Modulation of intracellular ATP influences seizure activity via the activity-dependent release of adenosine

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself under the supervision of Professor Bruno G. Frenguelli.

This thesis has not been submitted in any previous application for any degree. All sources of information are acknowledged in the form of references.

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Summary

A large number of patients with epilepsy have drug-resistant seizures. Therefore, there is a need for the development of new therapies. The purine nucleoside adenosine is an endogenous anticonvulsant that acts to supress neuronal excitability via adenosine A_1 receptors. The aim of this thesis was investigate whether manipulating ATP bioenergenetics and importantly adenosine levels had any effects on activity-dependent release of adenosine and seizure activity. ATP bioenergetics and adenosine levels were manipulated by pre-treating rat hippocampal slices with a combination of the sugar backbone of ATP (D-ribose) and the free purine base adenine (RibAde) and the phosphate buffer creatine. The role that the adenosine A_{2A} receptor plays in relation to epileptiform activity was also investigated. Biosensors were used to measure the real-time release of adenosine. The K^+ channel blocker 4-aminopyridine (4-AP; 50 μM) in Mg^{2+}-free medium was the model used for inducing spontaneous bursting epileptiform activity. Additionally, homocysteine thiolactone (HTL) was used to "trap" intracellular adenosine to test if extracellular adenosine measured with biosensors, is released as adenosine per se and if this had any effects on seizure activity.

I show that during bursting epileptiform activity, the amount of adenosine released is increased in RibAde slices compared to creatine and untreated (control) slices and increased the time between seizures compared to both creatine and control slices. No differences was found between creatine and control slices. My data also suggest that adenosine A_{2A} receptors may partially contribute to seizure activity. HTL reduced adenosine release in a burst-dependent manner and also increased the frequency of seizures. HTL influenced the intensity of bursts in control but not RibAde-treated slices. This thesis provides evidence for the beneficial role of the ATP precursors ribose and adenine on reducing seizure activity and will hopefully contribute to on-going attempts to establish adenosine-based epilepsy therapies.

List of abbreviations

4-aminopyridine- 4-AP

2,8-dihydroxyadenine- DHA

3,5-dihydroxyphenylglycine- DHPG

5’-diphosphate- UDP

5'-phosphoribosyl-1'-pyrophosphate- PRPP

8-cyclopentyl-1, 3-dimethylxanthine- CPT

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors- AMPARs

Adenine phosphoribosyltransferase- APRT

Adenine receptors- AdeRs

Adenosine A_1 receptors- A_1Rs

Adenosine A_{2A} receptors- A_{2A}Rs

Adenosine A_3 receptors- A_3Rs

Adenosine A_{2B} receptors- A_{2B}Rs

Adenosine 5’-diphosphate- ADP

Adenosine kinase- ADK

Adenosine 5’-monophosphate- AMP

Adenosine 5’-triphosphate- ATP

Adenosine triphosphatase- ATPase

Adenylosuccinate- AS

Analysis of variance- ANOVA

Anti-epileptic drugs - AEDs

Artificial cerebrospinal fluid- aCSF

Blood-brain barrier- BBB

Calcium- Ca^{2+}

Calcium chloride- $CaCl_2$

Central nervous system- CNS

Chloride- Cl^-

Complementary deoxyribonucleic acid- cDNA

Cornu ammonis – CA

Concentrative nucleoside transporters- CNTs

Creatine kinase- CK

Cyclic adenosine 5'-monophosphate- cAMP

Dentate gyrus- DG

Deoxyribonucleic acid- DNA

Diacylglycerol- DAG

Ectonucleoside triphosphate diphosphohydrolases -E-NTPDases

Ectonucleotide pyrophosphatase and / or phosphodiesterase- E-NPPs

Entorhinal cortex- EC

Equilibrative nucleoside transporters- ENTs

Field excitatory post-synaptic potentials- fEPSP

γ-aminobutyric acid- GABA

Glucose 6- phosphate- G-6-P

G-protein coupled receptor- GPCR

Guanidinoacetate- GAA

Guanidinoacetate-methyltransferase- GAMT

Guanosine monophosphate- GMP

Guanosine triphosphate- GTP

High frequency stimulation- HFS

Hippocampal sclerosis- HS

Homocysteine- HCY

Homocysteine thiolactone- HTL

Hydrogen ion- H^+

Hypoxanthine- HX

Hypoxanthine guanine phosphoribosyltransferase- HGPRT

Ionotropic glutamate receptors- iGluRs

Inosine monophosphate- IMP

Inositol triphosphate- IP3

Inter burst interval- IBI

Inter ictal- II

International Business Machines Corporation- IBM

International league against epilepsy- ILAE

Inter spike interval- ISI

Knockout- KO

L-arginine- glycine aminotransferase- AGAT

Magnesium- Mg^{2+}

Magnesium Chloride- $MgCl_2$

Magnesium sulfate heptahydrate- $MgSO_4.7H_2O$

Messenger ribonucleic acid- mRNA

Metabotropic glutamate receptors- mGluRs

Methionine adenosyltransferase- MAT

Micromolar μM-

Milimolar- mM

Nicotinamide adenine dinucleotide- NAD

Nicotinamide adenine dinucleotide phosphate- NADP

N-methyl-D- aspartate receptors – NMDARs

Oxygen glucose deprivation – OGD

Phosphocreatine- PCr

Phosphokinase C- PKC

Phospholipase C- PLC

Phosphatidylinositol 4,5-bisphosphate- PIP2

Potassium- K^+

Potassium chloride- KCl

Pyrophosphate- PPi

Responsive neural stimulation- RNS

Ribonucleic acid- RNA

Ribose-5-phosphate- Rib-5-P

Ribose-1-phosphate- Rib-5-P

Ribose and adenine- RibAde

Ribulose 5-phosphate- Ru-5-P

S-Adenosyl-L-homocysteine- SAH

S-Adenosyl-L-homocysteine thiolactone- SAHTL

S-Adenosyl-L- methionine- SAM

SCH 58261- SCH

Seizure-like events- SLEs

Sodium- Na^+

Sodium bicarbonate- $NaHCO_3$

Sodium chloride- NaCl

Sodium Dihydrogen Phosphite/ Sodium Phosphite Dibasic- NaH_2PO_3

Standard error of the mean- SEM

Statistical Package for the Social Sciences- SPSS

Status Epilepticus- SE

Temporal lobe epilepsy- TLE

Transient opening calcium- T-type Ca^{2+}

United Kingdom- UK

Uridine 5'-triphosphate- UTP

Vagus nerve stimulation- VNS

Voltage-gated potassium channel- Kv

World Health Organisation- WHO

Xanthylate- XMP

1. Introduction

1.1. Epilepsy

1.1.1. Prevalence, incidence and mortality

Epilepsy is a debilitating chronic neurological disease that affects 1 in 103 people in the United Kingdom (UK) (Joint Epilepsy Council, 2011). The World Health Organisation (WHO) in 2005 reported that there are at least 50 million people worldwide with epilepsy (World Health Organisation, 2005). The WHO defined active epilepsy as an individual having at least one epileptic seizure within the previous five years. Active epilepsy accounts for 1% of the global burden of disease, and 80% of these incidences are in developing countries (World Health Organisation, 2006). The prevalence of epilepsy is similar to that of breast cancer in women, lung cancer in men, Alzheimer's disease, substance abuse, depression and other affective disorders. Not only do those with epilepsy have to contend with epileptic seizures, but they also have to cope with interacting medical, psychological, economic and social repercussions. These repercussions have a major impact on epilepsy patients and there is a growing need for novel treatments.

In the UK the prevalence rate of people that have been diagnosed with epilepsy and take anti-epileptic drugs (AEDs) is approximately 600,000, this equates to 1 in 103 people or 9.7 per 1,000 (0.97%) (Joint Epilepsy Council, 2011). These figures encompass a large range of population groups and includes children 4 years old and under and people 65 years old and over. The number of new cases in the UK is ever increasing due to in part the increase in the general population (Booth, 2010; Joint Epilepsy Council, 2011). The incidence of newly diagnosed cases of epilepsy in the UK is approximately 51 per 100,000 per year (Booth, 2010; Joint Epilepsy Council, 2011). Epilepsy affects many aspects of life, both socially and physically. In some

instances, epilepsy can be fatal and studies showed that 1,150 individuals died from epilepsy-related causes in the UK in 2009, this is approximately 42% or 480 avoidable deaths per year (Booth, 2010; Joint Epilepsy Council, 2011). These statistics highlight the fact that epilepsy adheres to no social, economic or age group although the recorded cases of epilepsy are lower in the under 5-age group. Each of these groups will not be responsive to all treatments nor is the same treatment appropriate for all. Therefore, it is important to develop new tailored novel treatments.

1.1.2. Definition of epileptogenesis, epilepsy and seizure

Epileptogenesis is the process during which molecular and structural changes occur following a brain insult that leads to the changes in the neuronal network leading to clinical epilepsy (Maguire, 2016; Sloviter and Bumanglag, 2013).

Epilepsy is a group of chronic neurological disorders characterised by an individual having epileptic seizures or abnormal excessive synchronised neuronal discharges. Epilepsy is categorised as two or more unprovoked seizures occurring > 24 hour apart (Fisher*, et al.*, 2014). Epileptic seizures are "transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher*, et al.*, 2014).

The International League Against Epilepsy (ILAE) has recently revised their classifications of seizures types and now classify seizures as focal or generalized (Fisher*, et al.*, 2016). Focal seizures can be further subdivided into seizures with motor and non-motor signs and symptoms. Likewise, generalized seizures can be subdivided into motor and absence seizures. Seizures can be accompanied by a loss of consciousness and control of the bowels or bladder function. Seizures can

manifest in different ways and can vary from very brief lapses of attention or muscle spasms, to severe and prolonged epileptic spasms. Epileptic seizures vary also in their frequency and are intractable in approximately 35% of patients with focal seizure with impaired awareness or complex partial seizures in the old terminology (Engelborghs*, et al.*, 2000). Focal seizures with impaired awareness have been found to originate from abnormality in the temporal lobe and is associated with a loss of consciousness (Shin and McNamara, 1994). Temporary symptoms of epilepsy include, but are not limited to, a loss of awareness or consciousness, disturbances of movement, sensation, mood or mental function. People who experience seizures typically present with impairment in motor responses, increased incidences of other disease or psychological disorders such as depression.

1.2. Epilepsy: a balance between excitation and inhibition

Typically, epileptic seizures arise as a result of abnormal, excessive electrical discharges in the brain (Cavazo and Sanchez, 2004) and different areas of the brain can be the initiation site for these discharges which leads to a sudden imbalance between excitatory and inhibitory processes in the neural circuitry (Engelborghs*, et al.*, 2000). This imbalance is in part due to the release of inhibitory and excitatory neurotransmitters that have opposing effects. In the CNS, there are two main neurotransmitter systems that maintain balance, glutamatergic and γ-aminobutyric acid (GABA)-ergic neurotransmission.

1.2.1. Glutamatergic neurotransmission

The amino acid glutamate is the main excitatory neurotransmitter in the mammalian CNS and has been implicated in physiological and pathophysiological conditions such as epilepsy where it has been shown to be involved in the generation and spread of epileptic discharges (Chapman, 2000). Glutamate carries out its actions on cells by binding to its two main types of receptors (Gereau and Swanson, 2008):

1. Ionotropic glutamate receptors (iGluRs) that include a diverse group of ion channels. There are three types of iGluRs historically characterised based on the actions of their synthetic agonists: N-methyl-D-aspartate receptors (NMDARs), α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and kainate receptors.

2. Metabotropic glutamate receptors (mGluRs) that belong to the class C G-protein coupled receptor family (GPCR). There are three groups of mGluRs (Groups I, II and III), which exert their actions via coupling to G-proteins.

1.2.1.1. NMDA receptors

NMDARs are found in most neurones of the brain and in other cell types such as glia (Gereau and Swanson, 2008). They are heterotetramers made up of various complexes of the subunits GluN1 (8 isoforms), GluN2A-D and GluN3A-B (Cull-Candy and Leszkiewicz, 2004; Paoletti, 2011; Paoletti*, et al.*, 2013; Traynelis*, et al.*, 2010). NMDARs are functionally distinguished by their high calcium (Ca^{2+}) permeability and the strong voltage-dependent block of their channel pore by extracellular magnesium (Mg^{2+}) although incorporation of the GluN3 subunit has been shown to be associated with a decrease in Mg^{2+} blockade and Ca^{2+} permeability

(Henson, *et al.*, 2010; Pachernegg, *et al.*, 2012; Paoletti, *et al.*, 2013). NMDARs are gated by glutamate and the co-agonist glycine or D-serine and also respond to changes in the membrane potential in the absence of Mg^{2+}. These factors allow the NMDAR to act as a detector of pre- and postsynaptic activity in sensing both the binding of glutamate receptors at the presynaptic cell and depolarisation at the postsynaptic cell. Upon activation Ca^{2+} passes through the channel into the cell and can act as a second messenger to modulate synaptic strength and alter neuronal function. The removal of the Mg^{2+} block allows the flow of sodium (Na^{+}) and potassium (K^{+}) through the channel (Bourinet, *et al.*, 2014; Lüscher and Malenka, 2012; MacDermott, *et al.*, 1986; Mayer, *et al.*, 1984). The Mg^{2+} block of NMDARs is influenced by structural elements located at or near the channels narrow constriction site and is mainly attributed to the GluN2 subunit. Activation of NMDARs can also lead to the activation of protein kinases and phosphorylating enzymes (Bernard and Zhang, 2015; Gereau and Swanson, 2008; Liu, *et al.*, 2015; Lucchesi, *et al.*, 2011; Wang, *et al.*, 2016).

1.2.1.2. AMPA receptors

AMPA receptors are heterotetramers made up of four subunits GluA1-3 in mammals (Gereau and Swanson, 2008; Henley and Wilkinson, 2016). AMPARs are expressed in all neuronal cell types and in glial cells and are responsible for the majority of the fast excitatory synaptic transmission in the mammalian brain. Due to its fast kinetics and small single- channel conductance it rapidly inactivates and desensitizes in the presence of its agonists glutamate or AMPA, allowing for precise detection of excitatory post synaptic potentials that is an important feature for information processing in cortical networks. AMPARs are expressed in neurones and glia and are

widely distributed in the adult brain. The GluA1 and GluA2 subunits are widely distributed in the adult brain whereas the distribution of the GluA3 and GluA4 subunits is restricted. In brain regions such as hippocampus, forebrain and cerebral cortex the GluA1 and GluA2 subunits are predominantly expressed with low levels of expression of GluA3 and GluA4 (Gereau and Swanson, 2008; Lu and Roche, 2012; Sampedro*, et al.*, 2014; Traynelis*, et al.*, 2010). In the forebrain the majority of fast glutamatergic synaptic transmission is driven by those receptors containing the GluA1 and GluA2 subunits (Geiger*, et al.*, 1995). In general, glutamatergic neurotransmission begins with a fast response mediated by AMPARs, which results in membrane depolarisation that allows NMDAR channels to open and Ca^{2+} to pass through leading to downstream effects. The contributions of both the AMPA and NMDA receptors in epileptogenesis have long been recognised. In humans, changes in NMDA and AMPA receptor binding have been found in brain tissue from epileptic patients (Dingledine*, et al.*, 1990; Isokawa and Levesque, 1991) and there are current anti-epileptic drugs that act as glutamate blockers targeting the AMPA and NMDA receptors (Brodie*, et al.*, 2016; Connock*, et al.*, 2006; Wilby*, et al.*, 2005).

1.2.1.3. Kainate receptors

Kainate receptors are glutamate-gated cation channels with many similarities in function to AMPA receptors (Gereau and Swanson, 2008; Traynelis*, et al.*, 2010). Kainate receptors are tetramers divided into 4 subtypes GluK1-5, which are permeable to Na^{+} and K^{+}. Kainate receptors modulate excitatory and inhibitory transmission and are found both pre- and postsynaptically (Gereau and Swanson, 2008; Traynelis*, et al.*, 2010). Presynaptic kainate receptors through their ability to

both depress and facilitate synaptic transmission in different synapses at different stages of development kainate receptors have the ability to influence the strength of both excitatory and inhibitory neurotransmission (Chittajallu*, et al.*, 1996; Jane*, et al.*, 2009; Lerma, 2003; Traynelis*, et al.*, 2010). In mossy fibre synapses postsynaptic kainate receptors have been shown to play a role in neuronal excitability through their influence on other ionotropic receptors such as GABA receptors (Lerma, 2003; Traynelis*, et al.*, 2010). Kainate receptors are widely distributed in the brain and in the hippocampus where they are found on CA1 interneurones of the hippocampus and at the mossy fibre synapses, where they involved in synaptic plasticity (Chittajallu*, et al.*, 1996; Jane*, et al.*, 2009; Lerma, 2003; Traynelis*, et al.*, 2010). The involvement of kainate receptors in epileptogenesis has been well documented and kainate has been routinely used a method of modelling human temporal lobe epilepsy (discussed later) in animal models is well established (Ben-Ari, 1985; Ben-Ari*, et al.*, 2008).

1.2.1.4. Metabotropic glutamate receptors

Metabotropic glutamate receptors are members of the C subclass of receptors within the GPCR superfamily. mGluRs modulate cell excitability and synaptic transmission in the nervous system where they are widely distributed in neurones and glial cells. Group I (mGluR1 and mGluR5) mGluRs couple to G_q and stimulate phospholipase C (PLC) and the release of intracellular Ca^{2+}. mGluR1 receptors are predominantly located at postsynaptic synapses, where they play a role in the modulation of excitatory neurotransmission by influencing receptors such as the AMPA receptor. Activation of GI mGluRs can lead to a reduction in the expression of AMPA

receptors, which can lead to long-term depression (Lüscher and Huber, 2010; Sanderson*, et al.*, 2016).

Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) couple to $G_{i/o}$ signaling pathways and the inhibition of adenyl cyclase. These mGluRs are found predominantly at the presynaptic terminal where they are generally involved in decreased cell excitability by inhibiting glutamate release. In rats activation of presynaptic and postsynaptic group II mGluRs was associated with a reduction in neuronal excitability in the thalamus (Hermes and Renaud, 2011). In addition, studies have shown stimulation of both the group II and group III mGluRs have been shown to supress seizures in kindled rats and cell lines (Attwell*, et al.*, 1998a; Attwell*, et al.*, 1998b; Gasparini*, et al.*, 1999; Wong*, et al.*, 2002). Although, preclinical investigations into the anti-epileptic effects following modulation of mGluRs have proved promising, to date there are no anti-epileptic drugs that target these receptors types (Connock*, et al.*, 2006; Wilby*, et al.*, 2005).

1.2.2. GABA-ergic neurotransmission

GABA is the main inhibitory neurotransmitter at synapses and binds to fast ionotropic $GABA_A$ receptors or the GPCR $GABA_B$ receptors. GABA acts at $GABA_A$ receptors to hyperpolarise neurones by increasing their membrane chloride ion (Cl^-) conductance and stabilises the resting membrane potential close to the Cl^- equilibrium potential. The action of GABA at pre-synaptic terminals at its $GABA_B$ receptors inhibits the release of glutamate (Bormann, 2000). The involvement of GABA in epilepsy is extensively researched (Avoli and de Curtis, 2011) and many anti-epileptic drugs target the enhancement of GABA-mediated inhibition (Connock*, et al.*, 2006; Wilby*, et al.*, 2005). Enhancement of the GABA system can be done by

binding directly to $GABA_A$ receptors, blocking presynaptic GABA uptake, inhibition of GABA transaminase that inhibits GABA metabolism resulting in increased accumulation of GABA at postsynaptic receptors, or by increasing GABA synthesis (Connock*, et al.*, 2006; Wilby*, et al.*, 2005).

1.3. Aetiology of epileptic seizures

There is no single mechanism underlying seizures, in fact there are many factors such as environmental, genetic, pathological and physiological factors that are involved in the development of seizures and epilepsy (Shorvon, 2011). The age of the patient and the type of seizure also influences aetiology. Factors contributing to the development of epilepsy include genetic predispositions, brain trauma, CNS infection, antenatal factors such as congenital defects and perinatal risk factors, cerebrovascular disorders, parasitic infections, exogenous chemicals (alcohol and drugs), degenerative disorders, febrile convulsions, hippocampal sclerosis, cerebral palsy, sedative drug withdrawal, stimulant drugs and in some cases stress.

Epileptic seizures are typically categorised as focal or generalized seizures (Berg*, et al.*, 2010; Fisher*, et al.*, 2016; Fisher*, et al.*, 2014). Focal and generalized seizures vary with age, where focal seizures are more common in younger and elderly patients. Focal seizures are seizures that are localised to a part of one hemisphere of the brain. Typically, generalized tonic-clonic seizures can occur at any period throughout the life-course and are seizures that affect both hemispheres of the brain.

1.4. The hippocampus

The hippocampus is an important part of the limbic system found deep within the mesial temporal lobe of the brain, where it is involved in learning and memory, in

particular long-term memory and is also important for the formation of new memory and in the detection of new surroundings, stimuli and occurrences. The hippocampus has been used to study many disorders and is attractive because of its unique and useful anatomical and neurobiological features. In addition, the basic layout of cells in the hippocampal formation and circuitry is conserved in mammals and birds. The hippocampal formation (Fig 1.1.) consists of the following: the dentate gyrus (DG), the hippocampus proper (*cornu ammonis* (CA) 1, 2 and 3), subiculum, pre- and para-subiculum and the entorhinal cortex (EC). The circuitry of the hippocampal formation is predominantly unidirectional between the different cortical regions (Basu and Siegelbaum, 2015; Neves*, et al.*, 2008). The EC receives much of the neocortical inputs into the hippocampal formation and can be viewed as the first step in the hippocampal circuit. Polymodal sensory information from neurons in layer II of the EC project along the performant pathway to the DG making excitatory synaptic contacts with the dendrites of the granule cells in the DG. The granule cells in the DG send axons called mossy fibres to the proximal apical dendrites of CA3 pyramidal cells, which in turn send projections via the Schaffer collateral pathway to the CA1 region. The Schaffer collateral pathway forms the major input to the CA1 region of the hippocampus. The CA1 region also receives input form the EC via the perforant pathway and likewise sends projections back to the EC. Axons in the CA1 region also project onto the subiculum, which in turn sends axons to the EC. The projections from the CA1 region and the subiculum form a unidirectional closed loop circuit that begins and ends in the EC (Basu and Siegelbaum, 2015; Neves*, et al.*, 2008).

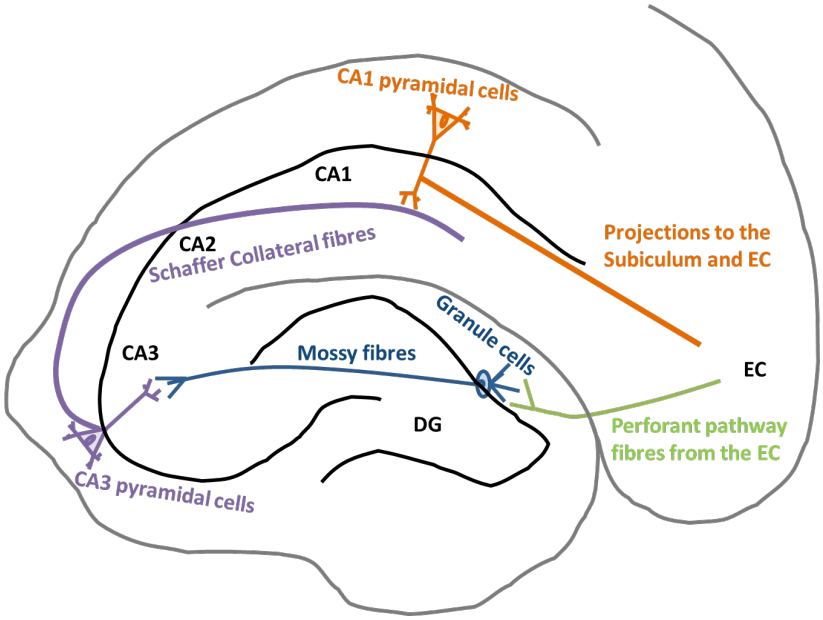

Figure 1.1. Simplified diagram of the hippocampus and tri-synaptic circuit. The hippocampus is divided into four regions: Cornus Ammonis 1-3 (CA1- CA3) regions and the dentate gyrus (DG). Input into the hippocampus from the entorhinal cortex (EC) via the perforant pathway synapse on the granule cells in the DG. This message is then passed along to the CA3 pyramidal cells via the axons of the granule cells called mossy fibres. Axons from the CA3 region synapse on CA1 pyramidal cells via the Schaffer collateral fibres. Finally, the axons of the CA1 pyramidal cells project to the subiculum and EC.

