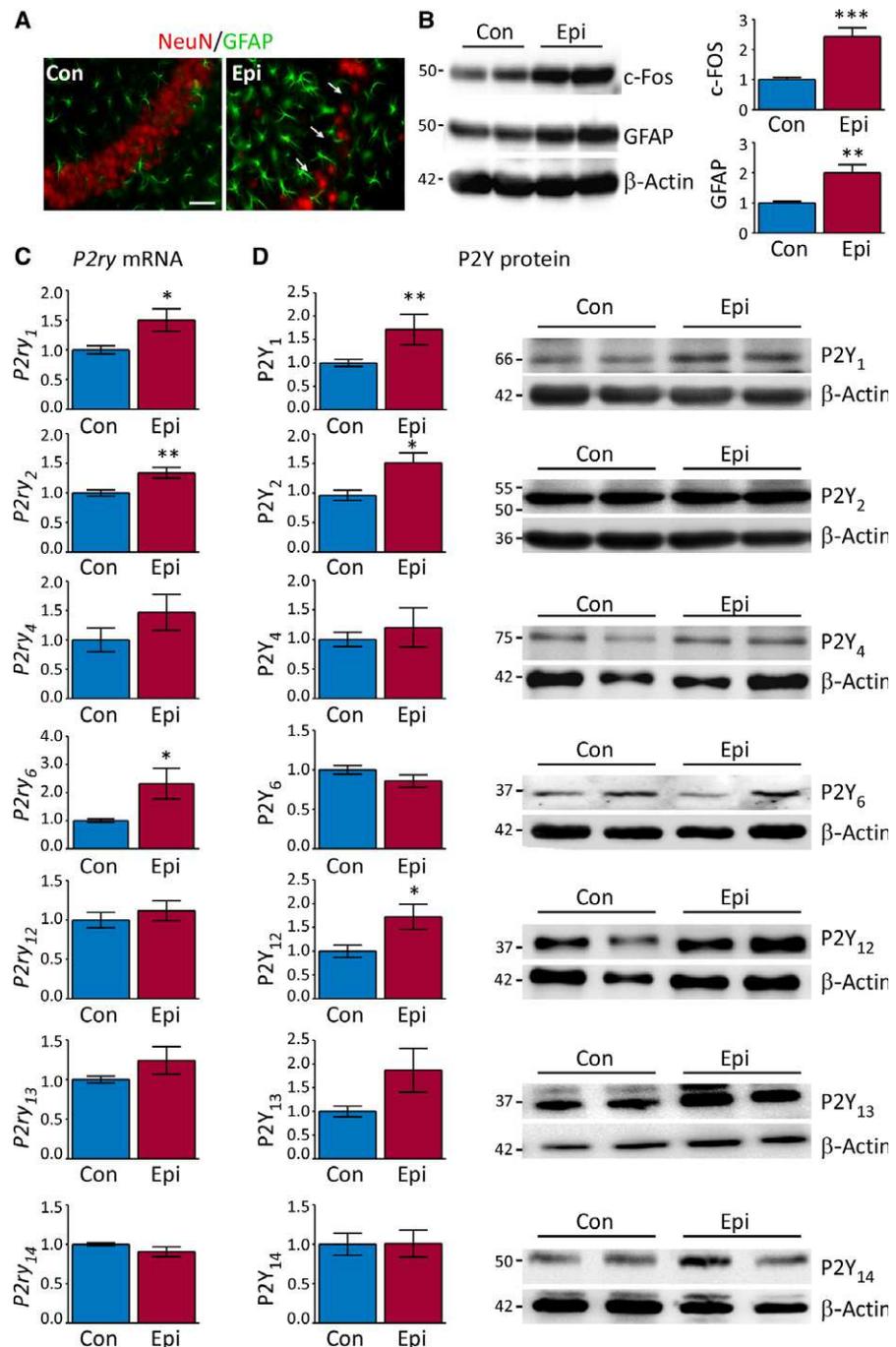
Finally, to establish the clinical relevance of our findings, we analyzed P2YR levels in surgically resected hippocampi from patients with drug-refractory TLE, compared to age- and gender-matched autopsy controls. Pathology reports

confirmed neuronal loss and gliosis, consistent with hippocampal sclerosis (Fig. S4; Table S1). Western blot analysis revealed higher levels of P2Y<sub>1</sub> and P2Y<sub>2</sub> in patient samples compared to controls, whereas levels of P2Y<sub>13</sub> were lower in patients than controls. The remaining P2YR including P2Y<sub>6</sub>, P2Y<sub>12</sub>, and P2Y<sub>14</sub> showed no significant differences except for P2Y<sub>4</sub>, which showed a modest, non-significant tendency to higher levels (Fig. 6A). Postmortem delay experiments confirmed P2YR levels being stable over the period corresponding to the maximal delay in the human control subjects (Fig. S3). These findings support the data from our experimental model showing an increase in P2YR levels within the damaged hippocampus in epilepsy.