1.5. Types of epilepsy

1.5.1. Mesial temporal lobe and the hippocampus

The hippocampus has been implicated in the pathophysiology of epilepsy. Increased hippocampal excitability is commonly found in human mesial temporal lobe epilepsy (TLE) (Avoli, 2007; D'Antuono, *et al.*, 2002). TLE is a type of focal epilepsy and the most common pathophysiology in patients with drug-resistant TLE is hippocampal sclerosis (HS) (Andersen, *et al.*, 2007; Blümcke, *et al.*, 2013). The international

league against epilepsy (ILAE) has classified HS into three types (Blümcke*, et al.*, 2013) based on hippocampal cell loss:

1) Type 1(most common form found in 60-80% of all TLE cases): Severe neuronal cell loss and gliosis in CA1 (> 80% cell loss), CA2 (30-50%), CA3 (30-90%) regions and the DG (50-60%).

2) Type 2 (affects 5-10% of all TLE surgical cases): Predominant neuronal cell loss and gliosis in CA1 region (80% of pyramidal cells), CA2 and CA3 (< 20% of principal cells)

3) Type 3 (rarest form and affects 4-7.4% of all TLE surgical cases): Cell loss and gliosis in DG (35%), CA3 (< 30%), CA2 (< 25%) and CA1 (< 20%).

Factors influencing the development of HS include: seizure frequency, severity and genetic susceptibility. The degree of HS can be influenced by the age at epilepsy onset and epilepsy duration (During and Spencer, 1992). HS can be caused by cerebral injury within the first few years of a person's life, typically in individuals with a genetic predisposition (Blümcke*, et al.*, 2013). As a consequence, patients with TLE who also manifest with HS have been shown to develop pharmaco-resistant focal seizures. This makes it very difficult to treat these individuals with drugs and other means of treatment such as surgery are explored. Studies have shown that following 2 years after surgical resection, seizures were absent in 60-80% of patients with drug-resistant TLE (Blümcke*, et al.*, 2013). Evidence has shown that TLE also presents with other disorders and there is increased comorbidity between TLE, and depression and psychosis (Andersen*, et al.*, 2007). An increase in mortality and neuropsychological deficits has also been associated with the location of the HS (Andersen*, et al.*, 2007). The coexistence of epilepsy with other underlining

neurological disorders means that it is often difficult to treat with drugs without having drug interactions and adverse side effects.

1.5.2. Rolandic epilepsy

Rolandic epilepsy is genetically determined and is the most common and well-known benign focal childhood epilepsy (Rugg-Gunn and Smalls, 2015). Rolandic epilepsy is found more commonly in males (1:5) and the age of onset ranges from 1-14 years with onset starting between 7–10 years in 75% of children (Rugg-Gunn and Smalls, 2015; Smith*, et al.*, 2015). The prevalence of rolandic epilepsy is approximately 15% in children aged 1–15 years with non-febrile seizures and the incidence rate is 10–20 in 100,000 children aged 0–15 years (Rugg-Gunn and Smalls, 2015).

The most common features of rolandic epilepsy are brief focal seizures lasting for 1–3 minutes. The focal seizures consist of unilateral facial sensory- motor symptoms in 30% of patients, oropharyngolaryngeal symptoms 53% of patients, speech arrest 40% of patients and hypersalivation 30% of patients (Rugg-Gunn and Smalls, 2015). Children may go on to develop usually mild and reversible linguistic, cognitive and behavioural abnormalities during the active phase of the disease, which might be worse in children where onset occurs before 8 years old (Rugg-Gunn and Smalls, 2015; Smith*, et al.*, 2015; Vannest*, et al.*, 2015). Less than 1% of rolandic epilepsy patients may go to develop into to more severe syndromes with linguistic, behavioural and neuropsychological deficits, such as Landau-Kleffner syndrome (Rugg-Gunn and Smalls, 2015; Smith*, et al.*, 2015; Vannest*, et al.*, 2015).

1.5.3. Juvenile myoclonic epilepsy

Juvenile myoclonic epilepsy is defined as myoclonic jerks or spasmodic jerky contraction of groups of muscles (mandatory criterion), without loss of consciousness predominantly occurring after awakening.

The pathogenesis of juvenile myoclonic epilepsy is not fully elucidated but it is generally thought of as a disorder of the thalamo-cortical circuit, but new evidence suggests that it might be more a disorder of the cortex (Park*, et al.*, 2017).

The main symptoms of juvenile myoclonic epilepsy are: bilateral, arrhythmic, irregular myoclonic jerks that which occurs predominantly in the arms usually occurring after awakening (Yacubian, 2016). The age of onset is between 10 and 25 years of age with equal prevalence in male and female (Rugg-Gunn and Smalls, 2015; Yacubian, 2016).

Juvenile myoclonic epilepsy accounts for 2.8-11.9% of all epilepsies and 26.7% of genetic generalized tonic-clonic seizures, a third of which have absence seizures (Rugg-Gunn and Smalls, 2015; Yacubian, 2016). Many go on to develop cognitive dysfunction that may have an impact on quality of life. Seizures can be successfully controlled with medication in 80% of patients with juvenile myoclonic epilepsy (Rugg-Gunn and Smalls, 2015; Yacubian, 2016).

1.5.4. Post-traumatic epilepsy

Traumatic brain injury carries an increased risk of developing epilepsy and this risk is correlated to the severity of the brain injury (Christensen, 2015; Rugg-Gunn and Smalls, 2015). Traumatic brain injury occurs more frequently in males than females and the risk of brain injury varies with age (greater in childhood, in the elderly, and in early adulthood) (Christensen, 2015; Rugg-Gunn and Smalls, 2015). The risk of

developing post-traumatic epilepsy after a traumatic brain injury is 30-50%, a 30 time increased risk of epilepsy compared to the general population (Larkin*, et al.*, 2016). Following a brain injury a cascade of events occur that leads to the development of an imbalance between excitatory and inhibitory processes. This imbalance further increases the risk of spontaneous epileptic events that are associated with excessive excitatory stimulation (Larkin*, et al.*, 2016). Post-traumatic epilepsy accounts for less than 10% of epilepsies (Christensen, 2015). Post-traumatic epilepsy is diagnosed when seizures occur late typically more than a week after the injury but the onset can take up to 10 years or longer to developing post-traumatic seizures (Christensen, 2015; Rugg-Gunn and Smalls, 2015). The risk of developing post-traumatic epilepsy is greatest in individuals older than 15 years old at the time of the brain injury and also higher if there is a familial history of epilepsy. Post-traumatic epilepsy is often difficult to treat but is preventable due to the long period of time (several years) between injury and the development of post-traumatic seizures (Christensen, 2015).

1.6. Treatment of epilepsy

Studies conducted in 2010 showed that whilst 52% of people with epilepsy in the UK are seizure-free (Moran*, et al.*, 2004), approximately 35% of patients with focal seizures with impaired awareness are drug resistant (Engelborghs*, et al.*, 2000). This leaves a treatment gap that is approximately 108,000 people with epilepsy who receive sub-optimal treatment but still have seizures. Given this treatment gap, there is a need for new, more effective treatments. The treatment of epilepsy is generally divided into four categories: anti-epileptic drugs (AEDs), neurostimulation, dietary interventions, and surgery.

1.6.1. Medication (AEDs)

Commonly used AEDs are believed to act primarily by the following main mechanisms (Connock*, et al.*, 2006; Wilby*, et al.*, 2005):

- The reduction in electrical excitability of the cell membrane, predominantly through inhibition of voltage-gated sodium channels
- The enhancement of the inhibitory actions of the neurotransmitter GABA: by enhancing the postsynaptic actions of GABA, inhibition of GABA transaminase, which inhibits GABA metabolism or by using drugs with similar modes of action as GABA agonists
- The inhibition of T-type calcium channels
- Inhibition of glutamatergic neurotransmission

The main drugs that are currently used for the treatment of epilepsy include: phenytoin, carbamazepine and valproate.

The main action of phenytoin is the inhibition of voltage-gated Na^+ channels and it can be used in the treatment of many types of epilepsy (Connock*, et al.*, 2006; Wilby*, et al.*, 2005).

Carbamazepine is a tricyclic antidepressant derivative and has a similar profile to that of phenytoin but fewer negative side effects (Connock*, et al.*, 2006; Wilby*, et al.*, 2005).

The mechanism of action of valproate is not clear but is believed to involve weak inhibition of GABA transaminase and an effect on voltage-gated Na^+ channels. Valproate may also be used to reduce low-voltage-activated T-type Ca^{2+} currents

(Kwan*, et al.*, 2001; Sills and Brodie, 2001), resulting in a reduction in the power of thalamocortical oscillations that are important in the generation of absence seizures (Connock*, et al.*, 2006; Wilby*, et al.*, 2005). Valporate is also used to treat seizures in juvenile myoclonic epileptic patients, with a high efficacy against generalized tonic-clonic and myoclonic seizures (Rugg-Gunn and Smalls, 2015; Yacubian, 2016).

All of the AEDs mentioned above have been used in the clinic in the treatment of tonic-clonic seizures and focal seizures and are normally the first line of treatment. Typically, it is preferable to give these drugs on their own as drug interactions occurs very commonly. Other AEDs include drugs that block the actions of glutamate receptors, such as felbamate and ketamine that block the actions of NMDARs (Connock*, et al.*, 2006; Kwan*, et al.*, 2001; Sills and Brodie, 2001; Sleigh*, et al.*, 2014; Wilby*, et al.*, 2005).

Newer drugs include vigabatrin, tiagabine and lamotrigine, and their modes of action are not well understood. Vigabatrin has been found to act by inhibiting GABA transaminase, which inhibits GABA metabolism and is useful because it can be used in patients who are unresponsive to traditional AEDs. Tiagabine is a GABA-uptake inhibitor where it acts to prevent the removal of GABA from the synaptic cleft. Lamotrigine has been found to inhibit voltage-gated Na^+ channels and has a broad therapeutic profile so can be used to treat a number of epilepsies.

The proportion of medically refractory patients remains at approximately 30% despite advances in the discovery of new drugs. It is clear that the need for non-drug treatment is justifiable and necessary. Although AEDs have been in use for many years there is still a need for new therapies for the treatment of drug-resistant seizures with improved efficacy or tolerability profiles. Also, there is a growing need for disease-modifying treatments that prevent or ameliorate the process of

epileptogenesis (Galanopoulou*, et al.*, 2012). Where AEDs are ineffective, other treatments are necessary. These include brain surgery, neurostimulation and the ketogenic diet.

1.6.2. Surgery

Brain surgery is performed in a small number of patients where drugs have proved to be ineffective and the epilepsy is severe. Brain surgery is an effective way of reducing or eliminating seizures in people with medically intractable epilepsy. Surgery is appropriate where seizures are localised to one part of the brain and the brain region is small (accounts for 3% of those who develop epilepsy) (National Institute for Health and Clinical, 2009). Temporal lobe epilepsy can be treated with an anterior mesial temporal resection. Complications include infections, and secondary neurological deficits that occur in 6% of patients (World Health Organisation, 2005). The proportion of epilepsy patients who remain seizure-free following surgery is 70%, and in many, a reduction in seizure frequency is what is generally experienced. Although the success rate of surgery is high, unfortunately it still remains inappropriate for those individuals where removal of brain tissue may prove to be debilitating.

1.6.3. Neurostimulation

Where AEDs have failed to control seizures, and epilepsy surgery is not viable, neurostimulation is often considered. All neurostimulation treatment is palliative and it is usually advised that it is preceded by a thorough surgical evaluation. There are currently a number of techniques, such as vagus nerve stimulation (VNS) and

responsive neural stimulation (RNS). VNS involves the surgical placement of a transcutaneous programmable device that provides mild and irregular electrical stimulation to the left cervical vagus nerve. The aim of VNS is to reduce the irregular electrical brain activity that leads to seizures. VNS decreases seizures by 50% in the high stimulation group compared to 8% in the low stimulation group (Chambers and Bowen, 2013). Double-blind randomised controlled trials reported a reduction range of 24.5%-28% in mean seizure (Chambers and Bowen, 2013). Frequent adverse effects include sleep-related decreases in respiratory airflow, hoarseness, throat pain and coughing that are due to the activation of the recurrent laryngeal nerve branch of the cervical vagus (Chambers and Bowen, 2013).

1.6.4. Ketogenic diet

The ketogenic diet can be used to treat epileptic seizures in children and adults where it is often difficult to treat seizures with AEDs (Kovac*, et al.*, 2013; Masino and Boison, 2012; Masino*, et al.*, 2013; Masino*, et al.*, 2009; Neal*, et al.*, 2008; Thammongkol*, et al.*, 2012). The ketogenic diet composed of a high-fat, low carbohydrate and protein diet, which requires working closely with a dietician. The ketogenic diet was originally designed to mimic the biochemical changes associated with fasting, which anecdotally has been shown to control seizure activity (reviewed in (Masino and Rho, 2012)). Under the ketogenic diet the body is not supplied with enough glucose for energy and stored fats can then be broken down for energy resulting in a build-up of acids called ketones within the body. The ketogenic diet produces ketone bodies, such as β-hydroxybutyrate, acetoacetate and acetone, which are products of fatty acid oxidation in the liver and reduced blood glucose levels. The ketone bodies can be used as an alternative substrate to glucose for energy utilisation.

When fatty acid levels are elevated as seen in the ketogenic diet and when the level of fatty acids exceed the metabolic capacity for the citric acid cycle, the citric acid cycle substrate acetyl-CoA is shunted to the production of ketone bodies. There is currently no primary mechanism of action for the ketone bodies in reducing seizure activity, although a number of mechanisms have been proposed (reviewed in (Masino and Rho, 2012)), but more supporting evidence is needed:

- Membrane hyperpolarisation through the activation of K^+ channels
- Enhancement of GABAergic neurotransmission
- A reduction in vesicular glutamate release
- Reduction in brain glucose consumption

In 2008 a randomised controlled trial of ketogenic diet (Neal*, et al.*, 2008) showed that 38% of children on the diet had a reduction in seizure frequency compared to those not on the diet, where seizure frequency increased. Side effects include vomiting, diarrhoea and constipation. Despite the benefits of this method of treatment, the ketogenic diet is not suitable for everyone and often families and patients find it difficult to maintain.

As outlined above the current treatments for the symptoms of epilepsy are failing a large cohort of epileptic patients. The mass majority of AED target the major inhibitory neurotransmitter GABA but none are currently used to target neuromodulators of synaptic transmission. Over the last few decades there has been growing interest in possible role of the neuromodulator adenosine. Adenosine is a purine nucleoside that has been implicated in the pathophysiology of epilepsy.

1.7. Models of seizures and epilepsy

Many different activity patterns are termed epileptiform activity and there are a number of models used to investigate the differing epileptiform activity. In addition, *in vitro* methods allow for investigation into epilepsy at the slice level where mechanisms of the generation, spread and termination of seizures can be studied at a level of detail often difficult to achieve in *in vivo* animal models. Animal models of epilepsy are useful in elucidating the underlying mechanisms of epileptogenesis and aid in the development and screening of novel treatments. Rodents, such as rats and mice, have brain regions that are similar in structure and function to that of humans and thus are useful tools for studying epilepsy. Brain slices, cell culture and molecular assays are advantageous because they represent reduced biological systems that allow insight into epilepsy.

1.7.1. Pharmacological *in vivo* animal models of status epilepticus

Chemoconvulsants are typically used to induce status epilepticus (SE) in rodents and include: kainic acid and pilocarpine (for review see (Reddy and Kuruba, 2013)). Chemoconvulsants are generally used to characterise the pathophysiology of epilepsy and to evaluate potential therapeutic interventions. Chemoconvulsants carry out their effects by enhancing glutamatergic neurotransmission, by blocking GABAergic inhibition or by enhancing the cholinergic system to induce seizures or SE.

1.7.1.1. Kainic acid model

The kainic acid model is the most widely used pharmacological model for inducing SE and have been administered in a number of rodent models. Kainic acid is a powerful neural excitant that acts as a high affinity agonist at kainate glutamate receptors. Typical experiments involve the implantation of two surface electrodes into brain regions such as the cortex and cerebellum, and one depth electrode into the hippocampus. In rats, administration of this potent convulsant subcutaneously/ intraperitoneally (8-12 mg/kg) leads to convulsions and progression of the development of SE (reviewed in (Levesque and Avoli, 2013; Reddy and Kuruba, 2013)). The convulsant dose of kainic acid is variable and depending on the strain (20-40 mg/kg, intraperitoneally) (reviewed in (Levesque and Avoli, 2013; Reddy and Kuruba, 2013)). Furthermore, intrahippocampal kainate injection in awake rats (0.4 μg) has also been successfully used to avoid the insult-modifying effects of anesthesia (Rattka*, et al.*, 2013). In rats, acute seizures can also be induced by intra-amygdaloid injections of kanic acid (0.4-2 μg) that produces symptoms that are similar to those observed following intrahippocampal injections (Ben-Ari*, et al.*, 1979; Gurbanova*, et al.*, 2008). Multiple injections are required in order for development of consistent SE, lesions and spontaneous seizures. This model results in widespread damage to brain regions such as the hippocampus (damage to the CA1, CA3 and hilar cells of the DG) and the amygdala. The extensive damage observed in this model is similar to lesions observed in human epileptogenic tissue. A major disadvantage of this model is that there is variable sensitivity that is dependent of the strain, age, sex and weight of the rats used. Also, kainic acid produces direct neuronal damage that occurs as a consequence of seizure-induced neuronal damage.

1.7.1.2. Pilocarpine model

This model is used to study the generation and spread of convulsive activity in the hippocampus and amygdala, as it produces consistent seizures associated with neurodegeneration (reviewed in (Reddy and Kuruba, 2013)). Pilocarpine is a muscarinic cholinergic agonist that is used to induce limbic seizures. Pilocarpine can be administered directly into the amygdala or hippocampus, or systemically to rats (400 mg/kg). Seizures are elicited by the activation of the cholinergic system and SE is believed to be due to repeated activation of the cholinergic system. Typical experiments involve the implantation of two surface electrodes into the brain such as the cortex and cerebellum and one depth electrode into the hippocampus. Rats are pre-treated with a muscarinic antagonist to prevent peripheral effects of pilocarpine. 30 minutes following administration of the muscarinic antagonist, pilocarpine is given and typically limbic seizures arise 30 minutes after the pilocarpine injection (reviewed in (Reddy and Kuruba, 2013)). The onset to seizures is dependent on the dose of pilocarpine. Similar to the kainic acid, pilocarpine produces widespread neuronal damage. A disadvantage of this model is that it induces greater neocortical damage than kainic acid.

1.7.2. *In vitro* animal models

There are different methods for studying epileptiform activity *in vitro* and I will briefly discuss the use of organotypic slice cultures and brain slices.

1.7.2.1. Organotypic slice cultures

Organotypic slice cultures are an attractive model for the study of epileptogenic changes in neural circuits. Organotypic slice cultures are capable of maintaining some of the intrinsic properties of the intact tissue and are used to overcome some of the issues associated with the use of slices (Routbort*, et al.*, 1999). This model allows for the study of epileptogenesis within individual cell types. One study used organotypic slice cultures from rat pups which experienced experimental febrile seizures to investigate the mechanisms underlying the emergence of ectopic granule cells (Koyama, 2013). An advantage that cell cultures have over slices is that they can be kept for weeks as opposed to days or hours. A disadvantage of using cell cultures is that they are typically taken from pups and are not representative of the adult brain (Heinemann*, et al.*, 2005).

1.7.2.2. Brain slices

Acutely prepared brain slices are the most commonly used preparation for studying epileptiform activity as they preserve the neural circuitry needed for the generation of seizures. Slices of 200-600 μm thickness are typically used, primarily to study acutely provoked electrographic seizures as opposed to chronic epileptogenesis, as slices do not survive for long periods of time. Seizure activity can either be recorded extracellularly or intracellularly with the use of microelectrodes. Seizure models include lowering the extracellular Mg^{2+} concentration in tissue (Anderson*, et al.*, 1986; Dreier and Heinemann, 1991; Gloveli*, et al.*, 1995; Hamon*, et al.*, 1987; Jones and Heinemann, 1988; Mody*, et al.*, 1987; Stanton*, et al.*, 1987; Walther*, et al.*, 1986; Zhang*, et al.*, 1995), increases in extracellular K^+ concentrations, typically from 3 mM to 10-12 mM (Fisher*, et al.*, 1976; Heinemann and Lux, 1977; Lothman

and Somjen, 1976; McNamara, 1994; Moody, *et al.*, 1974; Yaari, *et al.*, 1986), and decreases in extracellular calcium to 0.2 - 0.6 mM (Heinemann, *et al.*, 1977; Yaari, *et al.*, 1986). Low Mg^{2+} solutions have been shown to enhance NMDA receptor-dependent glutamatergic excitation through the removal of the Mg^{2+} block. Low Mg^{2+} preparations, sometimes used in conjunction with changes in the concentration of other ions such as K^+, can induce seizures in brain slices and are a widely adopted seizure model. In addition, pharmacological agents can also be used to induce seizure activity in acutely prepared brain slices (reviewed in (Avoli and Jefferys, 2016)) and ex vivo brain slices from chronically epileptic animals and human epileptic patients are also used to study epileptiform activity (Antonio, *et al.*, 2016; Jones, *et al.*, 2016).

1.8. K^+ channels and epilepsy

Epileptogenesis can also arise as a consequence of an imbalance in K^+ levels in the brain (Fisher, *et al.*, 1976; Moody, *et al.*, 1974). K^+ channels regulate neuronal excitability by regulating presynaptic neurotransmitter release (Johnston, *et al.*, 2010). K^+ currents play an active role in membrane potential, repolarisation and hyperpolarisation, ultimately acting to limit neuronal excitability.

K^+ channels are not only involved in normal physiological responses, but have also been implicated in the pathophysiology of epilepsy and mutations encoding the K^+ channels dysfunction has been linked to inherited epilepsy in humans and animal models (Cooper, 2012; Villa and Combi, 2016). Antagonism of K^+ channels prolongs the action potential duration, which leads to enhance release of neurotransmitters. Voltage-gated presynaptic K^+ (Kv) channels with subunits Kv1, Kv2, Kv3 and Kv4 can be blocked with 4-aminopyridine (4-AP; 2-10 mM)

(Johnston, *et al.*, 2010), to facilitate the release of neurotransmitters such as serotonin, norepinephrine, acetylcholine dopamine, glutamate and GABA (Morris, *et al.*, 1996). Kv channels are a group of diverse channels (Table 1.1.) that are involved in mediating outward K^+ currents that play a role in normal and pathological processes in neurones (reviewed in (McNamara, 1994; Reddy and Kuruba, 2013; Shah and Aizenman, 2014)). The loss of Kv1 or Kv7 has been shown to promote neuronal hyperexcitability, which can then have a major effect in disorders such as epilepsy (reviewed in (Shah and Aizenman, 2014)). In a mouse model of TLE, seizures trigger an upregulation of K_v1.1 channels transcription in dentate gyrus granule cells, was associated with an increased response delay of the dentate gyrus cells, which suggests a Kv1 channels-mediated anticonvulsive and neuroprotective mechanism to scale DG output (Kirchheim, *et al.*, 2013). Application of the K_v1antagonist dendrotoxin into rat hippocampus induces neuronal excitability, seizures and cell death (Bagetta, *et al.*, 1992; Lalic, *et al.*, 2011). Mutations in the Kv7 channel family have also been implicated in epilepsy, where mice expressing the dominant negative mutant Kv7.2 channel display spontaneous seizures and increased hippocampal neuronal excitability and cell death (Peters, *et al.*, 2004). In humans, treatment with retigabine, a Kv7 channel activator, enhances channel activation by inducing a hyperpolarisation effect on voltage-gated channel activation, and has been found to be effective in reducing epileptic seizures (Tatulian, *et al.*, 2001; Wuttke, *et al.*, 2005). These studies have demonstrated the importance of dysfunction of K^+ channels and thus the action of K^+ in epilepsy.

Table 1.1. K_v channel families. Gene names shown are those assigned by the IUPHAR and HGNC in addition to some other commonly used names. Taken form (Gutman*, et al.*, 2005).

IUPHAR	HGNC	Other
K_v1.1	*KCNA1*	*Shaker*-related family
K_v1.2	*KCNA2*	
K_v1.3	*KCNA3*	
K_v1.4	*KCNA4*	
K_v1.5	*KCNA5*	
K_v1.6	*KCNA6*	
K_v1.7	*KCNA7*	
K_v1.8	*KCNA10*	
K_v2.1	*KCNB1*	*Shab*-related family
K_v2.2	*KCNB2*	
K_v3.1	*KCNC1*	*Shaw*-related family
K_v3.2	*KCNC2*	
K_v3.3	*KCNC3*	
K_v3.4	*KCNC4*	
K_v4.1	*KCND1*	*Shal*-related family
K_v4.2	*KCND2*	
K_v4.3	*KCND3*	
K_v5.1	*KCNF1*	Modifier
K_v6.1	*KCNG1*	Modifiers
K_v6.2	*KCNG2*	
K_v6.3	*KCNG3*	
K_v6.4	*KCNG4*	
K_v7.1	*KCNQ1*	*KVLQT*
K_v7.2	*KCNQ2*	*KQT2*
K_v7.3	*KCNQ3*	
K_v7.4	*KCNQ4*	
K_v7.5	*KCNQ5*	
K_v8.1	*KCNV1*	Modifiers
K_v8.2	*KCNV2*	
K_v9.1	*KCNS1*	Modifiers
K_v9.2	*KCNS2*	
K_v9.3	*KCNS3*	
K_v10.1	*KCNH1*	*eag1*
K_v10.2	*KCNH5*	*eag2*
K_v11.1	*KCNH2*	*erg1*
K_v11.2	*KCNH6*	*erg2*
K_v11.3	*KCNH7*	*erg3*
K_v12.1	*KCNH8*	*elk1, elk3*
K_v12.2	*KCNH3*	*elk2*
K_v12.3	*KCNH4*	*elk1*

1.9. Purinergic signalling

Purines are one of two families of nitrogen-containing molecules termed nitrogenous bases (Fig 1.2.). Nitrogenous bases are needed to make the genetic material in every living organism. Purines are double-ringed structures that consist of a six-membered ring fused to a 5 membered ring heterocyclclic molecule.

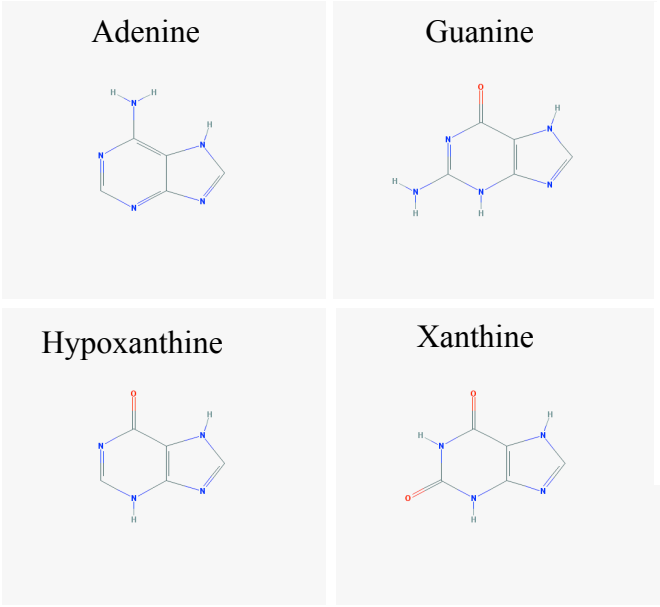

Figure 1.2. Structure of nitrogenous bases. Adenine and guanine are constituents of nucleotides occurring in nucleic acids. Hypoxanthine and xanthine are important intermediates in the synthesis and degradation of purine nucleotides. Taken from PubChem, URL: https://pubchem.ncbi.nlm.nih.gov

Geoffrey Burnstock first postulated in the 1970's the concept of purinergic neurotransmission (Burnstock, 1972; Burnstock*, et al.*, 1972a; Burnstock*, et al.*, 1972b), where adenosine 5'-triphosphate (ATP) was shown to be a neurotransmitter at various nerve terminals. Today, it is now accepted that ATP can act as a neurotransmitter and or as a neuromodulator in many physiological processes. Purines also play a major role in many pathophysiological processes such as epilepsy and are important molecules for both intracellular and extracellular signalling (Burnstock, 2009; Burnstock, 2013; Dale and Frenguelli, 2009; Dunwiddie and Masino, 2001). Purines such as ATP and the purine nucleoside adenosine are important signalling molecules that activate their own receptors. ATP as well as its nucleotide adenosine 5'-diphosphate (ADP), ADP-sugars, or the pyrimidines uridine 5'-diphosphate (UDP) and uridine 5'-triphosphate (UTP) can activate P2 receptors

whilst adenosine activates P1 receptors (Abbracchio, *et al.*, 2009; Fredholm, *et al.*, 2011; Fredholm, *et al.*, 1994; Fredholm, *et al.*, 2001a; Ralevic and Burnstock, 1998).

1.9.1. ATP

ATP is used as an energy source for nearly all cellular activity. ATP is a nucleoside consisting of three phosphate groups esterified to the sugar moiety ribose and the purine base adenine (Fig 1.3). The breaking of ATP during hydrolysis releases energy to form its nucleotides ADP and adenosine 5'-monophosphate (AMP). AMP is quickly recycled into ADP and ATP by the enzyme adenylate kinase for energy use. Intracellular ATP and its nucleotides ADP and AMP are constantly interconverted, providing a constant supply of energy.

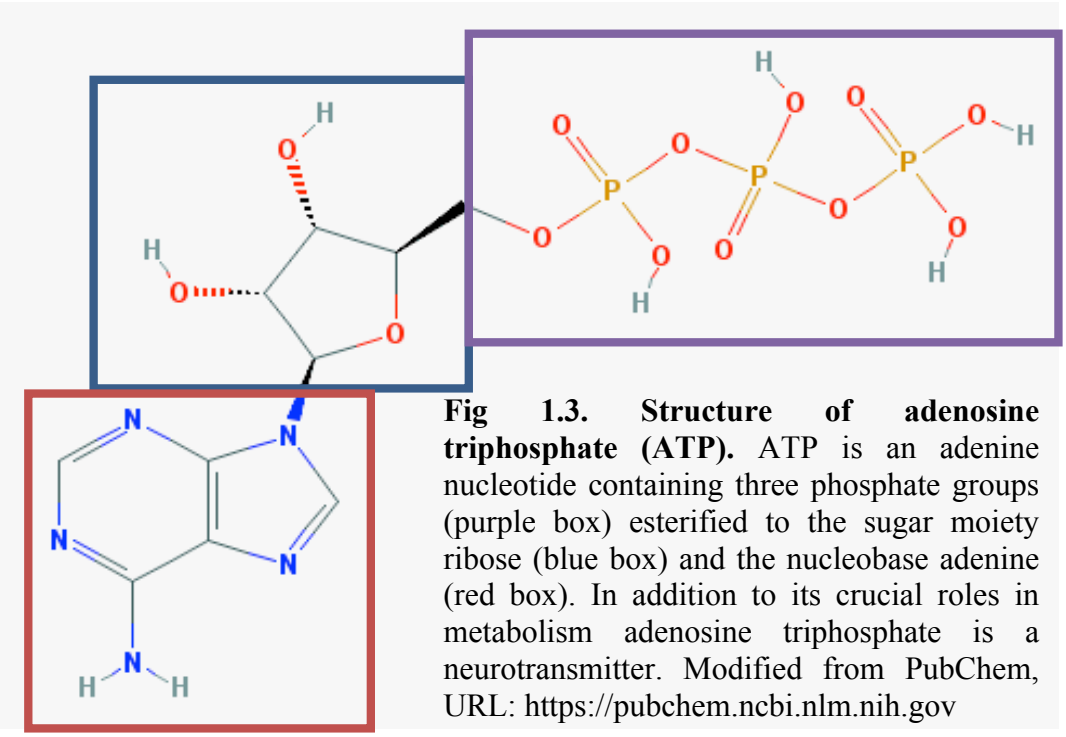

Fig 1.3. Structure of adenosine triphosphate (ATP). ATP is an adenine nucleotide containing three phosphate groups (purple box) esterified to the sugar moiety ribose (blue box) and the nucleobase adenine (red box). In addition to its crucial roles in metabolism adenosine triphosphate is a neurotransmitter. Modified from PubChem, URL: https://pubchem.ncbi.nlm.nih.gov

1.9.1.1. ATP synthesis

ATP is a major source of energy use in the brain. Energy homeostasis requires that there is a balance between cellular ATP consumption and ATP production. The reductions in ATP and increases in ADP, AMP and phosphates occur when ATP is used during both glycolytic and oxidative reactions. In addition to the enzymes involved in mitochondrial synthesis and glycolytic ATP production, adenylate kinase and creatine kinase (Fig 1.4.) also play an important role in the maintenance of high cytosolic ATP levels (Ames, 2000; Dzeja and Terzic, 2003). Both adenylate kinase and creatine kinase reactions play an important role in the buffering of the ATP/ADP (Ames, 2000; Dzeja and Terzic, 2003).

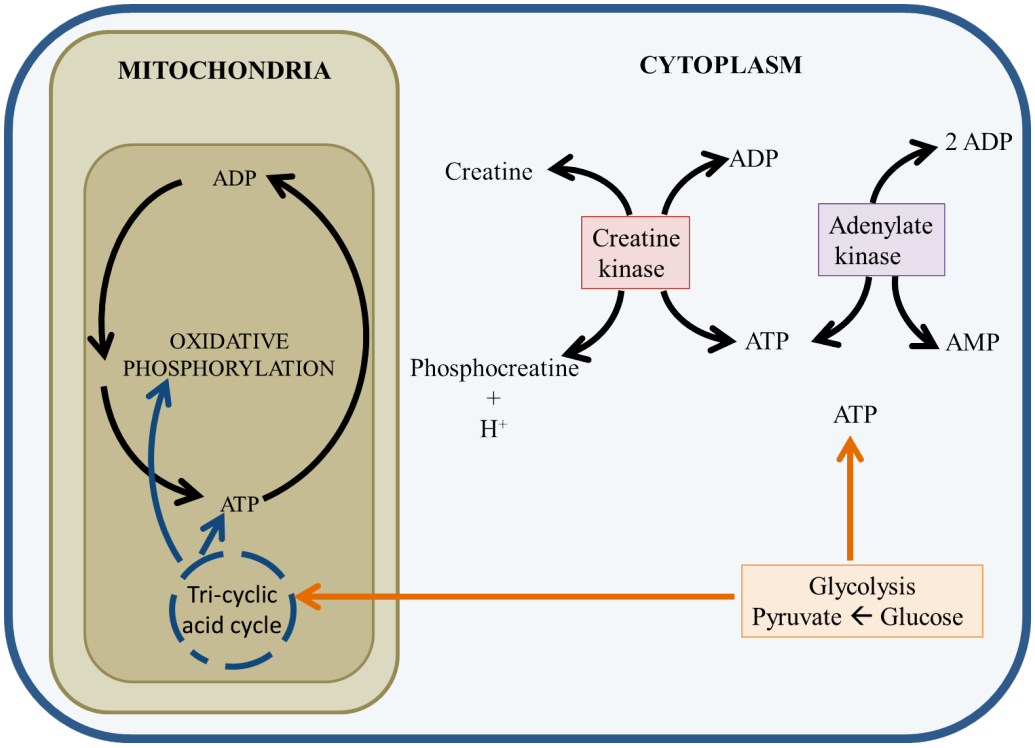

Fig 1.4. ATP synthesis pathways. ATP is synthesised in the mitochondria by oxidative phosphorylation. In the cytoplasm, ATP production can be done by the creatine kinase/phosphocreatine system and the adenylate kinase system. Glycolysis also produces ATP via anaerobic mechanisms in the cytoplasm. The glycolytic product pyruvate can further be used to generate more ATP via the recruitment of the tri-cyclic acid cycle and oxidative phosphorylation. Adapted from Dahout-Gonzalez et al., 2006.

1.9.1.2. ATP storage and release

Studies have shown that ATP and other nucleotides are taken up and stored in secretory and synaptic vesicles where it can be co-stored and co-released with other neurotransmitters (Burnstock*, et al.*, 2012). ATP is released from many cells as a result of physiological and pathophysiological response to stimuli such as mechanical stress, hypoxia and inflammation. Additionally, reduction in ATP levels have been shown in patients with temporal lobe epilepsy and may point to a role of reduced ATP concentration in brain tissue and the pathophysiology of epilepsy (Chu*, et al.*, 1998; Williamson*, et al.*, 2005). In the brain, ATP can function as an extracellular signalling molecule between neurones and glial cells. In glial cells, ATP acts as a widespread gliotransmitter, where the release of ATP from astrocytes whether by exocytosis or through membrane channels has been observed (reviewed in (Abbracchio*, et al.*, 2009)). Astrocytic release of ATP may play an important role in triggering cellular responses to trauma and ischaemia. It does this by initiating and maintaining astrogliosis (Boison*, et al.*, 2010). Astrogliosis can be defined as the pathological proliferation and hypertrophy of astrocytes, and this dysfunction in gliotransmission has been implicated in the pathophysiology of epilepsy (reviewed in (Boison, 2013; Boison*, et al.*, 2010)).

1.9.1.3. ATP receptors

ATP P2 receptors have been characterised on the basis of their mechanism of action, pharmacology and molecular cloning (reviewed in (Burnstock*, et al.*, 2012; Ralevic and Burnstock, 1998)). P2 receptors are divided into P2X ionotropic and P2Y metabotropic receptor subtype families. There are seven P2X receptor subtypes ($P2X_{1\text{-}7}$) and eight P2Y subtypes ($P2Y_{1,2,4,6,11,12,13 \text{ and } 14}$). In the CNS *in situ*

hybridisation of P2 receptor subtypes mRNA and immunohistochemistry of receptor subtype protein expression has shown that the P2 receptor subtypes are heterogenously expressed.

1.9.1.3.1. P2X

P2X receptors are ligand gated channels where upon ATP binding enables the flow of Na^+ and K^+ through their integral membrane channels . P2X receptors form trimers made up of individual subunits encoded by the seven distinct genes ($P2X_{1\text{-}7}$). All P2X subunits are expressed in neural cells and are widely distributed in the brain such as in the hippocampus where all the P2X subtypes are expressed. $P2X_1$ subtypes are also expressed in the cerebellum and the $P2X_3$ has been shown to be distributed in the brain stem (reviewed in (Burnstock, 2006)). P2X receptors are expressed in a segregated manner on glial cells (e.g. P2X1/5 are found in the cortex but not in other brain regions).

1.9.1.3.2. P2Y

P2Y receptors can form homodimers or heterodimers with other P2Y receptors or other transmitter receptors. P2Y receptors are part of the GPCR family and are further divided into two subgroups 1) $P2Y_{1,2,4,6 \text{ and } 11}$ subgroup and 2) $P2Y_{12,13, \text{ and } 14}$ subgroup. Subgroup 1 are coupled to $G_{q/11}$ to activate PLC and inositol triphosphate (IP3) endoplasmic reticulum Ca^{2+}-release signalling pathways (reviewed in (Abbracchio*, et al.*, 2009)). $P2Y_2$ and $P2Y_4$ may activate G_i, whilst $P2Y_{11}$ has also been shown to couple to G_s. Subgroup 2 are coupled to $G_{i/o}$ which inhibits adenylyl cyclase and modulates ion channels (reviewed in (Abbracchio*, et al.*, 2009)). P2Y

receptors are expressed on both neurones and glia, and in the hippocampus, $P2Y_{1,2,4,6 \text{ and } 12}$ receptors are expressed.

1.9.1.4. ATP and epilepsy

Recent studies have highlighted a potential role of ATP in seizure activity in (Dale and Frenguelli, 2009; Kumaria*, et al.*, 2008; Reid*, et al.*, 2014). During seizures there is a shift towards an increase in the metabolic state of the brain that results in the consumption of great amounts of energy and a reduction in ATP levels. This altered state results in a temporary insufficient supply of energy in the brain, which induces status epilepticus and the dysregulation of brain function. Studies have shown that there is a reduction in ATP levels during intense neuronal activity that might contribute to epilepsy in both humans (Chu*, et al.*, 1998) and in rats (Sanders*, et al.*, 1970). It has been proposed that anticonvulsive effect of the ketogenic diet might be mediated through its ability to enhance energy reserves and thus lead to the stabilisation of synaptic function, membrane potential and a reduction in seizure activity. One proposed mechanism of the ketogenic diet on the enhancement of energy reserves is the elevations in ATP levels in the brain that might enhance and or prolong the activity of the Na^+/K^+ ATPase pump. This is supported by evidence showing that adult rats fed a ketogenic diet for 3 weeks have higher brain concentrations of ATP (DeVivo*, et al.*, 1978).

Activation of ATP receptors may result in hyperactivity of neurons and thus, induce a positive feedback mechanism, progression of seizure activity and the prolongation of seizure duration (reviewed in (Puchałowicz*, et al.*, 2014)). An increase in the expression of $P2X_7$ receptors in hippocampal neurones (DG and CA1 cells), neocortex and microglia was found after epileptic seizures, as a result of activation of

microglial cells and as well as its increased sensitivity to ATP (reviewed in (Puchałowicz*, et al.*, 2014)). In microglia, an increase in the expression of $P2X_4$ receptors was found after the occurrence of status epilepticus. In contrast, a decrease in the expression of the $P2X_2$ receptors was found in mouse hippocampal neurones in a kainic acid model of epilepsy (reviewed in (Puchałowicz*, et al.*, 2014)). There is evidence that gives support to the action of ATP at its P2 receptors in epilepsy although this is unclear. In particular, there is evidence for the role of the $P2X_7$ receptors in epileptogenesis in several animal models (reviewed in (Dale and Frenguelli, 2009; Kumaria*, et al.*, 2008; Reid*, et al.*, 2014)). However, one study showed a limited role of the ATP P2 receptors in modulating electrographic seizures induced by electrical stimulation (Lopatář*, et al.*, 2011). Although this study saw a limited effect of ATP at the P2 receptors on seizure activity, the breakdown product of ATP adenosine was found to have pronounced inhibitory effects on seizure activity.

1.9.2. Adenosine

Adenosine is a signalling messenger molecule in the brain, where it is implicated in many normal and pathophysiological processes such as sleep and epilepsy (reviewed in (Masino, 2013)). Adenosine can act to either reduce the activity of excitable tissues or increase the delivery of metabolic substrates, thereby helping to couple the rate energy expenditure to the energy demand. Under normal conditions adenosine is continuously formed both intracellularly and extracellularly. Intracellularly production of adenosine can occur either as breakdown a product of ATP, or as a consequence of the hydrolysis of S-adenosylhomocysteine (SAH), which forms both homocysteine and adenosine. Extracellular adenosine can be formed from

ectonucleotidase-mediated hydrolysis of ATP and both neurones and glia can also directly release adenosine.

1.9.2.1. Adenosine synthesis

Adenosine is a neuromodulator that is involved in physiological and pathophysiological processes in the CNS and in peripheral organs such as the heart (reviewed in (Boison, 2006; Latini and Pedata, 2001; Masino, 2013)). The coupling of adenosine to its receptors activates various downstream signalling pathways, which is dependent on the receptor subtype activated which will be discussed in more detail in the sections below (reviewed in (Dunwiddie and Masino, 2001; Fredholm*, et al.*, 2005; Masino and Boison, 2012; Masino, 2013)). Adenosine plays an important role in the fine-tuning of synaptic transmission. Adenosine influences synaptic transmission in a number of ways: presynaptic inhibition facilitated by the inhibitory adenosine A_1 receptors or facilitation through its A_{2A} receptors. Adenosine also acts postsynaptically to modulate the actions of neurotransmitters such as glutamate and GABA and it also directly influences the hyperpolarisation or depolarisation of neurones by modulating the actions of ion channels (reviewed in (Dunwiddie and Masino, 2001; Fredholm*, et al.*, 2005; Masino and Boison, 2012; Masino, 2013)).

As mentioned above, intracellular adenosine is formed in 2 ways: 1) dephosphorylation of AMP by 5'-nucleotidase or 2) hydrolysis of SAH by SAH hydrolase.

1) In the cell, AMP is dephosphorylated by 5'-nucleotidase to make adenosine, which is the main route for making intracellular adenosine or deaminated to inosine monophosphate (IMP). Adenosine can either be further degraded to inosine by

adenosine deaminase or re-phosphorylated to AMP by adenosine kinase. Inosine cannot be re-phosphorylated to IMP, unlike adenosine due to the lack of a specific kinase (Mascia*, et al.*, 2000). Therefore, inosine is either released or further broken down by purine nucleosidase phosphorylase to hypoxanthine and the sugar moiety ribose-1-phosphate (Rib-1-P). Hypoxanthine can be reconverted to IMP and subsequently to AMP via salvage pathway (to be discussed in more detail below). Hypoxanthine can be released or through the actions of xanthine oxidase can further degrade hypoxanthine and its breakdown product xanthine to uric acid and hydrogen peroxide. Xanthine and uric acid, unlike hypoxanthine cannot be reconverted to purine nucleotides and are therefore, lost from the purine nucleotide pool.

2) In addition to the formation of adenosine from the catabolism of AMP, adenosine can be made as a product of the transmethylation pathway (Fig 1.5.). In the methyltransemethylation and transsulfuration pathways, S-adenosylmethionine (SAM) is converted to S-adenosylhomocysteine (SAH) by the actions of the methyltransferases (Cantoni*, et al.*, 1989; Lloyd*, et al.*, 1988; Lu, 2000; Mandaviya*, et al.*, 2014; Mudd*, et al.*, 2007). S-adenosylmethionine (SAM) acts as an important methly donor for many reactions. SAM is used by DNA methytransferase enzymes to transfer methyl groups to DNA and S-adenosylhomocysteine (SAH) is made as a by-product. SAH can be further hydrolysed to homocysteine and adenosine by SAH hydrolase. Homocysteine (HCY) can either be converted back to SAM where folate-vitamin B12-independent (betaine-homocysteine methyltransferase) and folate-vitamin B12-dependent (methionine synthase) first convert HCY to methionine (Mandaviya*, et al.*, 2014). Methionine is then converted to SAM by the actions of methionine adenosyltransferase (MAT), which helps to fuse methionine and ATP to make SAM. HCY can also be used in the transsulfuration pathway to make

glutathione that is used to remove toxins from the body (Cantoni, *et al.*, 1989; Lloyd, *et al.*, 1988; Lu, 2000; Mandaviya, *et al.*, 2014; Mudd, *et al.*, 2007).