## DISCUSSION

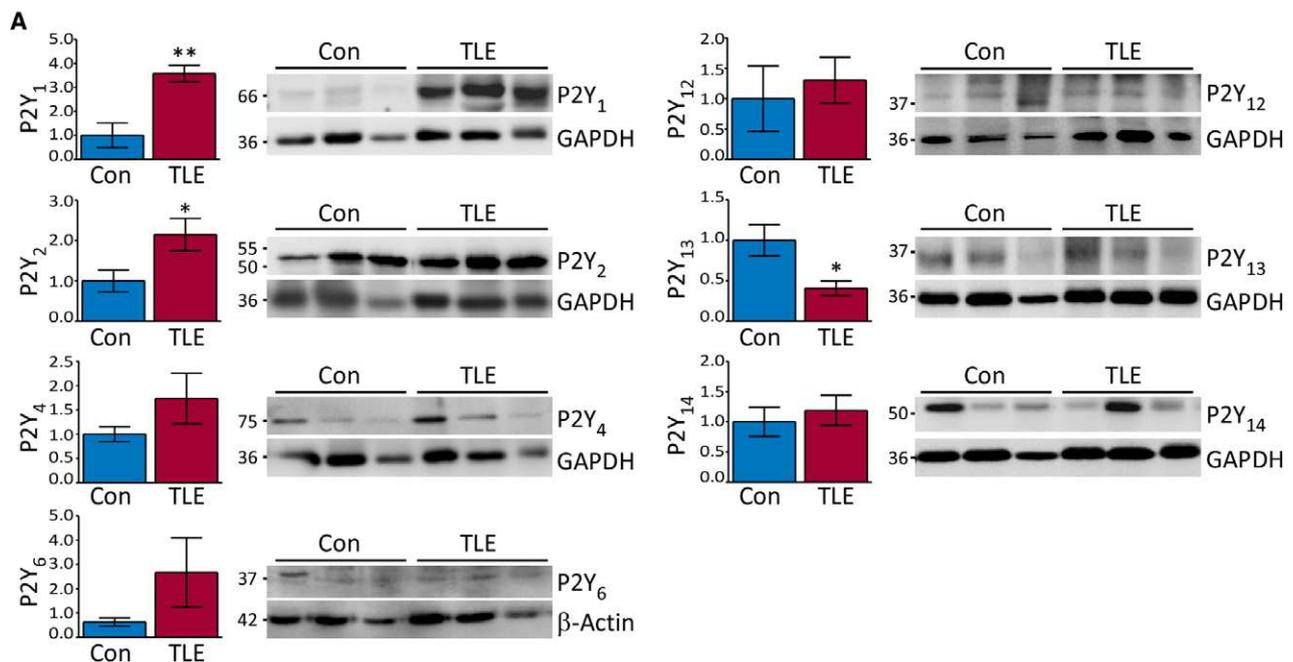
The main findings in the present study are that experimental status epilepticus causes a selective acute downregulation of adenosine-sensitive P2YR transcription and upregulation of uracil-sensitive P2YR transcription. Suggesting opposing functional roles for both receptor subtypes during seizures, ADP treatment increases seizure severity and UTP treatment decreases seizure severity. We also report select upregulation of P2YRs in experimental and human epilepsy. These findings support a functional role for P2YRs in status epilepticus and potentially new therapeutic targets for seizure control or disease-modification in epilepsy.

Although the expression and function of P2YRs has been well established in experimental status epilepticus and epilepsy, the P2YR subfamily has received minimal prior attention.<sup>10</sup> This has been due partly to a lack of suitable



tools (e.g. drugs) and animal models.<sup>22</sup> Only few studies have attempted to determine seizure-induced P2YR changes in the brain and a possible causal role of these receptors in seizure generation and during epilepsy. One of the first studies describing changes of the P2YR family after seizures used the intraperitoneal KA mouse model of status epilepticus. Results from these studies are in line with our results showing an early increase in *P2ry*<sub>6</sub> transcription and decrease in *P2ry*<sub>12</sub> and *P2ry*<sub>13</sub> transcripts following status epilepticus.<sup>11</sup> However, in contrast to our results showing

increased *P2ry*<sub>6</sub> receptor levels with no changes in *P2ry*<sub>12</sub> and *P2ry*<sub>13</sub> at later time-points following status epilepticus, the transcription of all three receptors was upregulated in the intraperitoneal KA model.<sup>11</sup> Differences in seizure-induced neurodegeneration or postseizure inflammation responses might account for the observed discrepancies.<sup>23</sup> During chronic epilepsy, the main response was an upregulation of P2YRs. This is in line with a recent study showing increased levels of P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> in brain tissue from patients with epilepsy due to cortical dysplasia.<sup>13</sup> We