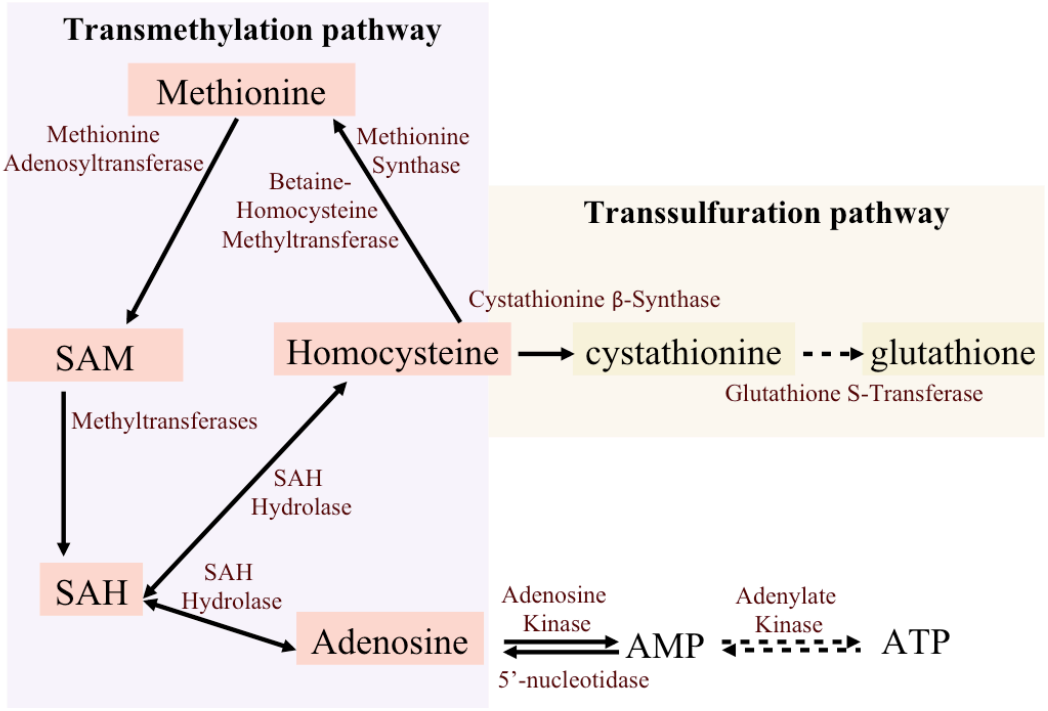

Figure 1.5. Transmethylation and transsulfuration pathways in the brain. S-adenosylmethionine: (SAM) is converted to S-adenosylhomocysteine (SAH) by the actions of methyltransferases. SAH is further broken down to adenosine and homocysteine through the actions of SAH hydrolase. Adenosine can be further recycled to make ATP in a multi-step process through the action of the enzymes adenosine kinase and adenylate kinase. Homocysteine can either be converted back to methionine by the actions of methionine synthase and betaine-homocysteine methyltransferase. Methionine and adenosine triphosphate (ATP) is then further converted to SAM by the actions of methionine adenosyltransferase. Homocysteine can also be used to make glutathione via the transsulfuration pathway in a 2-step process, where by it is converted to cystathionine by cystathionine β-synthase, which is further converted to glutathione by the actions of glutathione S-transferase. Adapted from (Lu, 2000; Mandaviya, *et al.*, 2014).

1.9.2.1.1. The purine salvage pathway

The purine salvage pathway (Fig 1.6.) is a pathway in which pre-formed purine bases can be returned to the nucleotide pool and is the predominant route in the brain (Allsop and Watts, 1980; Barsotti and Ipata, 2002; Gerlach, *et al.*, 1971; Mascia, *et*

al., 2000) and heart (Pauly, *et al.*, 2003; Zimmer, 1998) for the restoration and maintenance of adenine nucleotides.

The enzymes hypoxanthine guanine phosphoribosytransferase (HGPRT, EC2.4.2.8) and adenine phosphoribosyltransferase (APRT, EC2.4.2.7) catalyzes the transfer of the ribose phosphate from PRPP (5-phosphoribosyl-1-pyrophosphate) to free purine bases. PRPP is formed through the actions of PRPP synthetase, which activates carbon 1 of ribose-5-phosphate (Rib-5-P) by transferring it to the pyrophate moiety of ATP and Rib-5-P is an intermediate of the pentose phosphate pathway. HGPRT is found in all tissues and is highly expressed in the brain (Allsop and Watts, 1980; Murray, 1971) where it catalyses the transfer of the Rib-5-P from PRPP to the purine guanine or hypoxanthine producing pyrophosphate (PPi), guanosine monophosphate (GMP) or IMP respectively (Murray, 1971). Similar to HGPRT, APRT is also expressed in the brain (Allsop and Watts, 1980), where it catalyses the PRPP-dependent phosphoribosylation of the purine base adenine to AMP and PPi.

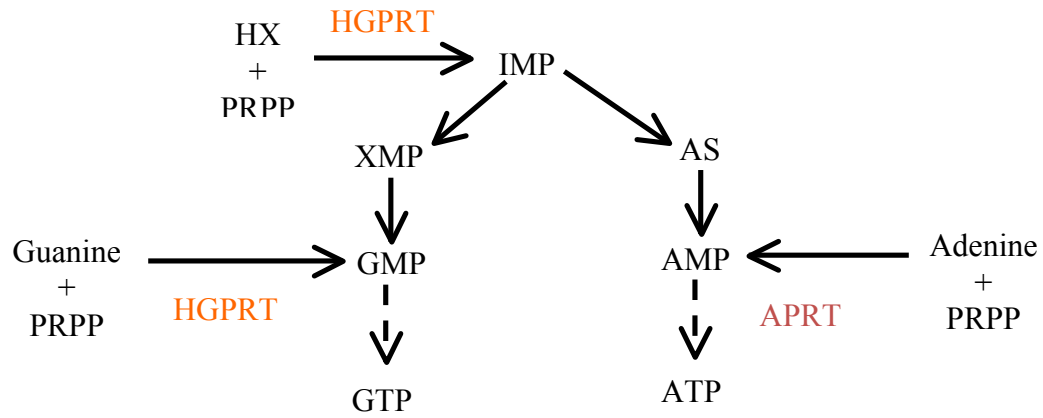

Fig 1.6. Purine salvage pathways. PRPP (5-phosphoribosyl-1-pyrophosphate) is converted to a nucleotide (AMP and GMP) by the addition of a nucleobase (adenine, guanine and hypoxanthine (HX)) through the actions of the phosphoribosyl transferases hypoxanthine guanine phosphoribosytransferase (HGPRT, EC2.4.2.8) and adenine phosphoribosyltransferase (APRT, EC2.4.2.7). IMP- inosime monophosphate; AS- adenylosuccinate; XMP xanthylate.

1.9.2.1. Adenosine release

The regulation of intracellular adenosine concentrations greatly influences the extracellular adenosine concentrations. Tight regulation between intracellular and extracellular concentrations of adenosine are maintained in two ways: i) the transport of adenosine in and out of the cell via equilibrative nucleoside transporters (ENTs), and ii) adenosine kinase which maintains adenosine concentrations in such a way as to drive extracellular adenosine inward.

Extracellular adenosine concentration occurs as a result of direct transport out of the cell by transport proteins (Cunha*, et al.*, 1996; Gu*, et al.*, 1995; Jonzon and Fredholm, 1985; Lovatt*, et al.*, 2012; Wall and Dale, 2013; White and MacDonald, 1990) or as a result of ATP exocytosis (Klyuch*, et al.*, 2012; Wall and Dale, 2013). Adenosine formed intracellularly is transported into the extracellular space mainly by specific bi-directional transporters through facilitated diffusion that maintains homeostatic control between intracellular and extracellular concentrations of adenosine (Cunha*, et al.*, 1996; Gu*, et al.*, 1995; Jonzon and Fredholm, 1985; Lovatt*, et al.*, 2012; Wall and Dale, 2013; White and MacDonald, 1990). Nucleoside transporters are located on both neurones and astrocytes. These comprise equilibrative nucleoside transporters (ENTs; composed of 4 isoforms ENT1-4), as well as concentrative nucleoside transporters (CNTs; composed of 3 isoforms CNT1-3) which are transport proteins capable of maintaining high adenosine concentrations against a concentration gradient. ENTs are Na^+ independent diffusion-limited channels, whereas CNTs are Na^+-dependent transporters (Isakovic*, et al.*, 2008). The ENT1 is widely distributed in the brain where the expression of ENT1 mRNA expression correlates with the distribution of adenosine A_1 receptors (Anderson*, et al.*, 1999).

Extracellular adenosine can arise from rapid extracellular metabolism of ATP (Wall and Dale, 2013). Extracellular ATP and its nucleotides undergo rapid degradation by ectonucleotidases to provide a readily available pool of adenosine. Ectonucleotidases such as ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase and / or phosphodiesterase (E-NPPs) are expressed in the brain (Belcher*, et al.*, 2006; Bjelobaba*, et al.*, 2006; Bjelobaba*, et al.*, 2007; Cognato*, et al.*, 2008; Langer*, et al.*, 2008; Shukla*, et al.*, 2005; Stefan*, et al.*, 2005). In the extracellular space E-NTPDases and E-NPPs hydrolyses ATP and ADP to AMP (Kukulski and Komoszyński, 2003). AMP is further hydrolysed to adenosine by ecto-5'-nucleotidase. Once formed in the extracellular space adenosine can exert its downstream effects by activating its receptors. When extracellular adenosine levels are high it can be transported into the cell by means of transporters. Once inside the cell it can be phosphorylated to AMP by adenosine kinase or degraded to inosine, thereby maintaining an inward gradient for adenosine (Boison, 2006).

1.9.2.2. Adenosine receptors

Adenosine receptors are highly conserved between species and all known four receptor subtypes have been cloned from rodents and humans (reviewed in (Fredholm*, et al.*, 2001a)). The adenosine receptors belong to the G protein-coupled family of receptors (class A). Currently only four types of adenosine receptor have been characterised: adenosine A_1 receptors (A_1R), A_3 receptors (A_3R) A_{2A} and A_{2B} ($A_{2A}R$ and $A_{2B}R$ respectively). Inosine has been shown to be a partial agonist at A_1 and A_3Rs (Fredholm*, et al.*, 2011).

1.9.2.2.1. A_1

Adenosine A_1Rs are highly expressed in brain regions such as the hippocampus and cortex where they couple to G-protein coupled $G\alpha_{i1/2/3}$ and $G\alpha_o$ leading to a decrease in cyclic adenosine 5'-monophosphate (cAMP), increase in PLC, and arachidonate activity. Activation of presynaptic A_1Rs prevents the release of glutamate via presynaptic inhibition of Ca^{2+} channels and direct interference with the vesicle release machinery. Postsynaptically, activation of these receptors leads to activation of K^+ channels coupled with hyperpolarisation. The pre-and post-synaptic actions of adenosine at these receptors will act to reduce the effect of the excitatory glutamate receptor activity coupled with a reduction in Ca^{2+} influx and depolarisation (Cunha, 2001; de Mendonça*, et al.*, 1995; Ribeiro*, et al.*, 2003; Ribeiro*, et al.*, 2002), which would further help to reduce the excitotoxic damage caused by Ca^{2+} influx and the energy demand of cells, thereby helping to preserve ATP levels (Dunwiddie and Masino, 2001). Overall, activation of the adenosine A_1R is associated with the inhibition of the release of many neurotransmitters where the most prominent inhibitory actions are generally on excitatory glutamatergic systems and adenosine A_1R-mediated inhibition of the glutamatergic system has been associated with anticonvulsant effects (Dunwiddie and Masino, 2001; Masino and Boison, 2012; Masino*, et al.*, 2009; Masino, 2013). Studies have shown that A_1R agonist have potent anticonvulsant effects (De Sarro*, et al.*, 1999; Gouder*, et al.*, 2003) where activation of these receptor subtypes lead to a suppression in seizure activity in a mouse model of drug-resistant epilepsy (Gouder*, et al.*, 2003).

1.9.2.2.2. A_{2A} and A_{2B}

The A_{2A} differs to the A_{2B} receptors in that it has a larger COOH end terminal domain. A_{2A}Rs are expressed in high levels in GABAergic neurones and the olfactory bulb but have low level of expression in other brain regions. A_{2A}Rs couple to G-protein coupled $G\alpha_o$ and $G\alpha_s$. Activation of these receptor subtypes and the coupling to its GPCR results in an increase in the production of cyclic AMP (cAMP), which is synthesised from ATP by adenylyl cyclase (Dunwiddie and Masino, 2013; Fredholm*, et al.*, 2001b; Masino and Boison, 2012; Schulte and Fredholm, 2000). In addition A_{2A}Rs also couple to $G\alpha_{olf}$ and $G\alpha_{15/16}$, which is associated with increased IP3. Activation of these receptor subtypes and the coupling to its GPCR results in the enhancement of PLC activity, which in turns catalyses phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG and IP3 further directly influences phosphokinase C (PKC) and intracellular Ca^{2+} levels respectively (Dunwiddie and Masino, 2013; Fredholm*, et al.*, 2001b; Masino and Boison, 2012; Schulte and Fredholm, 2000). The A_{2B}Rs are highly distributed in the large intestine and bladder, with low levels of expression in the brain where they couple to $G\alpha_s$ and (increased cAMP) and $G\alpha_{q/11}$(increased PLC). The activation of the A_2 receptors in particular the A_{2A}Rs, is generally associated with an enhanced release of several neurotransmitters such as glutamate and acetylcholine, in contrast GABA release can either be enhanced or inhibited by A_{2A}R activation (Sebastião and Ribeiro, 1996). A_{2A}Rs modulate many excitatory effects. The activation of these receptor subtypes by its agonist CGS 21680 can also lead to the enhancement of synaptic transmission in the CA1 region of the hippocampus (Sebastião and Ribeiro, 1992). The effects of the A_{2A}R on epilepsy are controversial where both anticonvulsant (De Sarro*, et al.*, 1999; Zhang*, et al.*, 1994) and

proconvulsant (Morgan and Durcan, 1990) effects have been reported. In addition the $A_{2A}R$ antagonist ZM 241385 was found to shorten the duration of epileptiform activity in rat hippocampal slices (Etherington and Frenguelli, 2004). This evidence suggests that the $A_{2A}R$ effects on seizure activity might be region specific.

1.9.2.2.3. A_3R

A_3Rs are mainly expressed in the cerebellum and hippocampus, with low levels of expression in most other brain regions. A_3Rs couple to G-protein G $\alpha_{i2,3}$ where it leads to decreased cAMP levels, and couples to the $G\alpha_{q/11}$ where it leads to increased PLC. Because A_3Rs and A_1Rs both couple to $G\alpha_i$ G-proteins, they are believed to mediate similar responses such as a reduction in transmitter release by blocking transient Ca^{2+} channels, hyperpolarise neurones by increasing K^+ conductance and lower cAMP levels (Fredholm, 2011a).

1.9.2.3. Adenosine and epilepsy

Adenosine was initially observed as a naturally occurring anticonvulsant in 1984 (Barraco*, et al.*, 1984), in which it was found that the anticonvulsant properties were mediated by the adenosine A_1Rs. The A_1Rs function by inhibiting excessive neuronal activity in the epileptic brain. The importance of these receptors types is also supported with genetic mouse models where A_1Rs knockout (A_1Rs KO) mice showed an increased susceptibility to developing seizures and developed lethal status epilepticus (SE) after experimentally-induced brain injury (Kochanek*, et al.*, 2006). These data provide strong evidence for the adenosine A_1Rs as potential therapeutic targets for the treatment of epilepsy.

In addition to the anticonvulsant properties of adenosine, dysfunction in the regulation of adenosine has been implicated in the pathophysiological processes of epilepsy (Dale and Frenguelli, 2009; Dunwiddie, 1999; Świąder*, et al.*, 2014). Any type of injury or stress leads to an acute surge of micromolar levels of adenosine that is beyond normal levels (Adén*, et al.*, 2004; During and Spencer, 1992; Lopatář*, et al.*, 2011). This acute surge in adenosine is a consequence of increased ATP degradation and decreased adenosine clearance (Gouder, 2004; Pignataro*, et al.*, 2008). These high levels of adenosine can then lead to several downstream effects that lead to epileptogenesis such as astrogliosis (Boison, 2010). An increase in $A_{2A}R$ expression and a decrease in A_1R expression on astrocytes influences astrocyte proliferation and may contribute to the development of astrogliosis (Brambilla*, et al.*, 2003; Hindley*, et al.*, 1994; Rathbone*, et al.*, 1991). In animal models of epilepsy the presence of astrogliosis was associated with increased levels of adenosine kinase and the presence of spontaneous seizures (reviewed in (Boison, 2013)).

Astrocytes play an important role in the regulation of extracellular adenosine and ATP. ATP and adenosine is involved in the regulation and coordination of synaptic strength and synaptic networks and any dysfunction in this pathway can have major impact on the system. Astrogliosis also has been linked to adenosine kinase (ADK) levels and ADKs regulation of extracellular adenosine concentrations (Aronica*, et al.*, 2011; Boison, 2010; Boison, 2013; Boison*, et al.*, 2010; Li*, et al.*, 2008). Because of the major role astrocytes and adenosine kinase play in not only the removal of adenosine from extracellular space but also the modulation of the levels of ATP and its nucleotides they may serve as possible targets in the treatment of epilepsy. Indeed, research has shown that in rat hippocampal slices a reduction of basal adenosine tone by overexpressing ADK leads to the development of seizures

(reviewed in (Boison, 2013)). In the CA1 region of the hippocampus ADK was found not to limit the activity-dependent release of adenosine (Etherington*, et al.*, 2009). In addition studies with genetic mice shows that in the hippocampus ADK is an important upstream regulator of adenosine-based homeostatic function of the brain where it exerts control of both the strength and dynamic range of synapses (Diógenes*, et al.*, 2014).

Given that ATP is the primary source of both intracellular and extracellular adenosine, it can be postulated that any change in the ATP pool is likely to influence adenosine production and release. In support of this, experiments have shown that not only can adenosine be released under conditions where ATP levels are preserved by pre-treating slices with creatine (Doolette, 1997), incubating slices with compounds that help to boost ATP levels also result in greater adenosine release (zur Nedden*, et al.*, 2014; zur Nedden*, et al.*, 2011).

1.10. Creatine

Creatine, also known as N-aminoiminomethyl-N-methylglycine, is a guanidine compound first extracted from meat by Michel Eugene Chevreul in 1832 (reviewed by (Salomons and Wyss, 2007)). In humans, the creatine pool is maintained by nutritional intake and endogenous synthesis. Foods such as fresh red meat and fish are high in creatine, at concentrations ranging from 3-10g per kg wet weight (reviewed in (Salomons and Wyss, 2007)). Creatine is absorbed from the small intestines where it enters the portal circulation and gets transported to the liver. The ingested creatine and the creatine made in the liver, is then transported into the systemic circulation and distributed around the body, by crossing the cell membrane via a specific creatine transport system against a large concentration gradient

(Wallimann, *et al.*, 2007; Wallimann, *et al.*, 2011). The creatine transporter is a member of the Na^+-dependent neurotransmitter family comprising of 12 membrane-spanning domains and cDNA clone from human brain places it at ≈ 70.5 kDa in size (Sora, *et al.*, 1994). Creatine is transported with at least 2 Na^+ and 1 Cl^-. Creatine transport is driven by the Na^+ gradient established by the Na^+/K^+-ATPase (Dai, *et al.*, 1999) and can be enhanced by hormones such as insulin that activate the Na^+/K^+-ATPase, which increases the driving force for creatine uptake. An inverse relationship exists between intracellular creatine concentrations and creatine uptake (Dodd, *et al.*, 1999) and an elevation in the extracellular concentration results in an initial increase intracellularly followed by a down regulation. The regulation of creatine transport across the membrane can occur acutely or chronically (reviewed in (Brosnan and Brosnan, 2007)). Acute transport is regulated by changes in creatine concentration, in the Na^+ gradient or changes in the insertion of the transporter into the plasma membrane. Chronic transport is regulated by changes in the level of gene expression, translation or post-translational modifications. On such example is the effect of increased intracellular concentration of creatine on the expression of AMP-activated protein kinase, which would initiate a signalling pathway leading to changes in gene expression (reviewed in (Brosnan and Brosnan, 2007)).

Specific creatine transporters are required for the uptake of creatine into muscle and brain (Fitch, *et al.*, 1968; Rebaudo, *et al.*, 2000). Oral supplementation of creatine to healthy patients showed a dose-dependent increase in blood plasma levels (Harris, *et al.*, 1992). In the brain, it is believed that creatine supply from the circulating blood is limited due to the presence of the blood-brain barrier (BBB), since continuous oral administration of creatine for weeks is needed to raise brain creatine levels (Dechent, *et al.*, 1999; Lyoo, *et al.*, 2003). One study suggests that brain creatine transporters

play a dual role of 1) uptake of circulating creatine across the BBB and 2) the neuronal uptake of creatine that is synthesized in glia (Lunardi*, et al.*, 2006). In contrast, one study suggests that there is little evidence of mRNA for creatine transporters in astrocytes, which are associated with the BBB, and further suggests that the brain receives the bulk of its creatine via endogenous synthesis found in every cell in the CNS that is capable of creatine synthesis (Braissant*, et al.*, 2001). In mammals creatine can be synthesized in tissues such as skeletal and cardiac muscle, spermatozoa, brain, and retina from amino acids arginine, glycine and methionine (Andres*, et al.*, 2008). There are two steps in the biosynthesis of creatine in mammals (Figue 1.7): 1) the formation of guanidinoacetate (GAA) from arginine and glycine catalysed by L-arginine- glycine aminotransferase (AGAT; EC2.1.4.1); 2) methylation of the GAA to form creatine by the enzymatic action of guanidinoacetate-methyltransferase (GAMT; EC2.1.1.2).

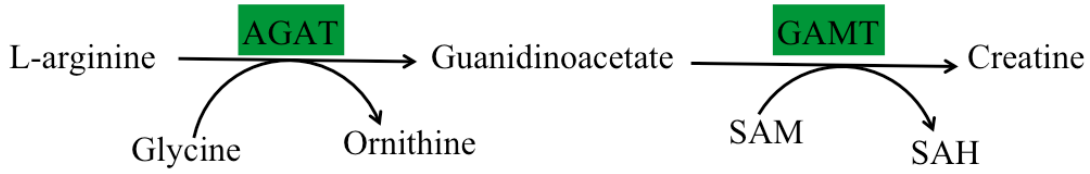

Figure 1.7. Biosynthesis of creatine. L-arginine-glycine aminotransferase (AGAT; EC2.1.4.1 catalyses the amidino group transfer from L-arginine to glycine to form guanidinoacetate and ornithine. Methyl group transfer from S-adenosyly-L-methionine (SAM) to guanidinoacetate to form creatine and S-adenosyl-L-homocysteine (SAH).

Both AGAT and GAMT are found in neuronal subpopulations, astrocytes and oligodendrocytes (Rae and Bröer, 2015). Following absorption, creatine can be excreted as creatinine in the urine. Creatinine is mainly excreted in urine and its daily excretion is directly proportional to total body creatine where it is estimated in muscle mass it is (20–25 mg/kg/24 h) in children and adults and found in lower mass in infants younger than 2 years (Fernandes, 2006).

Creatine can be released in an action potential-dependent manner whereby electrically evoked release of labelled creatine and unlabelled creatine was abolished when Ca^{2+} was omitted from the medium in the presence of inhibition of the Na^{+} channel. Similarly, inhibition of the K^{+} channel with 4-AP enhanced electrically evoked release of labelled and unlabelled creatine (Almeida*, et al.*, 2006). The authors also suggest that this may possibly modulate the function of postsynaptic receptors for neurotransmitters such as GABA.

1.10.1. Creatine kinase (CK)

CK catalyzes the reversible transfer of the N-phosphoryl group from phosphocreatine (PCr) to ADP to generate ATP and stabilizes cellular ATP at approximately 3-6 mM depending on the cell type (Saks and Venturaclapier, 1994; Saks*, et al.*, 1996). In hippocampal slices, incubation with creatine has been found to increase creatine levels without increasing ATP (Lipton and Whittingham, 1982; Okada and Yoneda, 1983; zur Nedden*, et al.*, 2014). Alternatively CK can capture immediately available cellular energy by creating an ATP pool, whereby ATP levels are maintained at the expense of PCr (Doolette, 1997). As a result of this during periods of high energy demand such as those seen during ischemia, pre-treatment of brain tissue with creatine acts to delay the degradation of ATP to its metabolites (Balestrino*, et al.*, 2002; Balestrino M, 1999; zur Nedden*, et al.*, 2014).

In mammals and birds there are three isoforms (isoenzymes) of CK expressed in species-specific, developmental stage-specific and tissue-specific manners. These consist of: (1) CK-M the skeletal muscle isoform (most of PCr is found here); (2) CK-B the brain isoform; and (3) CK-MB the cardiac muscle isoform (reviewed in (Andres*, et al.*, 2008; Wallimann*, et al.*, 2011; Wyss and Kaddurah-Daouk, 2000). CK is expressed in all brain cells but is highly expressed and localised in Bergman

glia and Purkinje cells in the cerebellum, neuronal cells in the hippocampus and epithelial cells in the choroid plexus (Kaldis*, et al.*, 1996; Wallimann and Hemmer, 1994; Wallimann*, et al.*, 2011).

The interplay between cytosolic and mitochondrial CK isoenzymes accomplishes multiple roles in cellular energy homeostasis. Both isoenzymes contribute to the build-up of a large intracellular pool of PCr that represents an efficient temporal energy buffer and prevents a rapid fall in global ATP concentrations upon cell activation or sudden stress conditions (Andres*, et al.*, 2008; Wallimann*, et al.*, 2011).

1.10.2. The CK/PCr system for temporal and spatial energy buffering

The Cr/PCr shuttle provides an attractive means of buffering the ATP/ADP ratio. Creatine is taken up into the cell via creatine transporters and transformed into PCr by either mitochondrial CK coupled to oxidative phosphorylation or by cytosolic CK coupled to glycolysis (Andres*, et al.*, 2008; Wallimann*, et al.*, 2011). The cytosolic CK transphosphorylates glycolytically generated ATP into PCr that is susequently fed into the PCr pool, after which PCr is used to buffer cytosolic ATP/ADP ratios for local ATP consumption. During high workload such as that seen during epileptic seizures, high-phosphocreatine is shuttled from the mitochondria to sites of energy consumption associated with ATP-consuming processes (Andres*, et al.*, 2008; Wallimann*, et al.*, 2011). Creatine is then used by ATPases to regenerate ATP locally *in situ* to fuel these ATP-requiring processes and to keep local ATP/ADP ratios very high. Creatine would then diffuse back into the mitochondria to be recharged again by mitochondrial CK. Mitochondrial CK is located in the intermembrane space of mitochondria and transphosphorylates mitochondrially

generated ATP into PCr, which then leaves the mitochondria via a mitochondrial creatine transporter.

1.10.3. Creatine-associated diseases

New creatine-deficiency syndromes have been recently discovered in humans that affect either endogenous creatine synthesis or creatine transport. Patients with these syndromes have been shown to have an almost complete lack of creatine in the brain and present with severe neurological symptoms such as developmental delay, epileptic seizures, autism and severe mental retardation (Stockler*, et al.*, 2008). Creatine depletion in brain is associated with disruption of neuronal functions and changes in mitochondrial structure (Andres*, et al.*, 2008; Wallimann*, et al.*, 2011). *In vitro studies* using rat hippocampal slices showed that there was an increase in the creatine levels in cells as a consequence of the long-term effects of pilocarpine-induced seizures after repeatedly induced seizures (Dulinska*, et al.*, 2012; Kutorasinska*, et al.*, 2013). These data provide evidence for an increase in creatine to meet energy demands by replenishing ATP from PCr during seizure activity. In addition it should be noted that these studies do not rule out that the increase in creatine deposits could be a consequence of seizure-induced change in CK enzymatic activity. Thus, the increase in creatine deposits may also reflect the role of creatine in the pathological process of epilepsy.

Given creatine's ability to buffer ATP levels and delay its degradation to its metabolites, in this thesis experiments with creatine will be used for comparative purposes with the main focus being on D-ribose and adenine supplementation.

1.11. Ribose/Adenine and the salvage pathway

1.11.1. Ribose

Ribose is the pentose sugar backbone of ATP. Ribose is able to bypass rate-limiting steps of the oxidative pentose phosphate pathway that generates ATP (Zimmer and Gerlach, 1978). The pentose phosphate pathway has both oxidative and non-oxidative branches. The oxidative pentose pathway branch, glucose 6- phosphate (G-6-P) derived from either glycolysis or gluconeogenesis is converted to ribulose 5-phosphate (Ru-5-P) in 3 reactions. G-6-P conversion to Ru-5-P is catalysed by G-6-P dehydrogenase the first and rate limiting enzyme, producing 2 molecules of NADPH. NADPH is used for the synthesis of free fatty acids and for the conversion of oxidised to reduced glutathione.

Ribose is transformed into one of its phosphorylated derivatives such as ribose-5-P (Rib-5-P) part of the pentose phosphate pathway in order to be incorporated into purine nucleotides without cleavage of its ribofuranosidic ring (Fig 1.8.). Ribose phosphate synthesis occurs through two main pathways 1) through the oxidative branch of the pentose phosphate pathway, 2) through the phosphorylitic cleavage of the N-glycosidic bond of ribonucleosides. Rib-5-P and ribose-1-phosphate (Rib-1-P) are the two major pentose phosphates that can be readily interconverted by phosphopentomutase (Camici*, et al.*, 2006; Zimmer, 1996). Pentose phosphates can be used as energy source in their own right in both bacteria and eukaryotes. Rib-5-P is the core structure of ribonuceotides and is mainly synthesised from either glucose-6-phosphate in the oxidative branch of the pentose phosphate pathway or via ribokinase-mediated phosphorylation of free ribose. When used as a source of energy, Rib-5-P through the non-oxidative branch of the pentose phosphate pathway

can be converted to glycolytic intermediates to be used in the glycolytic pathway. Purine nucleoside phosphorylase and uridine phosphorylase are examples of nucleoside phosphorylases that are involved in the synthesis of Rib-1-P. Rib-1-P can be further recycled for nucleoside interconversion via its conversion to Rib-5-P and the pentose phosphate pathway (Camici*, et al.*, 2006; Zimmer, 1996).

In the brain adenine salvage via ribose is a PRPP synthase dependent process (Mascia*, et al.*, 2000). This process starts by the phosphorylation of ribose by ribokinase to produce the ribose phosphate Rib-5-P via the recruitment of deoxy ATP, in a reversible reaction phosphorylation of ATP by PRPP synthase leads to production of PRPP. PRPP plays an integral part in both *de novo* and *salvage* synthesis of nucleotides (Camici*, et al.*, 2006). An increase in PRPP is believed to be involved in ribose-enhanced synthesis of ATP (Barsotti and Ipata, 2002) and this balance between PRPP and ATP is what is believed to play a major part in modulating adenine salvage in the rat brain (Barsotti and Ipata, 2002).

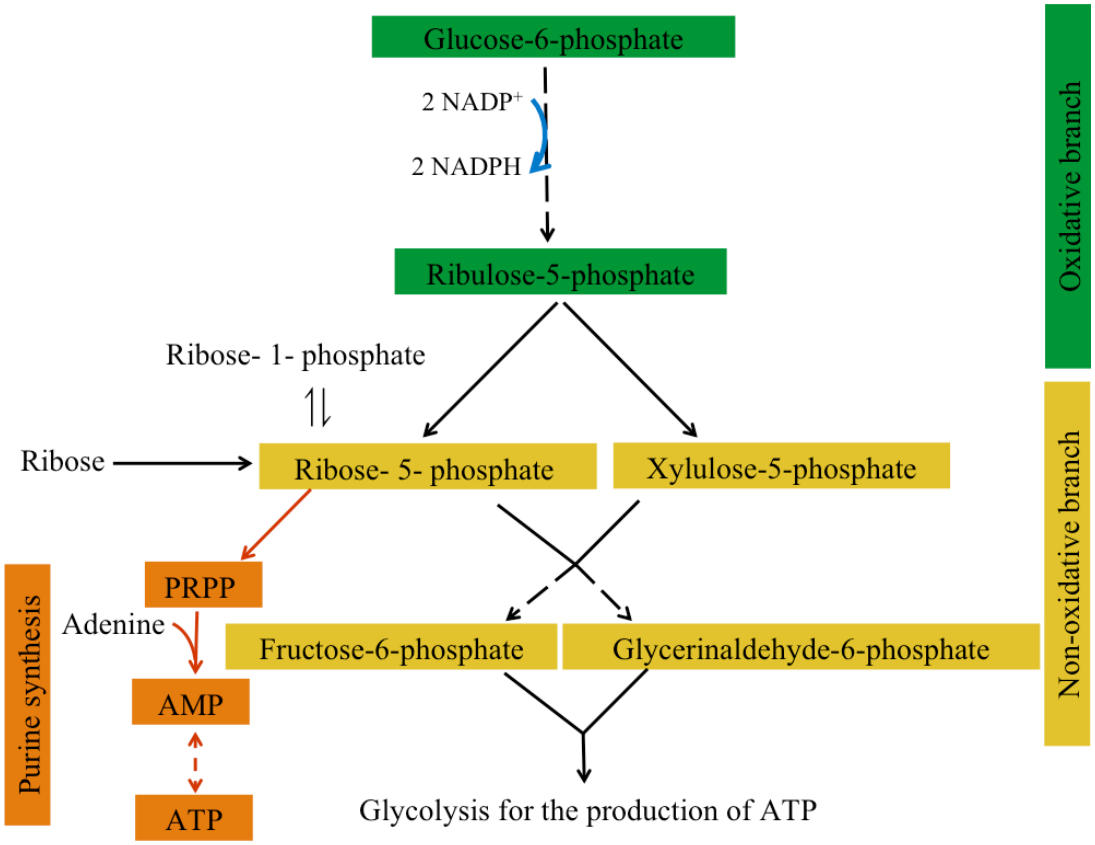

Figure 1.8. Schematic representation of the pentose phosphate pathway and its connection to glycolysis and purine synthesis. Glucose-6-phosphate is converted to ribulose-5-phosphate by glucose-6-phosphate dehydrogenase, gluconolactone and 6-phosphogluconate dehydrogenase in the oxidative branch producing NADPH. In the non-oxidative branch ribulose-5-phosphate is converted to ribose-5-phosphate and xylulose-5-phosphate by ribulose-5-phospahte isomerase and ribuloase-5-phopshate epimerase, respectively. These two pentose sugars are further reconverted to the glycolytic intermediates fructose-6-phosphate and glycerinaldehyde-3-phosphate via two reactions catalyses by transketolase and transaldolase Ribose-5-phosphate can also be synthesised by phosphorylation of ribose by ribokinase. It is then anabolized to PRPP (5-phosphoribosyl-1-pyrophosphate) by PRPP synthase, or interconverted with Rib-1-phosphate by phosphopentomutase. PRPP is subsequently used for purine salvage as an activated sugar donor. Adapted from (Camici*, et al.*, 2006; Zimmer, 1996; Zimmer, 1998)

1.11.2. Adenine

The nucleobase adenine is an important core component of ATP and is derived from the cleavage of methylthioadenosine a byproduct of polyamine synthesis by methylthioadenosine phosphorylase, to adenine and methylthioribose-1-phosphate (Della Ragione*, et al.*, 1986; Ipata*, et al.*, 2011). De novo synthesis of adenine occurs as a by-product of intracellular polyamide synthesis (Kamatani and Carson, 1981). In order to exert its actions at its receptors, adenine must be transported from the cytosol to the extracelluar space (reviewed in (Thimm*, et al.*, 2015)). Plasma concentrations of adenine found in humans range in the nano molar range (31-700 nM) (Marlewski*, et al.*, 2000; Slominska*, et al.*, 2002). Adenine is mainly metabolised by cytosolic APRT to generate nucleotides via the purine salvage pathway (section 1.8.2.1.1.) (Kamatani and Carson, 1981; Murray, 1971). Adenine is more extensively retained in body tissues than orally administered guanine, hypoxanthine and xanthine. When adenine concentrations are high adenine can be partly excreted from the kidneys without being metabolised (Bartlett, 1977). If metabolised, adenine can be metabolised to 2,8-dihydroxyadenine (DHA) by xanthine oxidase, which can precipitate in the kidney and cause damage (reviewed in (Claramunt*, et al.*, 2015)).

Adenine receptors (AdeRs) were originally identified in rat (Bender*, et al.*, 2002), since then two adenine receptors have been characterised in mice mAde1R and mAde2R and one receptor in the Chinese hamster cAdeR (Thimm*, et al.*, 2013; von Kügelgen*, et al.*, 2008). Although only two AdeR have been characterised, pharmacological data indicate that other receptor types might exist in humans and pigs (Borrmann*, et al.*, 2009; Wengert*, et al.*, 2007). Adenine receptors have been classified as P0 receptors and are part of the GPCR super family (class A), and all

known adenine receptors have been found to be G_i coupled (reviewed in (Thimm, *et al.*, 2015). Adenine receptors are highly expressed in the nervous system and in the rat moderate to high expression has been found in the heart testes, kidney and spleen (Bender, *et al.*, 2002; von Kügelgen, *et al.*, 2008; Watanabe, *et al.*, 2005).
AdeRs have been shown to be involved in the normal physiological and pathophysiological function of the kidney where they have been shown to inhibit Na^+/K^+-ATPase activity in functional experiments using isolated pig proximal tubule (Wengert, *et al.*, 2007).

1.11.3. Ribose/Adenine as a possible therapeutic treatment of epilepsy

Therapeutic use of ribose has been studied extensively in the cardiac system (Brookman and St Cyr, 2010; Omran, *et al.*, 2003; Pauly, *et al.*, 2003; Pauly and Pepine, 2000; Perkowski, *et al.*, 2011; Pliml, *et al.*, 1992). Ribose has also been used as a metabolic supplement where administration of oral D-ribose in patients with severe coronary artery disease improved tolerance to temporary ischaemia induced by exercise tests (Pliml, *et al.*, 1992). Additionally, studies have shown that ATP repletion during recovery from reversible ischaemia is enhanced if nucleobases such as adenine are co-administered with ribose (Zimmer, 1996; Zimmer, 1998). In human adenylosuccinase deficiency, an autosomal recessive disorder is associated with psychomotor retardation, epilepsy and in some cases autistic features (Stone, *et al.*, 1992; Verginelli, *et al.*, 1998). Administration of ribose led to a reduction in seizure frequency (Salerno, *et al.*, 1999). However, the effect of ribose as a treatment of epilepsy was not supported by studies conducted by (Jurecka, *et al.*, 2008) where

ribose treatment was not found to be effective in the treatment of epilepsy. Given the conflicting results further work is needed to clarify the effects of ribose as a treatment for epilepsy.

Adenine has also been administered in humans. However, because adenine can be metabolised by xanthine oxidase (Bendich*, et al.*, 1950), and the by-product DHA can then lead to the development of kidney stones (Van Acker*, et al.*, 1977), adenine is given with the xanthine oxidase inhibitor allopurinol (Edvardsson*, et al.*, 1993; Greenwood*, et al.*, 1982; Simmonds, 1986) to prevent the formation of kidney stones. Adenine and allopurinol have been given for the treatment of metabolic disorders (Balasubramaniam*, et al.*, 2014; Jinnah*, et al.*, 2013). Although adenine has yet to be found to provide any beneficial effects in the treatment of metabolic disorders, other than a noted acceleration in growth in a patient with adenylosuccinate lyase deficiency, no adverse effects are yet to be reported. The use of ribose/adenine as a treatment for epilepsy needs to further investigated and will be the main focus of this thesis.

Previous studies from our laboratory have shown that a loss of ATP precursors is responsible for the decreased ATP content of brain slices. By supplementing the artificial cerebrospinal fluid (aCSF) with a combination of (1 mM) D-ribose and (50 μM) adenine (RibAde), the recovery of tissue ATP levels can be improved. This improvement of ATP levels also resulted in an increased activity-dependent release of adenosine and, via activation of adenosine A_1Rs, the threshold for the induction of long-term potentiation was raised (zur Nedden*, et al.*, 2011). We have also shown that it is possible to modulate the decline and recovery of the intracellular ATP associated with metabolic stress by increasing the pre-oxygen glucose deprivation (pre-OGD) tissue ATP levels with RibAde or by buffering ATP metabolism with

creatine. Creatine treatment was found to delay the degradation of ATP, sustained energy charge during OGD, maintained post-OGD ATP levels and resulted in a nearly complete recovery of synaptic transmission after OGD, which might be associated to the reduction in adenosine release found in creatine-treated slices (zur Nedden*, et al.*, 2014). We also showed that in contrast, RibAde treatment resulted in increased tissue ATP levels under basal conditions and after OGD, increased adenosine release and improved cell viability even when administered in the post-OGD period (zur Nedden*, et al.*, 2014).

Ribose/Adenine salvage (Fig 1.9.) may be an effective strategy for the improvement of cell survival and function and may have therapeutic implication in the treatment of epilepsy.

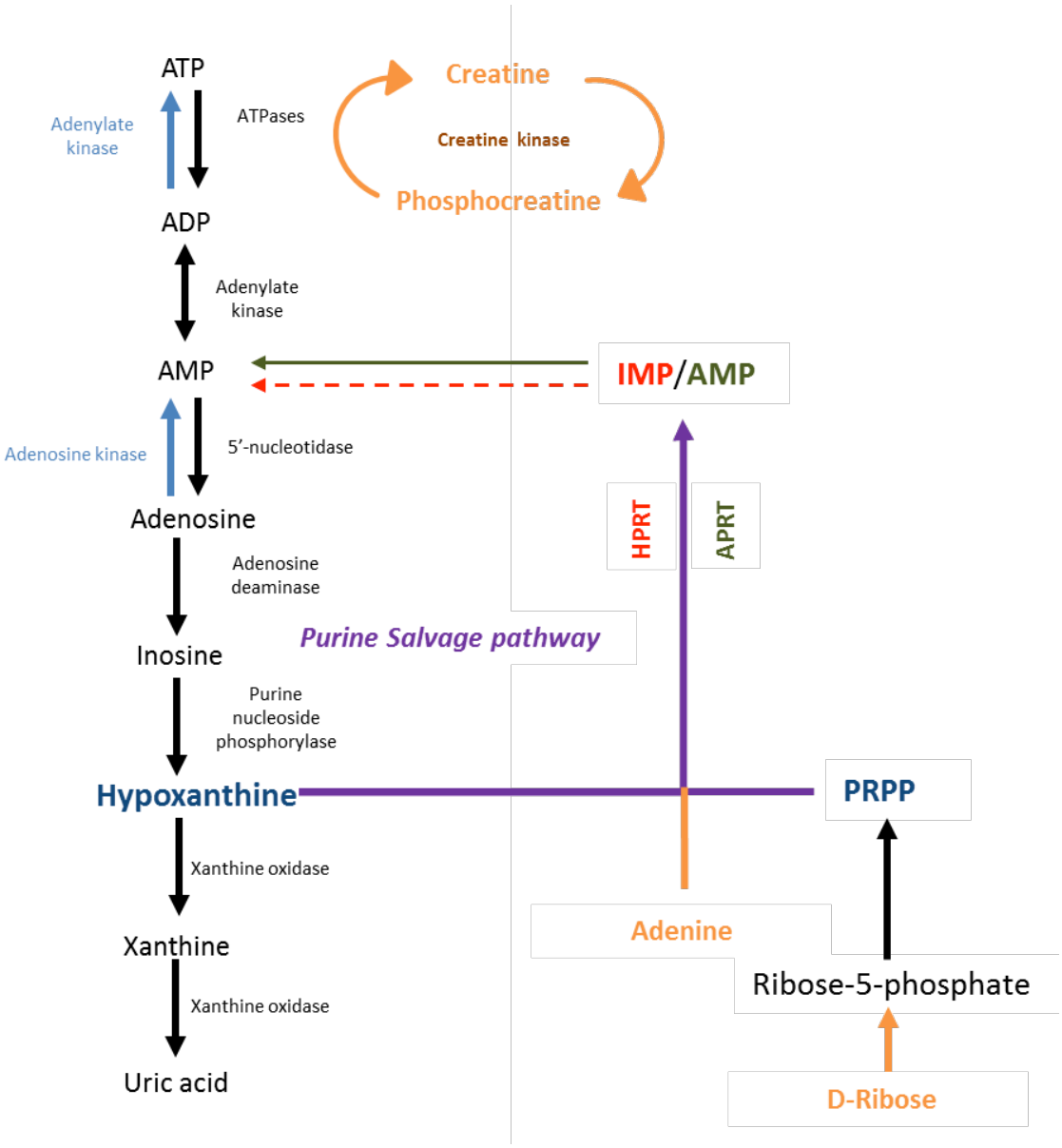

Fig 1.9. Schematic representation of ribose and adenine salvage for the synthesis of purine nucleotides and its connection to the ATP degradation pathway. Exogenously applied ribose and adenine (RibAde) helps to restore the ATP pool in the brain. The purine-salvage pathway re-uses hypoxanthine and adenine to restore purine nucleotides. Exogenous application of creatine helps to delay the breakdown of ATP via the actions of creatine kinase. ATP- adenosine triphosphate; ADP- adenosine diphosphate; AMP- adenosine monophosphate; IMP- inosine monophosphate; PRPP- 5-phosphoribosyl-1-pyrophosphate; HPRT- Hypoxanthine-guanine phosphoribosyltransferase; APRT- Adenine phosphoribosyltransferasess.

1.11. Aims

In this thesis I test the influence of modulation of intracellular ATP on adenosine release during seizure activity

Firstly, I test the hypothesis that pre-incubating rat hippocampal slices with RibAde and creatine influences intracellular adenosine levels and thus the activity-dependent release of adenosine. Additionally, I investigate the effect of these compounds on seizure activity. I also measure the release of adenosine during seizure activity using enzyme-based microelectrode biosensors and test if this release of adenosine acts via the A_1Rs to modulate seizure activity.

Two models of seizure activity were utilised: 1) Mg^{2+}-free aCSF with increased K+ concentrations (6 mM and 9 mM); and 2) Mg^{2+}-free aCSF with 4-aminopyridine (50 μM; 4-AP).

2. Materials and Methods

2.1. Drugs and Chemicals

Creatine, D-ribose, adenine, 8-cyclopentyltheophylline (CPT), 4-aminopyridine (4-AP), hydroxytryptamine hydrochloride (5-HT), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261) and L-homocysteine thiolactone hydrochloride (HTL) were purchased from Sigma Aldrich. CPT was dissolved in 0.1 M NaOH. All salts for the aCSF for hippocampal slice experiments were purchased from Fisher Scientific.

2.2. Preparation of hippocampal slices

17-23 day old male Sprague-Dawley rats were used and killed by cervical dislocation and then decapitated in accordance with Schedule 1 the UK Government Animals (Scientific Procedures) Act 1986 and with local Ethical Review procedures. The brain was quickly removed and placed in ice-cold aCSF containing (mM): NaCl (124); KCl (3), $CaCl_2$ (2), $NaHCO_3$ (26), NaH_2PO_3 (1.23), D-glucose (10), and $MgSO_4.7H_2O$ (1) with an additional $MgCl_2$ (10); pH 7.4. Parasagittal hippocampal brain slices (400 μm) were cut on a vibratome (Microm HM 650 V microtome) (zur Nedden*, et al.*, 2011) and kept at 34 °C gassed with 95% O_2/ 5% CO_2 for at least 3 hours before use in an incubation chamber (100 or 250 ml) (Edwards*, et al.*, 1989). Slices were incubated in either standard 1 mM Mg^{2+}-containing aCSF or in a standard aCSF containing either 1mM Creatine aCSF or 50 μM adenine + 1mM D-ribose (RibAde).