**Figure 6.**

P2YR expression in the hippocampus of patients with TLE. **(A)** Representative Western blot ( $n = 1/\text{lane}$ ) and corresponding graphs showing P2YR expression levels in the hippocampus of TLE patients when compared to control (Con) ( $n = 6/\text{group}$ ). GAPDH or  $\beta$ -Actin are shown as guide to loading. \* $p < 0.05$ , \*\* $p < 0.01$ .

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have now extended these data, showing no significant changes for the remaining receptors in a TLE patient cohort, with the only exception being P2Y<sub>13</sub> displaying lower levels. Of interest, P2Y<sub>13</sub> activation has been shown to exert neuroprotective effects *in vitro*<sup>24,25</sup>; therefore, the reduction in P2Y<sub>13</sub> levels could contribute to increased vulnerability to seizure-induced neurodegeneration. We did observe some differences between our mouse model of epilepsy and the TLE patient brain. In particular, where P2Y<sub>13</sub> expression was decreased in human TLE, P2Y<sub>13</sub> levels were increased in the mouse model. Furthermore, P2Y<sub>12</sub> showed only a minor, nonsignificant increase in TLE patient samples, but was significantly upregulated in experimental epilepsy. These discrepancies may be due to differences in seizure severity and/or seizure frequency (animals vs. patients), time of disease stage (early disease stage in animal model vs. late stage of disease in TLE patients), or treatment effects (e.g., AEDs). A key limitation of the present study is that we only analyzed samples from epileptic mice 2 weeks after status epilepticus. It is therefore possible that progressive aspects of the model such as neurodegeneration, gliosis, and synaptic remodeling would continue to influence P2YR expression and function. This could be addressed in the future by analysis of later (e.g., 3, 6 month) time points. Regardless of these differences, most P2YRs seemed to be upregulated during epilepsy. Another limitation of our study to consider is that it is also possible that some of the changes observed in P2YR changes reflect overt cellular changes

such as reduced levels of specific P2YRs expressed by neurons and increased expression among those expressed by glia, as these two populations change in the intra-amygdala KA model.<sup>16</sup> Therefore, differences in the composition of cell types present in the analyzed tissue according to disease stage may also contribute to differences in the observed changes in P2YR induction and expression. Furthermore, to date, P2YRs have been shown to be expressed throughout the brain with different P2YRs predominant on specific cell types including neurons (all P2YRs), astrocytes (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>12</sub>, and P2Y<sub>14</sub>), microglia (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>12</sub>, and P2Y<sub>14</sub>), oligodendrocytes (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub>), and endothelial cells (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, and P2Y<sub>12</sub>).<sup>4</sup> Our study does not provide information, however, on absolute levels of the receptors or their cell type-specific expression following status epilepticus or during epilepsy. This is important and must be addressed in future studies.

A key observation in the present study is that the direction of changes in P2YR subtypes after status epilepticus correlates closely with the known receptor agonist profile and G protein-coupling of the receptors. Most strikingly, whereas adenine-sensitive receptors were downregulated, most uracil-sensitive receptors, with the UDP-glucose sensitive P2Y<sub>14</sub> being the only exception, are upregulated following status epilepticus. Nuclear factor kappa B (NF- $\kappa$ B) has been shown to drive the transcription of P2Y<sub>2</sub> during inflammatory conditions.<sup>26</sup> However, to date, we do not know what