2.3. Electrophysiology recordings and drug application

Post recovery (at least 3 hrs), individual slices were placed on a submerged mesh in a recording chamber, secured with a platinum harp with nylon threads and

were perfused with oxygenated aCSF at a rate of ~ 6.0 mL/min and maintained at 32.3 ± 0.5 °C. Slices underwent a period of electrical stimulation of the Schaffer collateral-commissural pathway in hippocampal area CA1 prior to experiments to ensure viability of the slice, stability of the recording and to measure the effects of drugs on basal synaptic transmission. All pharmacological agents were applied at the desired concentration via the aCSF. A twisted bipolar Teflon-coated tungsten wire (50 μm diameter) electrode (100 μm overall diameter) was placed in the stratum radiatum of the CA1 region of the hippocampus. Extracellular field excitatory postsynaptic potentials (fEPSP) were evoked using square-wave pulses (0.1 ms in duration) at 10 s intervals. A glass microelectrode filled with aCSF described previously (Frenguelli*, et al.*, 2007; zur Nedden*, et al.*, 2011) was used to record fEPSPs (both evoked and spontanueous) and was also placed in the stratum radiatum of the CA1 region of the hippocampus. Constant current stimulation was given over the range of (50-300 μA) and the current eliciting a fEPSP of approximately half the maximal slope was used for the remainder of the experiment. Stimulus parameters and acquisition and analysis of fEPSPs were under control of LTP software (Anderson and Collingridge, 2001). Once stability and tissue viability was established a baseline of 10 minutes was recorded, after which 0 mM Mg^{2+} aCSF was applied. All drugs were added to the 0 mM Mg^{2+} aCSF. Once in 0 mM Mg^{2+} aCSF increases in fEPSP slope were allowed to asymptote (typically 15 min). Electrical stimulation either persisted throughout the experiment or was switched off to allow spontaneous seizures to be observed. Recordings lasted between 2-3.5 hours.

2.4. 6 mM and 9 mM K^+ recordings

Many previous studies have shown that an increase in extracellular potassium ion (K^+) concentration is sufficient for inducing seizures. In these experiments increases in K^+ in the aCSF was used to induce seizures. To investigate the effect of increases in K^+ concentration on seizure activity, K^+ concentration was increased to 6 mM and 9 mM K^+. Experiments were carried to investigate whether these concentrations of K were capable of producing seizures and to characterise the different seizures produced at these concentrations.

2.5. 4-aminopyridine recordings

4-aminopyridine a voltage-gated potassium channel blocker, was used in these experiments to induce spontaneous seizures in hippocampal slices. 50 μM 4-AP was applied after washout of Mg^{2+} from slices. In some experiments to assess the role of the adenosine A_1 and A_{2A} receptors on seizure activity the receptor antagonists CPT (A_1 receptors) and SCH 58261 (A_{2A} receptors) were applied first in 0 mM Mg^{2+} after which CPT (1 μM) + 4-AP or SCH 58261 (50 nM) + 4-AP was applied and seizure activity was measured.

2.6. Homocysteine thiolactone recordings

Homocysteine thiolactone (HTL) was used in these experiments as a method for trapping intracellularly formed adenosine as S-adenosylhomocysteine thiolactone (SAHTL). 100 μM HTL was added to both standard aCSF and 0 mM Mg^{2+} aCSF then bath applied according to the same protocol used for 50 μM 4-AP/ 0 mM Mg^{2+} seizure model.

2.7. Continuous RibAde recordings

Continuous application of RibAde to bath solutions was used in these experiments to test whether continually perfusing slices with RibAde produced any changes in the release of adenosine. RibAde prepared as indicated above was added to both standard aCSF and 0 mM Mg^{2+} aCSF then bath applied according to the same protocol used for 50 μM 4-AP/ 0 mM Mg^{2+} seizure model. Continuous application of RibAde aCSF to the adenosine biosensor produced a response that is due to purine nucleoside phosphorylase metabolism of adenine, which gives rise to the production of inosine that is then detected by the adenosine biosensors. This finding is in line with what has been previously shown where purine nucleoside phosphorylase shows intrinsic activity to adenine in the presence ribose (Zimmerman*, et al.*, 1971). In this thesis the effect of continuous application of RibAde on adenosine biosensor response was measured. New microelectrode biosensors were used and calibrated with 10 μM and 100 μM adenosine in standard aCSF (Fig 2.1 A). After which, the application of RibAde aCSF was found to produce a rapid increase in current on the adenosine biosensor (Fig 2.1 B) that decreased over time on both the adenosine and null biosensors. This response to RibAde aCSF was greater than that recorded for the 10 μM adenosine calibration but less than that for the 100 μM adenosine calibration in standard aCSF. Following 4 hours in RibAde aCSF, calibrations with 10 and 100 μM adenosine were made in RibAde solutions (Fig 2.1 C). In comparison to the calibration made at the start in standard aCSF, the response with 10 μM adenosine was slightly smaller than the minimum threshold value of 1 nA used for previous experiments, therefore the mimimum threshold value was relaxed to 0.9 nA. Overall, these data suggest that prolonged exposure of the adenosine biosensor to RibAde-

containing bathing solutions was capable of producing a response that did not mask the effect of additional adenosine release.

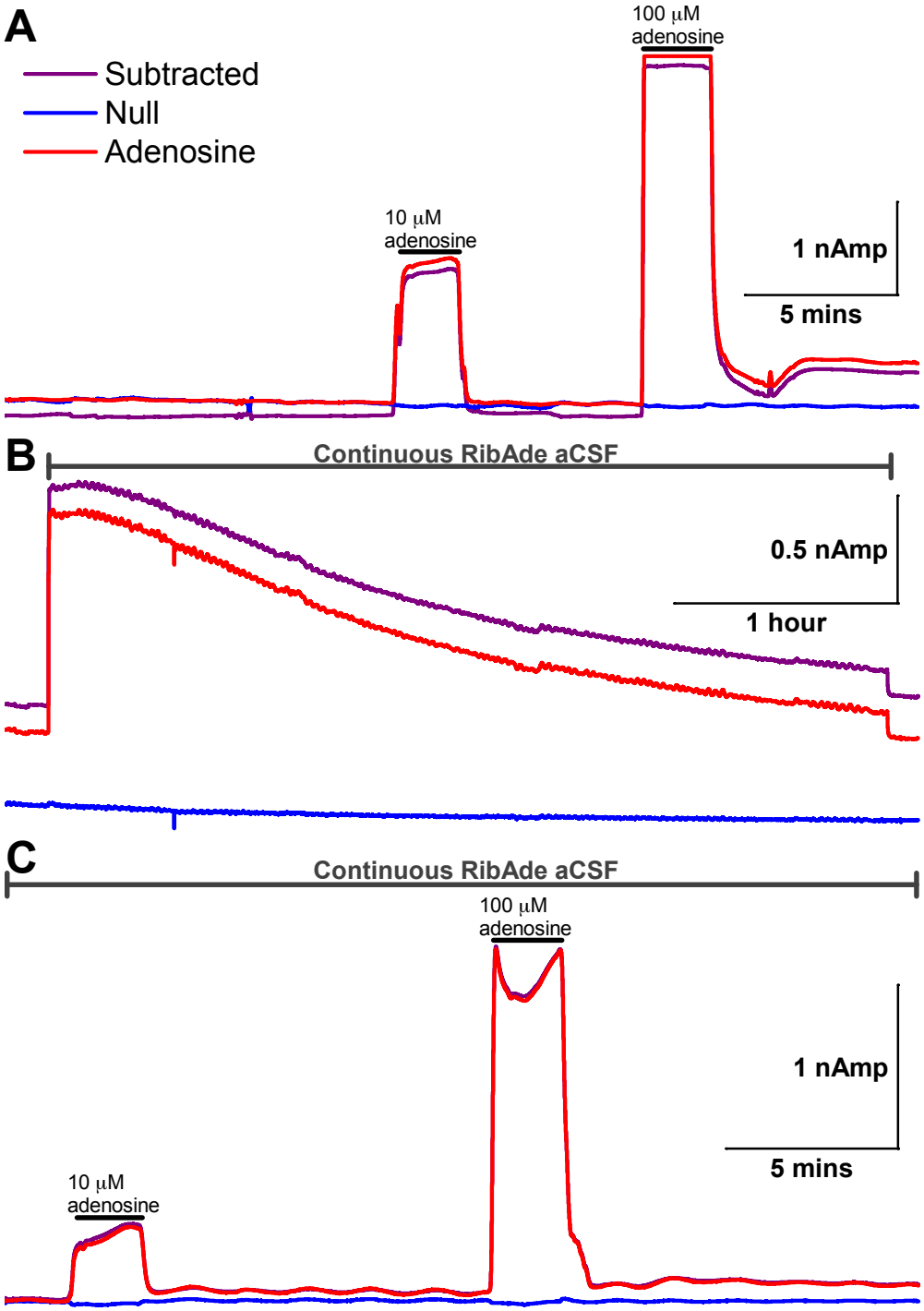

Figure 2.1. Real-time measurements of RibAde bathing solution on microelectrode biosensors. Calibrations with new biosensors (n = 1) were made in normal bathing solutions in 10 and 100 μM adenosine (A). Once a stable baseline was established (B) bathing solutions containing 1 mM ribose and 50 μM adenosine (RibAde) was washed on for 4 hours (n = 2). RibAde bathing solution produced a rapid increase in current measured on the adenosine biosensor Note the decrease in current over time on both the adenosine and null biosensors. Calibrations were made after 4 hours in RibAde bathing solution (C) with 10 and 100 μM adenosine (n = 1). A response to 10 and 100 μM adenosine was not masked by the presence of the RibAde solution.

2.8. Biosensor recordings

Microelectrode biosensors have been routinely used *in vivo* and *in vitro* to accurately measure the release of various analytes including adenosine (Dale and Frenguelli, 2012; Dale*, et al.*, 2002; Dale*, et al.*, 2000; Etherington*, et al.*, 2009; Frenguelli*, et al.*, 2003; Frenguelli and Wall, 2016; Frenguelli*, et al.*, 2007; Gourine*, et al.*, 2007; Gourine*, et al.*, 2002; Lindquist and Shuttleworth, 2014; Llaudet*, et al.*, 2003; Lopatář*, et al.*, 2011; Wall*, et al.*, 2010; zur Nedden*, et al.*, 2011). The benefits of using microelectrode biosensors over more traditional microdialysis probes is that biosensors give real-time measurements that are sensitive and stable in both *in vivo* and *in vitro* preparations and respond to adenosine concentrations from 100 nM to 20 μM (Dale and Frenguelli, 2012; Dale*, et al.*, 2002; Dale*, et al.*, 2000; Etherington*, et al.*, 2009; Frenguelli*, et al.*, 2003; Frenguelli and Wall, 2016; Frenguelli*, et al.*, 2007; Gourine*, et al.*, 2007; Gourine*, et al.*, 2002; Lindquist and Shuttleworth, 2014; Llaudet*, et al.*, 2003; Lopatář*, et al.*, 2011; Wall*, et al.*, 2010; zur Nedden*, et al.*, 2011).

Adenosine and null biosensors were used in these experiments to measure real-time release of purines. Adenosine and null biosensors (Pt/Ir wire of 50 μm in diameter and 500 μm in length) were purchased from Sarissa Biomedical Ltd (Coventry, UK) and were inserted into the slice in area CA1. The biosensors contain specific enzymatic cascades able to break down adenosine to produce hydrogen peroxide. The enzymes (xanthine oxidase, purine nucleoside phosphorylase and adenosine deaminase) are deposited on the screening layer where they are entrapped in a polymer matrix as described in (Frenguelli and Wall, 2016; Llaudet*, et al.*, 2003). Due to the nature of the enzymatic cascade

present in the adenosine biosensors they are also capable of detecting not only adenosine but also its metabolites inosine and hypoxanthine. Therefore, the values provided here not only reflect adenosine but also its metabolites. The hydrogen peroxide is oxidised on the surface of the polarised Pt/Ir wire to give a rise to a current linearly related to the concentration of the measured analyte.

Null sensors contain no enzymes and measure only non-specific electroactive signals. After each experiment, sensors were withdrawn from slices and calibrated with 10 μM adenosine. The values from adenosine biosensors are given as micromolar prime (μM′) to reflect that the adenosine signal is a composite signal of adenosine and its metabolites. The release of adenosine was measured over a given time period of 15 minutes in zero Mg^{2+}, after the appearance of 3 bursts or 10 minutes in 4-AP and 10 minutes in CPT, to account for this the integral measurements are given as μM′s. A 10 μM serotonin solution was also used to assess the patency of the electro-active interferent screening layer of the sensors. Biosensor measurements were only accepted and further processed if serotonin response did not exceed 150 pA. The current response of the simultaneously recorded null sensors was subtracted from adenosine signal to reveal net purine signal. Biosensor reading was taken once the release had stabilized.

2.9. Data analyses

Traces of extracellular recordings were rectified, the number of bursts was counted and inter burst interval (IBI) was calculated. Within a burst the inter spike interval (ISI) was calculated and duration of the burst showing tonic (ISI < 0.09) and clonic (ISI > 0.1). Bursting seizure activity was defined as synchronous bursting seizures with periods of quiescence (IBI). The IBI is defined as the time

between seizures. The end of seizure is taken when the Inter Spike Interval ISI is less than 1.5 second apart. The ISI is the time between spikes. Basal synaptic transmission measurements were made, which included the measurement of slope for paired pulses given 50 ms apart, fibre-volley amplitude and slope measurements during input/output curves at 50 μA increments from 50-300 μA. A paired-pulse interval of 50 ms was chosen because it produced maximal paired-pulse facilitation in slices treated with the same concentrations of ribose and adenine used in this thesis (zur Nedden*, et al.*, 2011). The maximum percentage change in fEPSP slope from baseline time during washout of Mg^{2+} from slices was measured. The area under the curve was measured for the biosensor data for a given measurement and the values expressed as μM´ s.

2.10. Statistical analysis

Values are expressed as mean ± SEM. N values represent number of slices per condition, which is equivalent to the number of animals per condition. If more than two groups were to be assessed, a one-way ANOVA was used. Where interactions were found, post hoc Bonferroni Tests were made. For comparison of fEPSP slope during input/output curves repeated measures ANOVA was applied. A Kolmogorov-Smirnov test was used to investigate differences in the distribution of ISI between treatments. For comparison of ISI before and after application of different drugs a paired t-test was applied. Graphs were drawn and statistical analyses were performed in OriginPro 8.5 software. Statistical significance was taken as $p < 0.05$.

3. Optimisation of a rat hippocampal seizure model of human temporal lobe epilepsy

3.1. Introduction

Animal models of epilepsy are useful in elucidating the underlying mechanisms of epileptogenesis and aid in the development and screening of novel treatments. Many different activity patterns are termed epileptiform and this makes it difficult to make comparisons between different epilepsy models. *In vitro* models of epilepsy play an important role in allowing investigators to validate approaches and compare similarity between in vitro epileptiform activity and in vivo findings. Most models of epileptic seizures rely on activity similar to seizures observed *in vivo*. Such equivalents or seizure-like events (SLEs) are characteristic changes in electrical activity and basal ionic net flow, measured either extracellularly or intracellularly. Acute brain slices, typically 200-600 μmeter thick are the most commonly used *in vitro* preparation for studying epileptic seizures (Coppola and Moshé, 2012; Galanopoulou, 2011; Pitkänen*, et al.*, 2005). One historical seizure model involves lowering the magnesium (Mg^{2+}) concentration (Dreier and Heinemann, 1991; Gloveli*, et al.*, 1995; Heinemann and Lux, 1977) to induce recurrent short electrical discharges (Anderson*, et al.*, 1986). Other seizure models, such as increasing the extracellular potassium (K^{+}) concentration, can be administered independently or combined with lowering the Mg^{2+} concentration (Balestrino*, et al.*, 1986; McNamara, 1994; Poolos and Kocsis, 1990; Poolos*, et al.*, 1987). Low Mg^{2+} solutions act by lowering neuronal firing threshold by modifying the actions of ion channels such as the NMDA receptor, K^{+} channels and voltage gated calcium channels (Coan and Collingridge, 1985; Herron*, et al.*, 1985a; Mody*, et al.*, 1987; Nowak*, et al.*, 1984; Stanton*, et al.*, 1987; Traub*, et al.*, 1994). Low Mg^{2+} solutions also act to reduce the surface charge screening of the membrane which would facilitate the activation of voltage-gated channels such as voltage gated calcium channels (Heinemann*, et al.*,

2005). The reduced surface charge screening at these channels could then go on to facilitate membrane depolarization and the generation of an action potential (Hartzell and White, 1989; Mody*, et al.*, 1987). In addition, by eliminating extracellular Mg^{2+}, the Mg^{2+} block of the NMDA receptor is effectively removed. Removal of the Mg^{2+} block allows for the activation of the NMDA receptor at resting membrane potentials, which would typically return to their resting state after activation, however, when Mg^{2+} is removed, this allows for repetitive depolarization of the membrane potential. Repetitive depolarization of membrane potential is sufficient to initiate and sustain seizure activity (Akiyama*, et al.*, 1992); indeed studies have implicated NMDA receptors in an increase in seizure susceptibility (Dingledine*, et al.*, 1986; Herron*, et al.*, 1985b; King and Dingledine, 1986; Nowak*, et al.*, 1984). Nominally Mg^{2+}-free solutions have been shown to enhance glutamatergic excitation, whereas high K^+ solutions act to increase intrinsic excitability. Using solutions where Mg^{2+} concentrations are lowered and K^+ concentration is increased should therefore act together to promote neuronal excitation. Studies have shown that stimulated and spontaneous epileptiform activity can be induced in the presence of low Mg^{2+} by the simultaneous application of elevated K^+ (3.3 – 7 mM) (Anderson*, et al.*, 1986; Balestrino*, et al.*, 1986; Dreier and Heinemann, 1991; Jones and Heinemann, 1988; Lopatář*, et al.*, 2011; Mody*, et al.*, 1987; Poolos and Kocsis, 1990; Poolos*, et al.*, 1987; Reddy and Kuruba, 2013; Stanton*, et al.*, 1987; Walther*, et al.*, 1986; Zhang*, et al.*, 1995). These studies give support for the use of 6 mM K^+ as a viable method for inducing spontaneous epileptiform activity, as a further comparison 9 mM K^+ was used as a means of producing a more robust epileptiform activity in this thesis. Subsequent research shows that the use of a low Mg^{2+} solution produces seizure activity that are similar to electrographic seizures observed in

humans and pharmacologically induced *in vivo* seizures in rat hippocampus slice. The role of adenosine in epilepsy has been extensively researched and studies have shown that incubation with high concentrations of K^+ such as 6 mM increases the spontaneous release of adenosine in slices (Lopatář*, et al.*, 2011; Shimizu*, et al.*, 1970).

The experiments carried out in the present study used the nominally Mg^{2+} free model in combination with either increasing the extracellular K^+ concentration or using the K^+ channel blocker 4-aminopyridine (4-AP).

3.2. Results

3.2.1. Nominally Mg^{2+} free model: test of model efficacy and parameters

3.2.1.1. 6 mM and 9 mM K^+/ 0 mM Mg^{2+}-induced seizures

Increasing K^+ concentration through bath application in slices has been routinely used to model human TLE (Pitkänen*, et al.*, 2005). Here, we found that increasing the K^+ concentration to 6 mM or 9 mM, failed to produce consistent reproducible spontaneous bursting seizures (Fig 3.1 middle panels; Table 3.1), this was associated with a low sample size for analysis of seizure parameters (Appendix 1). Percentages of observed seizure activity in slices treated with 6 mM / 9 mM K^+ are summarised in Table 3.1. All slices showed some form of activity. However, a large percentage of slices showed non-bursting seizure activity (bursting activity is defined in chapter 2.9). Seizure activity observed with the increased K^+ model also included slices showing inter-ictal activity (Fig 3.1; top panels) and status epilepticus (bottom panels).

A chi-square test of independence was carried out on the data (raw numbers) in Table 3.1. For the 6 mM K^+ data chi-square was found to be 13.098 and *p*-value = 0.011. This indicates that there is a relationship between treatment and the type of epileptiform activity observed.

For the 9 mM K^+ data set chi-square was found to be 1.629 and *p*-value = 0.804, suggesting that there is no relationship between treatment and the type of epileptiform activity observed.

Due to the inconsistencies in recorded seizures in both 6 mM and 9 mM K^+/0 mM Mg^{2+} seizure model for all the three treatment groups, a new approach was explored that would increase the reproducibility of seizures.

Table 3.1. Summary of seizure activity observed in 6- and 9mM K^+.

[K^+]	Treatment	Inter Ictal (%)	Bursting (%)	Status Epilepticus (%)	Total number of slices
6	Creatine	0	35 (n = 6)	65 (n = 11)	17
	Control	6 (n = 1)	76 (n = 13)	18 (n = 3)	17
	RibAde	33 (n = 6)	28 (n = 5)	39 (n = 7)	18
9	Creatine	0	76 (n = 13)	24 (n = 4)	17
	Control	16 (n = 3)	58 (n = 11)	26 (n = 5)	19
	RibAde	8 (n = 1)	69 (n = 9)	23 (n = 3)	13

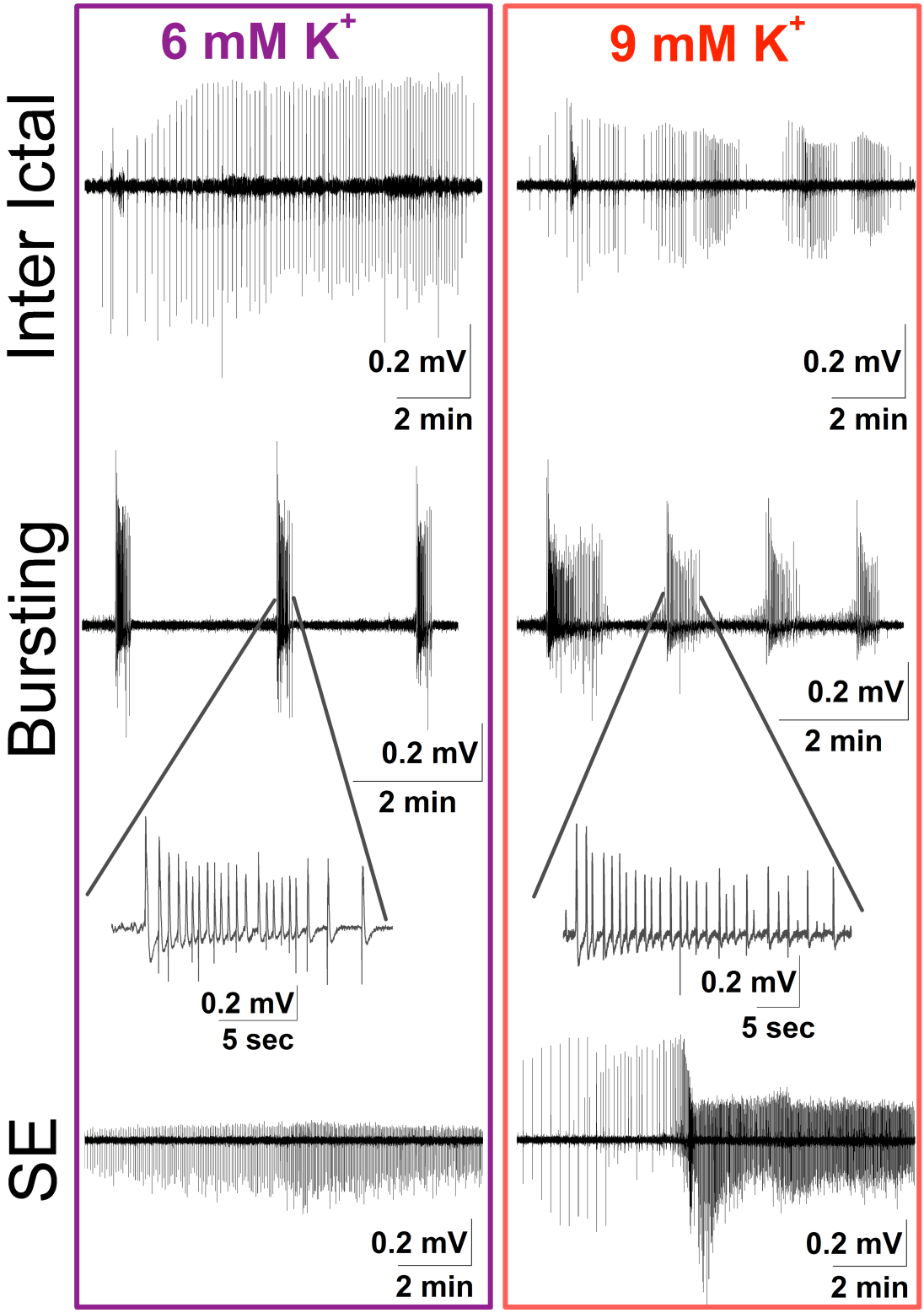

Fig 3.1. Representative seizure activity in 6 mM and 9mM K^+/zero magnesium model. Representative traces of seizures activity in control slices following challenge with 6 m and 9 mM K^+. Top panels show observed inter ictal seizure activity, defined as spiking frequency with intervals greater than 1.5 s. Middle panels show bursting seizure activity, defined as sequential seizure bursts with periods of quiescence between each burst. Zoom in shows burst 2 epileptiform activity. Bottom panels show status epilepticus (SE) activity, defined as prolonged intense spiking that lacks periods of quiescence.