drives or blocks transcription of P2YRs during seizures. Nevertheless, the clear distinction in the induction pattern of these receptors points toward common pathways from receptor activation to transcriptional control. The fact that ADP treatment increases seizure pathology and UTP treatment protects the brain from seizure damage further suggests that these are intracellular adaptations leading to the distinct post-status epilepticus P2YR induction profile. Why does ADP increase seizure pathology and why does UTP treatment protect the brain from damage? Previous data have shown that UTP reduces the neuronal firing rate of cortical and thalamic neurons.<sup>27</sup> Furthermore, UTP metabolites such as uridine have been shown to reduce epileptic seizures in patients with epileptic encephalopathy.<sup>28</sup> This would be consistent with our EEG observations. It is difficult, however, to narrow our results to a specific P2YR subtype with all uracil-sensitive P2YRs being upregulated after status epilepticus. In contrast to the uracil-sensitive P2YRs, it is tempting to speculate that the proconvulsive effects of ADP are mediated primarily through the P2Y<sub>1</sub> subtype. P2Y<sub>1</sub> is the only adenine-sensitive receptor showing increased protein levels following status epilepticus, despite a decrease in mRNA transcription. The reason for this remains elusive; however, there are several possible explanations, among them seizure-induced posttranscriptional changes including, for example, a reduction in P2Y<sub>1</sub>-targeting microRNAs and posttranslational alterations in P2Y<sub>1</sub> protein turnover. We have shown that experimental status epilepticus leads to an inhibition of the ubiquitin-proteasome system (UPS), thereby potentially increasing P2Y<sub>1</sub> levels. UPS function is also altered during chronic epilepsy in mice.<sup>29</sup> Seizure-induced changes in receptor stability or internalization may also account for the increase in P2Y<sub>1</sub> levels.<sup>30</sup> Although, to date, P2Y<sub>1</sub> has not been studied in the setting of epilepsy, several lines of evidence point toward a possible role of this receptor in epileptic pathology. P2Y<sub>1</sub> activation has been associated with changes in the release of neurotransmitters including glutamate and GABA.<sup>31,32</sup> P2Y<sub>1</sub> has also been shown to interfere with synaptic transmission by modulating neurotransmitter receptor function<sup>33</sup> and to mediate the propagation of Ca<sup>2+</sup> waves within astrocyte networks.<sup>34</sup> P2Y<sub>12</sub> represents a second adenine-sensitive receptor potentially having a causal role during seizure pathology. However, P2Y<sub>12</sub> knockout mice were found to display an exacerbated seizure phenotype in two kainate models,<sup>14</sup> suggesting P2Y<sub>12</sub> activation has anticonvulsive rather than a proconvulsive effects.

Another key finding of the present study is the increased expression of Gq-coupled P2YRs and the decreased expression of Gi-coupled P2YRs. The canonical Gq signaling pathway leads to an increase in phospholipase C (PLC) activation, and increased intracellular inositol 3 phosphate (IP<sub>3</sub>) levels resulting in the release of Ca<sup>2+</sup> from intracellular calcium stores and the formation of diacylglycerol (DAG), with the subsequent activation of protein kinase C (PKC).<sup>6</sup>

Notably, both PLC and PKC deficiency have been associated with a more severe epileptic phenotype, and therefore the changes in P2Y signaling may represent adaptive changes to limit epileptogenic changes.<sup>35,36</sup> On the other hand, Gi signaling inhibits adenylate cyclase, thereby decreasing cAMP production and PKA activation.<sup>6</sup> Again, in good agreement with protective mechanisms driving P2Y expression, elevated cAMP has been reported to be proepileptogenic and PKA inhibition reduced epileptiform activity.<sup>37,38</sup>

Probably the most likely cause for the observed neuroprotection via UTP is a reduction in seizure severity. However, UTP or its metabolites have been frequently described to provide neuroprotective effects in the brain against various pathologic insults such as ischemia or Alzheimer's disease.<sup>7,39</sup> It is unclear why ADP treatment did not translate into increased neurodegeneration despite exacerbating seizures. A possible explanation is the conversion of ADP into adenosine by extracellular ectonucleotidases.<sup>5</sup> Adenosine is a known anticonvulsive and neuroprotective molecule.<sup>40</sup> Whether treatment with ADP or UTP influences the development of epilepsy or the epileptic phenotype has not been investigated. A causal role is, however, likely with different P2YRs being up- or downregulated during chronic epilepsy. In particular, the potent anticonvulsive and neuroprotective effects provided by UTP during status epilepticus strongly suggest that drugs antagonizing uracil-sensitive P2YRs, may represent a new treatment strategy not only for status epilepticus but also to prevent the development and progression of epilepsy. However, the extremely short half-life of ADP or UTP in the brain<sup>5</sup> makes studies using this class of agonists extremely difficult, and more stable and centrally available specific P2Y agonists/antagonists will have to be used in future studies.

In conclusion, our present data demonstrate a specific induction and expression profile of the P2YR family following status epilepticus and during epilepsy. Further, functional studies using P2YR agonists modulated seizure pathology, identifying P2YRs as possible new targets for the treatment of status epilepticus and drug-refractory epilepsy.

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## DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Specificity of P2Y<sub>1</sub> antibody.

**Figure S2.** Enduring effects of P2Y agonists on seizure severity following lorazepam treatment.

**Figure S3.** Absence of postmortem effect on hippocampal P2Y<sub>1</sub> and P2Y<sub>2</sub> receptor levels.

**Figure S4: Table S1.** Autopsy control and TLE patient clinical and pathologic details.