3.2.1.2. 50 µM 4-aminopyridine/zero Mg^{2+}-induced seizures

The other model we use in the present study employs a K^+ channel blocker, 4-aminopyridine (4-AP). The blockade of voltage-gated presynaptic K^+ channels with 4-AP acts to lengthen action potential duration leading to further increases in the influx of extracellular calcium through voltage-sensitive calcium channels into the pre-synaptic terminal (Rudy, 1988; Thesleff, 1980). It is this increase in pre-synaptic calcium concentration that results in an enhanced release of neurotransmitters such as glutamate from the presynaptic terminal (Schechter, 1997; Somjen, 2002; Thompson*, et al.*, 2008).

In order to have a robust model to test the effects of creatine and RibAde on epileptiform activity, it was important to be able to record consistent reproducible epileptiform activity in brain slices. In the present study 4-AP at a concentration of 50 µM produced consistent reproducible epileptiform activity (Table 3.2; Fig 3.2). Initial experiments showed that the 4-AP model produced more consistent bursting seizure activity where all treatments showed 84-100% bursting activity (Table 3.2) compared to 28-76% with the high K^+ model. Chi-square test of independence on data from Table 3.2 showed that chi-square = 0.320 and *p*-value = 0.988, suggesting that there is no relationship between treatment and the type of epileptiform activity observed.

It should also be noted that the 4-AP model resulted in bursting seizures that were of longer durations than that in the high K^+ model.

Table 3.2. Summary of seizure activity observed in 50 µM 4-aminopyridine.					
Type of seizure	**Treatment**	**Inter Ictal (%)**	**Bursting (%)**	**Status Epilepticus (%)**	**Total number of slices**
Spontaneous	**Creatine**	0	100 (n = 10)	0	10
	Control	8 (n = 1)	84 (n = 10)	8 (n = 1)	12
	RibAde	8 (n = 1)	84 (n = 10)	8 (n = 1)	12

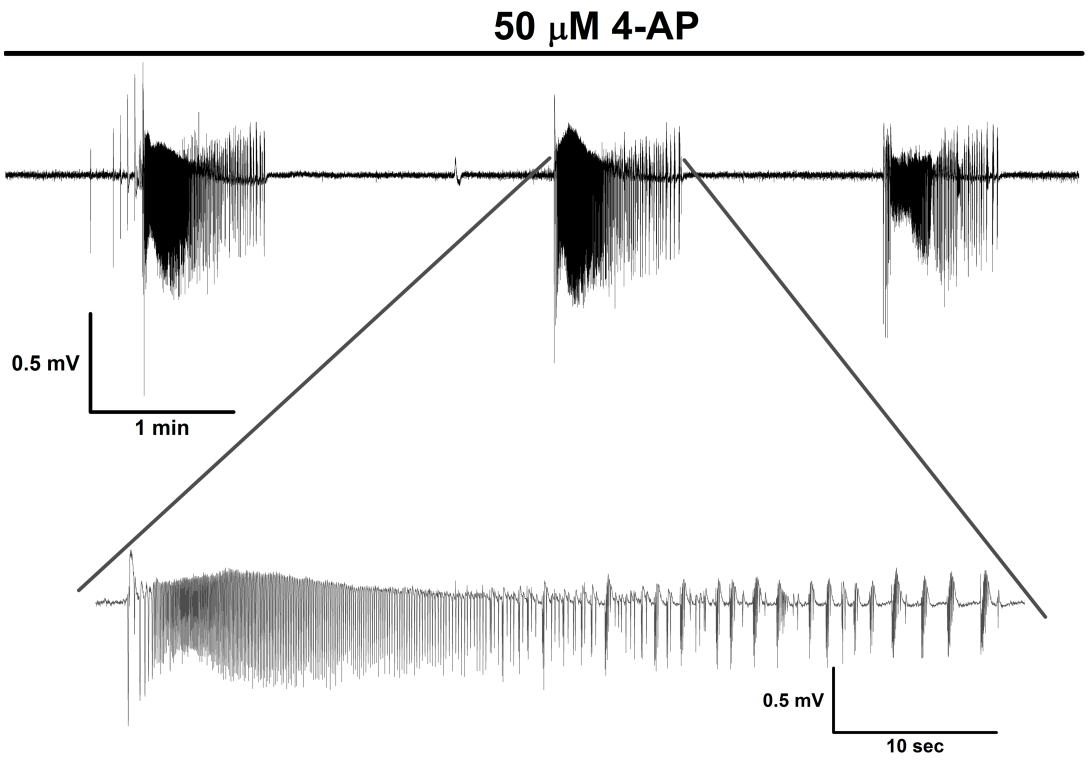

Fig 3.2. Representative bursting seizure activity in 50 μM 4-aminopyridine/zero magnesium model. Representative trace of spontaneous synchronous bursting seizure activity in a control slice following challenge with 4-aminopyridine (4-AP). Lower panel shows a zoom in of burst 2 epileptiform activity.

3.3. Discussion

In the present chapter I have shown that the 50 μM 4-AP/0 zero Mg^{2+} model was more effective at producing reproducible bursting seizures than the 6 mM and 9 mM K^+/zero Mg^{2+} models.

3.3.1. 6 mM and 9 mM K^+/zero Mg^{2+}-induced seizures

Extracellular K^+ concentrations were increased to 6 mM and 9 mM in zero Mg^{2+} aCSF. Chi-square test of independence indicated that there is a relationship between treatment and observed epileptiform activity when slices were challenged with 6 mM but not 9 mM K^+. This results suggests that challenging slices with 6 mM K^+ concentration produced a shift towards an increase in a more convulsive epileptiform activity (status epilepticus) in creatine treated slices (11/17) compared to those observed for control slices which had a lower tendency towards status epilepticus (3/17). Likewise in RibAde-treated slices compared to creatine slices there was a reduced tendency towards status epilepticus, whereby the distribution of observed epileptiform activity was evenly distributed between the three groups. When the K^+ concentration was further increased to 9 mM the frequency of observed epileptiform activity was independent of treatment, suggesting that there were no tendency towards either bursting or non-bursting epileptiform activity between the different treatments. At this concentration there was a higher number of bursting epileptiform activity in both creatine (from 35% to 76%) and RibAde-treated slices (from 28% to 69%) compared to that observed with a K^+ concentrations of 6 mM. However, in control slices a K^+ concentration of 9 mM decreased the frequency of bursting from 76% to 58% in 6 mM. Although increasing the K^+ concentrations to 6 mM and 9 mM did produce seizure activity similar to those reported in previous studies (Anderson*, et al.*, 1986; Lopatář*, et al.*, 2011; Mody*, et al.*, 1987), in this report

concentrations of 6 mM and 9 mM failed to reliably produce spontaneous bursting seizures (28-76%). Where previous studies using higher K^+ concentrations from standard bathing solutions to induce seizures were not focused on a single type of seizure activity, for the purposes of the present study it was important that the K^+ concentrations used consistently produced similar seizure activity. My data suggests that an increase in K^+ concentration beyond that used in the standard aCSF in conjunction with washout of Mg^{2+} from slices produces unreliable responses in synaptic and network excitability that make it an ineffective model to use to study the effects of RibAde and creatine on seizure activity.

3.3.2. 50 µM 4-aminopyridine/zero Mg^{2+}-induced seizures

Due to the inconsistencies in the seizures generated with the high K^+ models, another model was used as means of overcoming the difficulties found. For this we explored using the potassium channel blocker 4-AP. Initial investigative experiments showed that the 4-AP model produced more robust, stable and consistent seizure activity compared to the previous model. These findings are similar to epileptic spontaneous seizures recorded with a concentration of 50 µM 4-AP in rat hippocampal slices (Avoli*, et al.*, 1996; Easter*, et al.*, 2007; Yonekawa*, et al.*, 2003). Chi-square test of independence indicated that there was no relationship between treatment and observed epileptiform activity. Therefore, there was no preference within a treatment for bursting epileptiform activity vs. non-bursting epileptiform activity within the three treatments groups. Given this result the 4-AP model does result in a higher proportion of bursting epileptiform activity compared to the K^+ model. Therefore, given promising results from using the 4-AP model, the decision was taken to continue to use the 50 µM 4-aminopyridine/zero Mg^{2+} model.

In summary, this chapter deals with establishment of zero Mg^{2+} models of acute *in vitro* seizure-like events. All the models explored here are based in part on facilitating the excitability of tissue by omitting Mg^{2+} ions from the bathing solution. The resulting SLEs induced chemically with the additional application of 4-AP were reproducible, and therefore this model was chosen to test effects of purinergic modulation on seizure activity.

4. Modulation of intracellular ATP in *in vitro* models of seizure activity: effect on adenosine release

4.1. Introduction

The findings of the previous chapter provide support for the use of 0 mM Mg^{2+} aCSF/50 μM 4-aminopyridine as a reproducible seizure model. In the present chapter, this seizure model will be employed to investigate the effect of modulation of intracellular ATP on adenosine release.

ATP is the major energy source in the CNS. Following a brain insult ATP levels are depleted and the energy requirements (ATP stores or production) exceed the brain's ability to synthesise ATP. Due to this, the amount of ATP and its nucleotides such as ADP are reduced, but the breakdown product adenosine is increased.

During epilepsy, a surge in the ATP breakdown product adenosine occurs following the onset of seizure activity (Adén*, et al.*, 2004; During and Spencer, 1992; Lopatář*, et al.*, 2011). In addition to the anticonvulsant properties of adenosine, dysfunction in its regulation has been implicated in the pathophysiological processes of epilepsy (Boison, 2010; Dunwiddie, 1999; Świąder*, et al.*, 2014).

Given that ATP is the primary source of adenosine, it can be postulated that any change in the ATP pool is likely to influence adenosine production and release. In support of this, experiments have shown that incubating slices with creatine (1 mM) buffers ATP metabolism and results in a reduction in adenosine release in response to oxygen glucose deprivation (zur Nedden*, et al.*, 2014). In the same study, combination of ribose (1 mM) and adenine (50 μM) was shown to boost ATP levels and also result in greater adenosine release (zur Nedden*, et al.*, 2014; zur Nedden*, et al.*, 2011).

Given the importance of ATP as a source of adenosine and evidence which suggest a pro-convulsive role of P2 receptors as well as the involvement of adenosine in the

pathophysiology of epilepsy, the main focus of this chapter was to test whether modulation of intracellular ATP by (a) enhancing the purine salvage pathway or (b) delaying the degradation of ATP to its metabolites affects activity-dependent release of adenosine. This was achieved by pre-treating slices with either (a) a combination of 1 mM D-ribose and 50 μM adenine (RibAde) or (b) 1 mM creatine.

To date, little is know about the effect on RibAde on epileptic seizures. Due to the greater ATP pool in RibAde-treated slices (zur Nedden*, et al.*, 2014; zur Nedden*, et al.*, 2011) it would be expected that there might be a greater activity-dependent release of adenosine that might reduce seizure activity.

In comparison to RibAde, 1 mM of creatine would be expected to delay the degradation of ATP to adenosine through phosphocreatine's donation of a phosphate group to ADP, reactions (Chapter 1.9.) catalysed by creatine kinase. Therefore, it would be expected that pre-treating slices with creatine would result in a reduction in the release of adenosine and might result in an increase in seizure excitability.

In this chapter I will show that it is possible to modulate the amount and pattern of adenosine release during seizure activity as well as the frequency and intensity of seizures by modulation of intracellular ATP levels with substances that are well tolerated in humans.

4.2. Results

4.2.1. Real-time measurement of activity-dependent release of adenosine from rat hippocampal brain slices

In these studies, adenosine microelectrode biosensors were used to make real-time measurements of adenosine release in rat hippocampal slices.

4.2.1.1. Real-time recording of adenosine release

Figure 4.1 shows an example of an individual recording taken from a control slices. Real-time recordings are shown in the top panel and the associated changes on the extracellular recording electrode are shown on the lower panel following challenge with washout of magnesium from slices (0 mM Mg^{2+} aCSF), after 3 bursts during bursting seizures in 4-aminopyridine, a potassium channel blocker or after 10 minutes (50 μM 4-AP), and during challenge with the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (1 μM CPT).

The average release of adenosine is illustrated in figure 4.2 for creatine, control and RibAde-treated slices (creatine, n = 8; control, n = 11; RibAde, n = 13 slices).

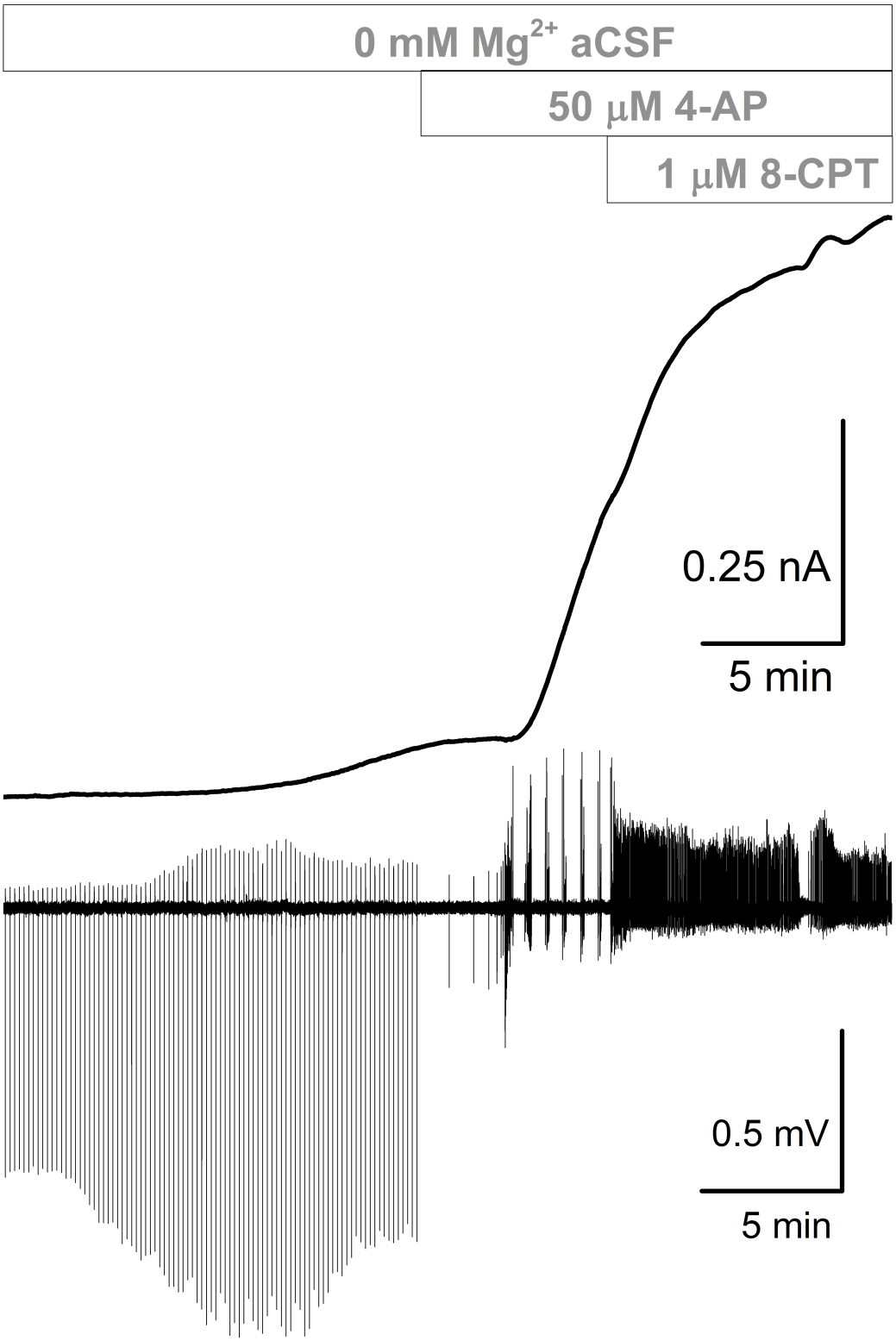

Figure 4.1. Representative example of epileptiform activity and associated activity-dependent release of adenosine in control slices. Micro-electrode recording of activity-dependent adenosine release are shown in the top panel and the associated changes on the extracellular recording electrode are shown on the lower panel following challenge with washout of magnesium from slices (0 mM Mg^{2+} aCSF), after 3 bursts during bursting seizures in 4-aminopyridine, a potassium channel blocker or after 10 minutes (50 μM 4-AP), and during challenge with the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (1 μM CPT).

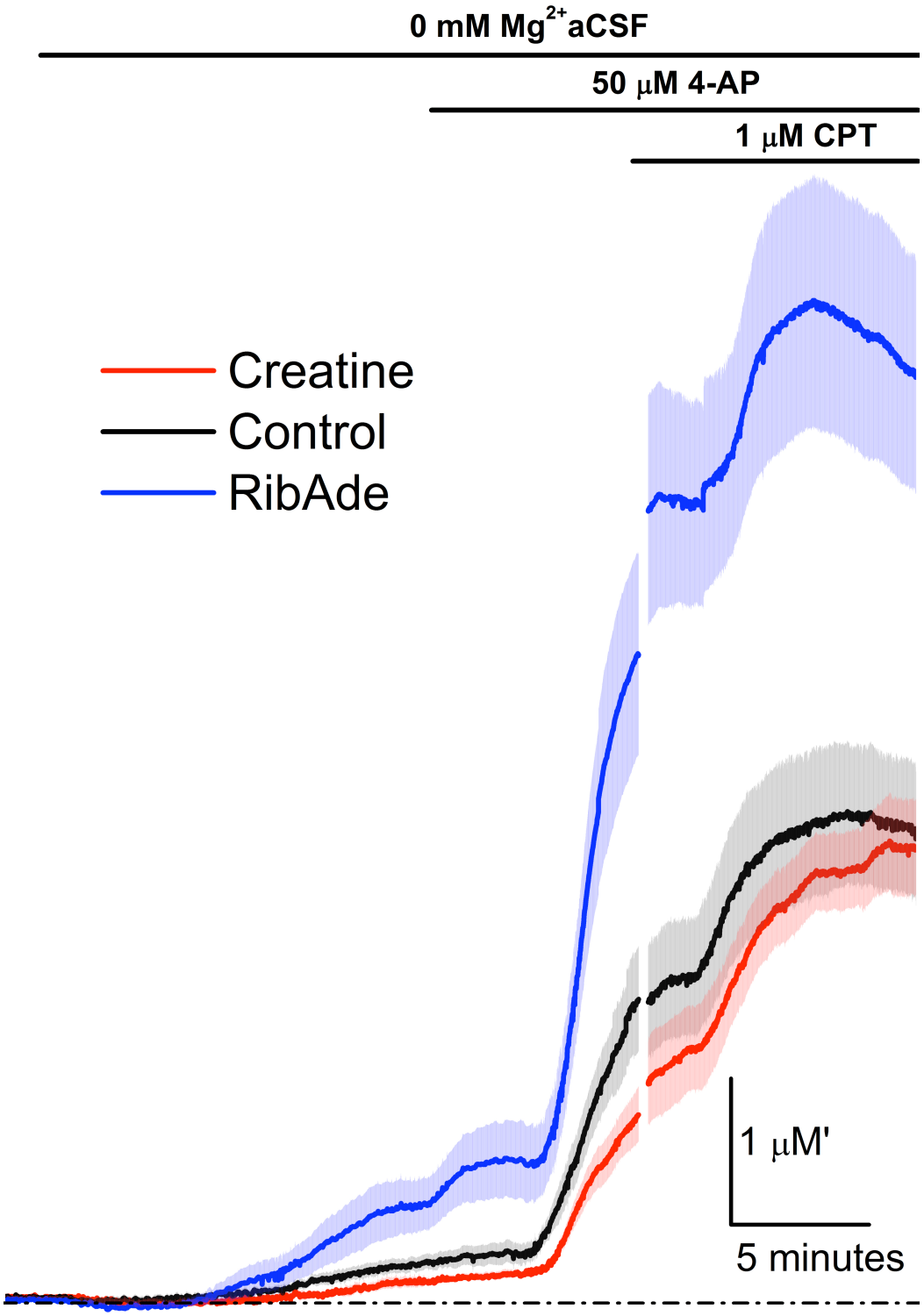

Figure 4.2. Real-time measurement of activity-dependent release using micro-electrode biosensors. Average biosensor traces show the activity-dependent release of adenosine during washout of Mg^{2+} from slices (0 mM Mg^{2+} aCSF), 50 μM 4-aminopyridine-induced seizures (50 μM 4-AP) and challenge with the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (CPT). Dotted line represents baseline prior to washout of Mg^{2+} from slices. CPT was added after the presentation of three bursts or after 10 minutes in 4-AP as shown by a break in the traces. Adenosine release in RibAde-treated slices (—, n = 13 slices) was greater than control (—, n = 11 slices) and creatine-treated slices (—, n = 8 slices). Data shown is mean ± SEM.

To further quantify the differences in adenosine release between treatments the integral of the area under the curve measurements were made (Figure 4.3 A-D). Adenosine release under the conditions listed above is summarised in Table 4.1. One-way ANOVA analysis reported a difference in the release of adenosine in Mg^{2+}-free aCSF (Fig 4.3 A). However, the post-hoc test did not reveal any significant differences (One-way ANOVA: $p = 0.04$; $F_{(2,28)} = 3.52$; Post-hoc Bonferroni: creatine vs. RibAde, $p = 0.07$).

During seizure activity in 4-AP, total adenosine release was increased (Fig 4.3 B) in RibAde slices compared to control and creatine slices (One-way ANOVA: $p = 0.001$; $F_{(2,28)} = 8.47$; Post-hoc Bonferroni: creatine vs. RibAde, $p = 0.003$; control vs. RibAde, $p = 0.009$).

In these experiments CPT increased the amount of adenosine released from slices directly following 4-AP induced seizures, but this release was not significantly different (Fig 4.3 C) between treatments (One-way ANOVA: $p = 0.846$; $F_{(2,25)} = 0.168$).

Total combined release (Fig 4.2 D) per slice was significantly increased (One-way ANOVA: $p = 0.006$; $F_{(2,29)} = 6.186$) in RibAde-treated slices compared to creatine-treated slices (Post-hoc Bonferroni, $p = 0.025$) and control slices (Post-hoc Bonferroni, $p = 0.013$).

These data suggest that modulating intracellular ATP levels by pre-treating slices with RibAde results in a significant increase in the activity-dependent release of adenosine. In comparison, pre-treating with creatine resulted in the least amount of activity-dependent release of adenosine, similar to observations made previously by zur Nedden *et al.,* (2014) during oxygen-glucose deprivation in hippocampal slices.

Table 4.1. Integrated measurements of activity-dependent release of adenosine. Measurements were made in creatine, control and RibAde-treated slices. One –way ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$ compared to RibAde-treated slices, n = 8-13 slices. Data is shown as mean ± SEM

	Creatine (µM´ s)	**Control (µM´ s)**	**RibAde (µM´ s)**
0 mM Mg^{2+} aCSF	0.13 ± 0.02 (n = 8)	0.20 ± 0.03 (n = 11)	0.44 ± 0.12 (n = 13)
50 µM 4-AP	0.95 ± 0.18** (n = 8)	1.31 ± 0.23** (n = 11)	3.15 ± 0.53 (n = 13)
1 µM CPT	1.76 ± 0.23 (n = 8)	1.56 ± 0.21 (n = 8)	1.77 ± 0.31 (n = 12)
Total release	2.84 ± 0.26* (n = 8)	2.85 ± 0.33* (n = 11)	5.22 ± 0.75 (n = 13)

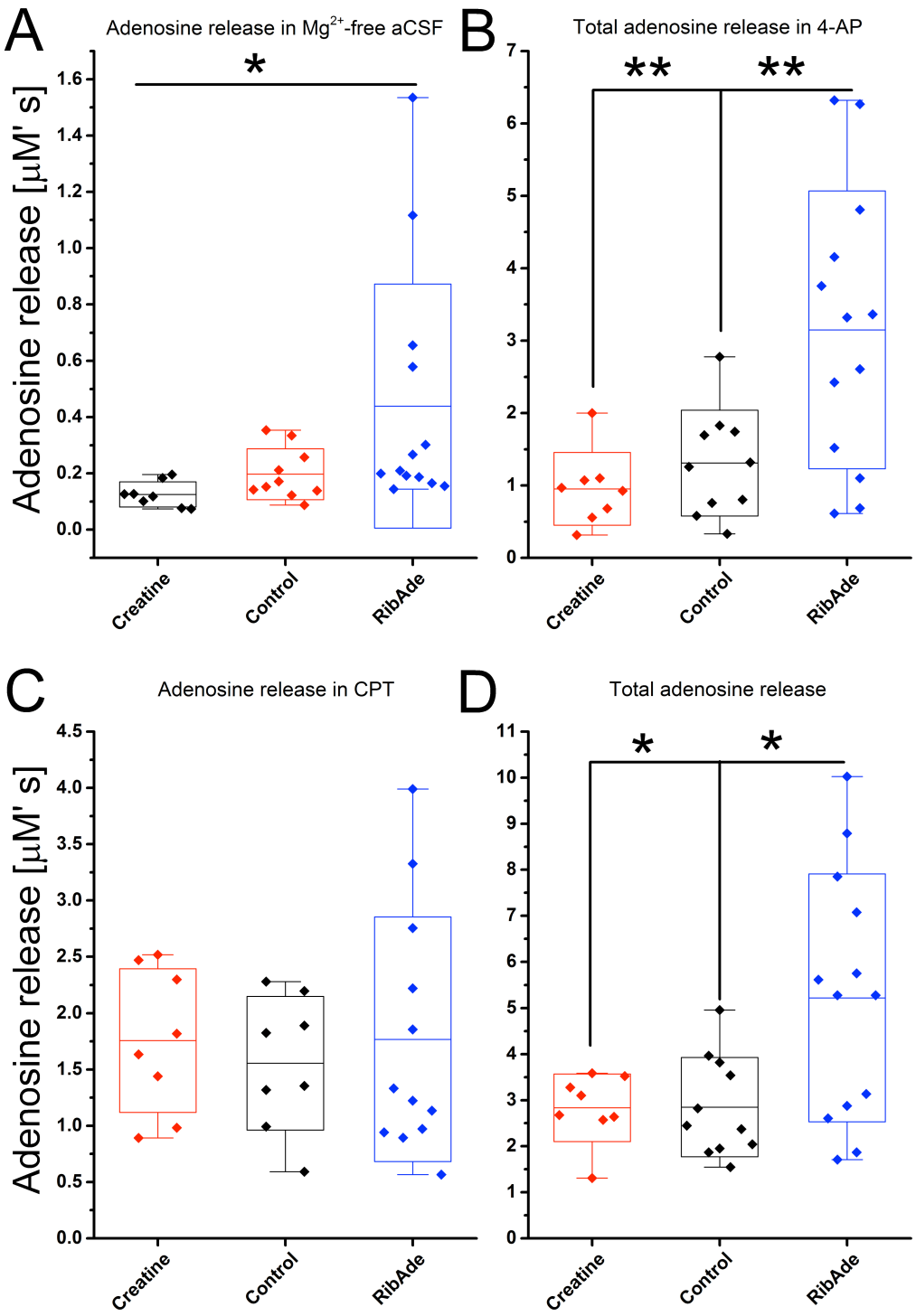

Figure 4.3. Integrated measurements of activity-dependent release of adenosine. The integral of the area under the curve measurements of adenosine release following 15 minutes in magnesium (Mg^{2+})-free solution (A) and during a maximum of three 50 µM 4-aminopyridine (4-AP) induced seizures (B) was increased in RibAde-treated slices (n = 13) compared to control (n = 11) and creatine-treated slices (n = 8). During seizure activity, the levels of adenosine release from slices was increased compared to that measured before. No differences (C) were found during application of the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (CPT; 1 µM). Total adenosine release was greatly increased in RibAde-treated slices (D). Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by $*p < 0.05$ and $**p < 0.01$.

4.2.1.2. Assessment of burst-dependent release of adenosine

The release of adenosine associated with each burst (Fig 4.4) was calculated during 4-AP-induced seizures. The increased release of adenosine in RibAde-treated slices observed in Fig. 4.3 B was in large part due to adenosine release during burst 1 (Fig 4.5; Table 4.2; One-way ANOVA: $p = 0.001$; $F_{(2,29)} = 8.527$; creatine, n = 8; control, n = 11; RibAde, n = 13 slices). The burst 1-associated increase in adenosine release in RibAde-treated slices was greater than creatine-treated (Post-hoc Bonferroni: $p = 0.005$) and control slices (Post-hoc Bonferroni: $p = 0.005$). No difference in the burst-dependent release of adenosine was found for burst 2 (One-way ANOVA: $p = 0.206$; $F_{(2,26)} = 1.682$; creatine, n = 6; control, n = 10; RibAde, n = 12 slices) and burst 3 (One-way ANOVA: $p = 0.601$; $F_{(2,19)} = 0.524$; creatine, n = 4; control, n = 9; RibAde, n = 9 slices).

The data shows that, in RibAde-treated slices, the greatest release of adenosine was associated with the initial seizure. In addition, unlike control and creatine, RibAde showed a change in the adenosine release profile between bursts whereby adenosine release is decreased in each subsequent burst. This suggests that the higher levels of burst-1 associated adenosine release in RibAde may be sufficient to dampen down subsequent seizure activity which may then affect its associated adenosine release.

Table 4.2. Summary of burst-dependent release of adenosine. Measurements were made in creatine, control and RibAde-treated slices. One-way ANOVA: $^{**}p < 0.01$ compared to RibAde-treated slices, n = 8-13 slices. Data shown as mean ± SEM

Burst number	Creatine (µM´ s)	Control (µM´ s)	RibAde (µM´ s)
1	0.48 ± 0.11** (n = 8)	0.66 ± 0.18** (n = 11)	2.43 ± 0.51 (n = 13)
2	0.72 ± 0.14 (n = 6)	0.79 ± 0.21 (n = 10)	1.12 ± 0.22 (n = 13)
3	0.43 ± 0.18 (n = 4)	0.56 ± 0.13 (n = 9)	0.40 ± 0.10 (n = 9)

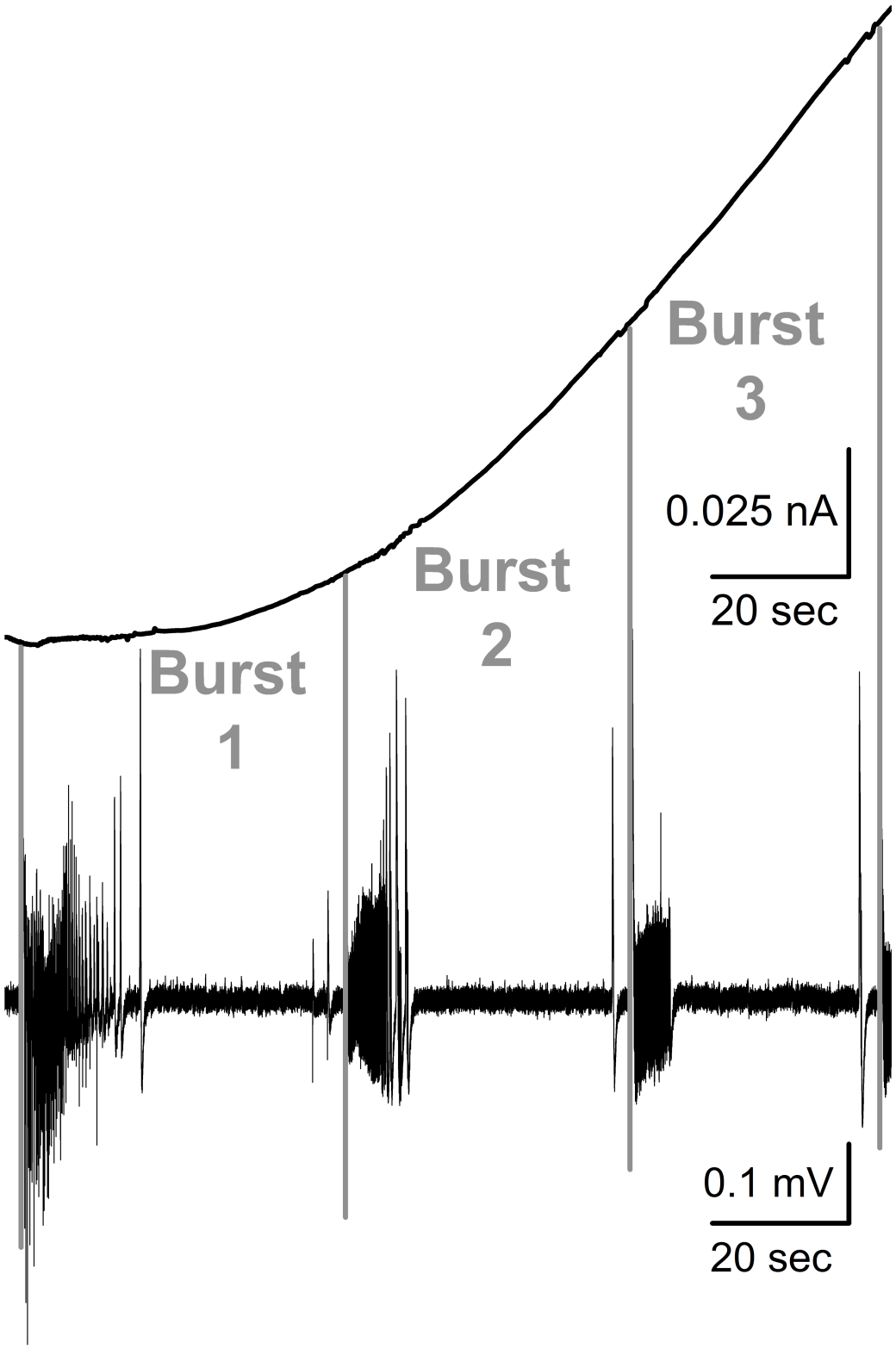

Figure 4.4. Representative example of 4-aminopyridine-induced bursting epileptiform activity and associated activity-dependent release of adenosine in control slices. Representative 4-AP-induced bursting epileptiform activity and the associated differential raw current recordings of representative adenosine release made with microelectrode biosensors in a control slice.

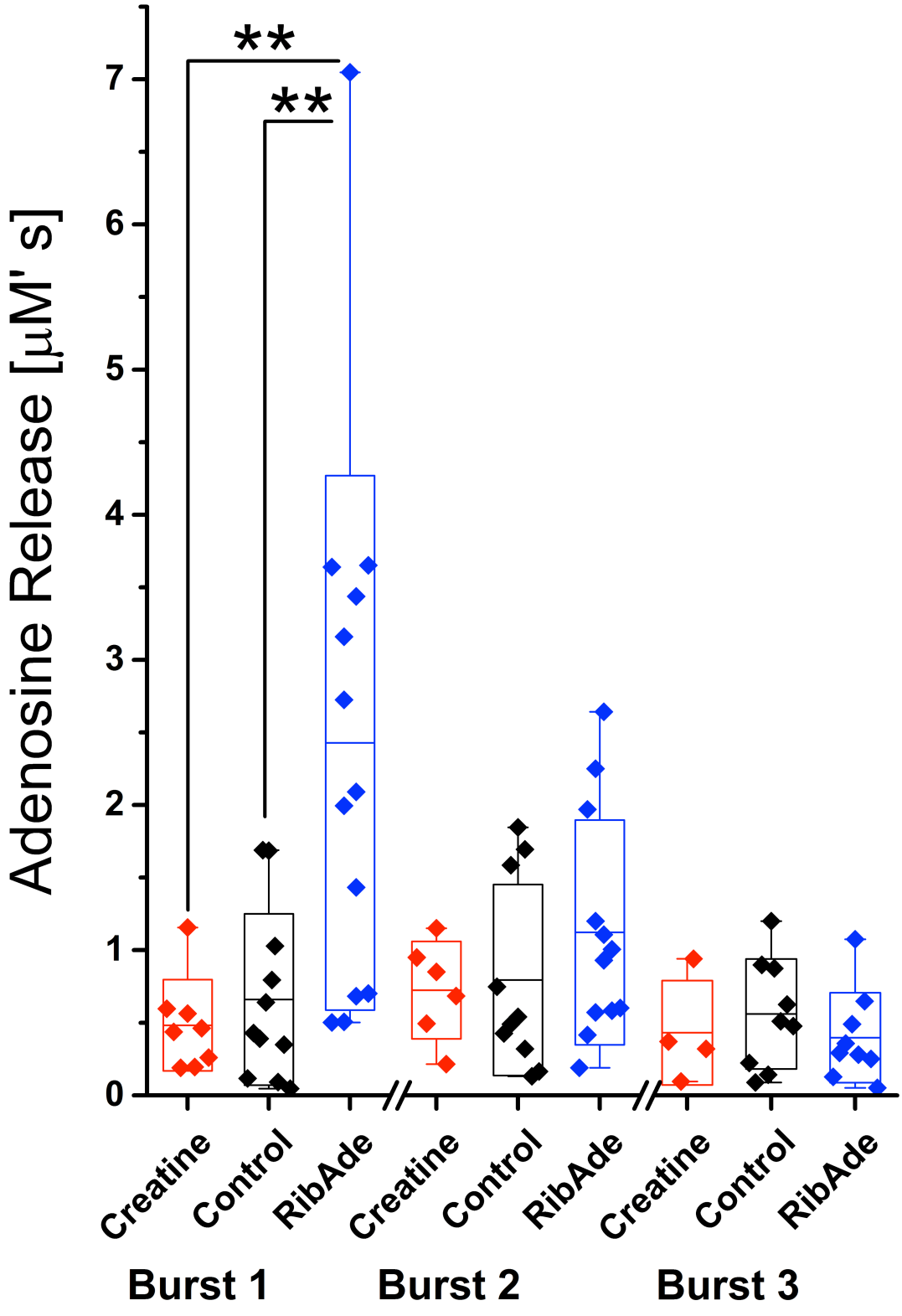

Figure 4.5. Adenosine release in slices pre-treated with RibAde but not creatine is burst-dependent. Burst 1 adenosine release was increased in slices pre-treated with RibAde (n = 13) compared to control (n = 11) and creatine slices (n = 8). No difference in the release of adenosine between the three groups was found for bursts 2 and 3. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits.. Significances indicated by $^{**}p < 0.01$.

4.2.1.3. Assessment of continuous application of RibAde on adenosine release

The burst-dependent release of adenosine data suggests that there may be adenosine depletion after burst 1. In order to test whether activity-dependent release of adenosine could be increased in RibAde-treated slices, continuous application of RibAde (Continuous RibAde) to the bathing solution was used.

4.2.1.3.1. Effect of Continuous RibAde on activity-dependent release of adenosine

Figure 4.6 shows the average release of adenosine for control, RibAde-treated and Continuous RibAde-treated slices (control, n = 11; RibAde, n = 13; Continuous RibAde, n = 8 slices).

Measurement of the integral of the area under the curve (Table 4.3) showed that the release of adenosine in Continuous RibAde-treated slices was increased compared to control and RibAde-treated slices during washout of $Mg^{2+}$from slices (Fig 4.7 A; One-way ANOVA: $p < 0.0001$; $F_{(2,28)} = 24.115$; Post-hoc Bonferroni: $p < 0.0001$). During 4-AP-induced seizures adenosine release in Continuous RibAde-treated slices were increased compared to control slices (Fig 4.7 B; One-way ANOVA: $p = 0.028$; $F_{(2,28)} = 4.064$; Post-hoc Bonferroni: $p = 0.034$). No difference during CPT was found (Fig 4.7 C; One-way ANOVA: $p = 0.290$; $F_{(2,25)} = 1.302$). Total adenosine release was increased in Continuous RibAde slices compared to control (Fig 4.7 D; One-way ANOVA: $p < 0.001$; $F_{(2,29)} = 11.539$; Post-hoc Bonferroni: $p < 0.001$) and RibAde-treated slices (Post-hoc Bonferroni: $p = 0.026$).

Table 4.3. Summary of the integrated measurements of activity-dependent release of adenosine with Continuous RibAde slices. Measurements were made in control, RibAde- and Continuous RibAde-treated slices. One –way ANOVA: $^{*}p < 0.05$, $^{***}p < 0.01$, $^{****}p < 0.0001$ compared to Continuous RibAde-treated slices, n = 6-13 slices. Data shown as mean ± SEM.

	Control (µM´ s)	**RibAde (µM´ s)**	**Continuous RibAde (µM´ s)**
0 mM Mg^{2+} aCSF	$0.20 \pm 0.03^{****}$ (n = 11)	$0.44 \pm 0.12^{****}$ (n = 13)	2.12 ± 0.40 (n = 8)
50 µM 4-AP	$1.31 \pm 0.23^{*}$ (n = 11)	3.15 ± 0.53 (n = 13)	3.96 ± 1.12 (n = 8)
1 µM CPT	1.56 ± 0.21 (n = 8)	1.77 ± 0.31 (n = 12)	2.25 ± 0.28 (n = 8)
Total release	$2.85 \pm 0.33^{***}$ (n = 11)	$5.22 \pm 0.75^{*}$ (n = 13)	8.33 ± 1.17 (n = 8)

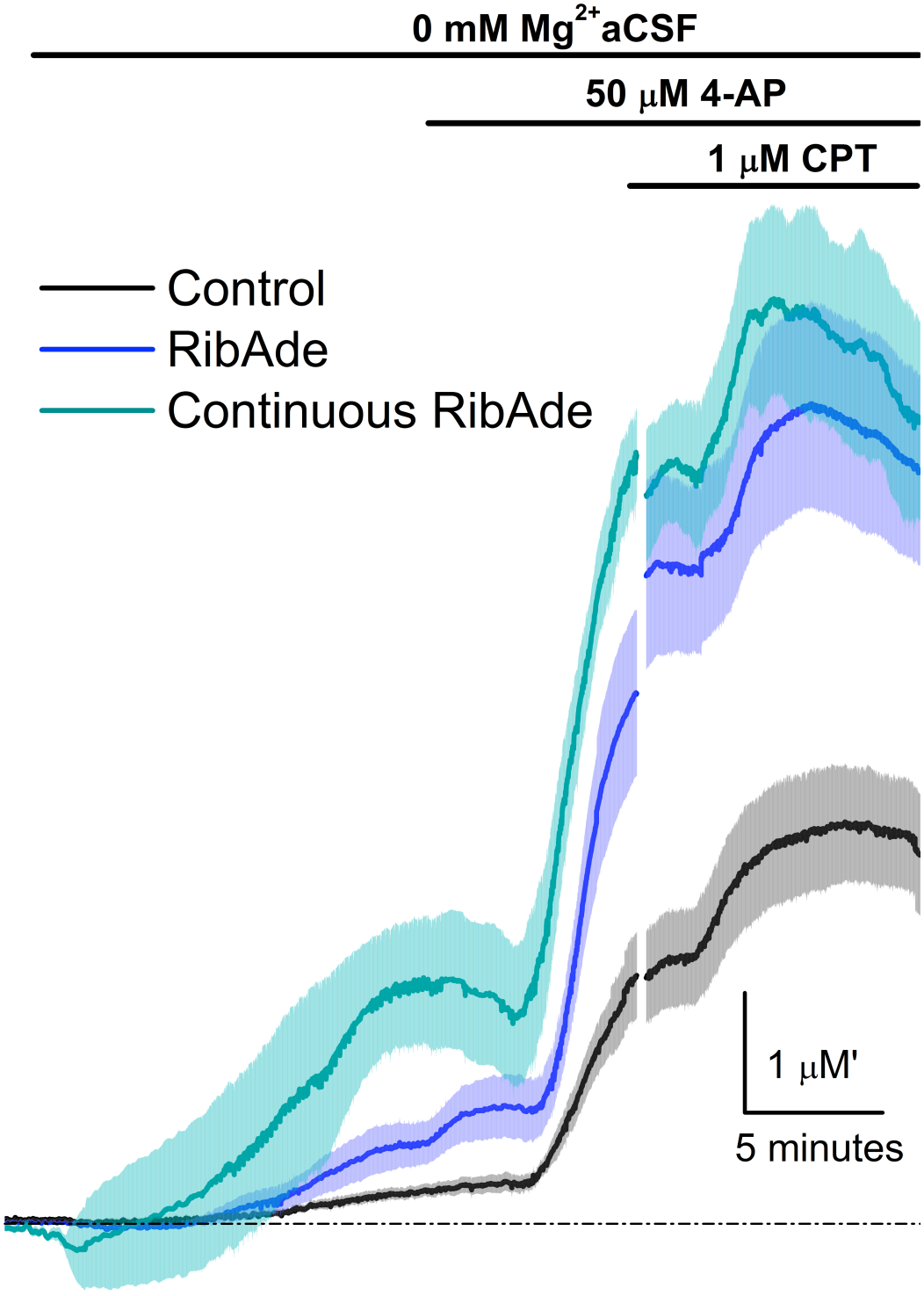

Figure 4.6. Average recordings of activity-dependent release of adenosine in control, RibAde and Continuous RibAde slices. Average biosensor traces for control (—, n = 11 slices), RibAde-treated slices (—, n = 13 slices) and Continuous RibAde-treated slices (—, n = 8 slices) show the activity-dependent release of adenosine during washout of Mg^{2+} from slices (0 mM Mg^{2+} aCSF), 50 μM 4-aminopyridine-induced seizures (50 μM 4-AP) and challenge with the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (CPT). Dotted line represents baseline prior to washout of Mg^{2+} from slices. CPT was added after the presentation of three bursts or after 10 minutes in 4-AP as shown by a break in the traces. Data shown is mean ± SEM.

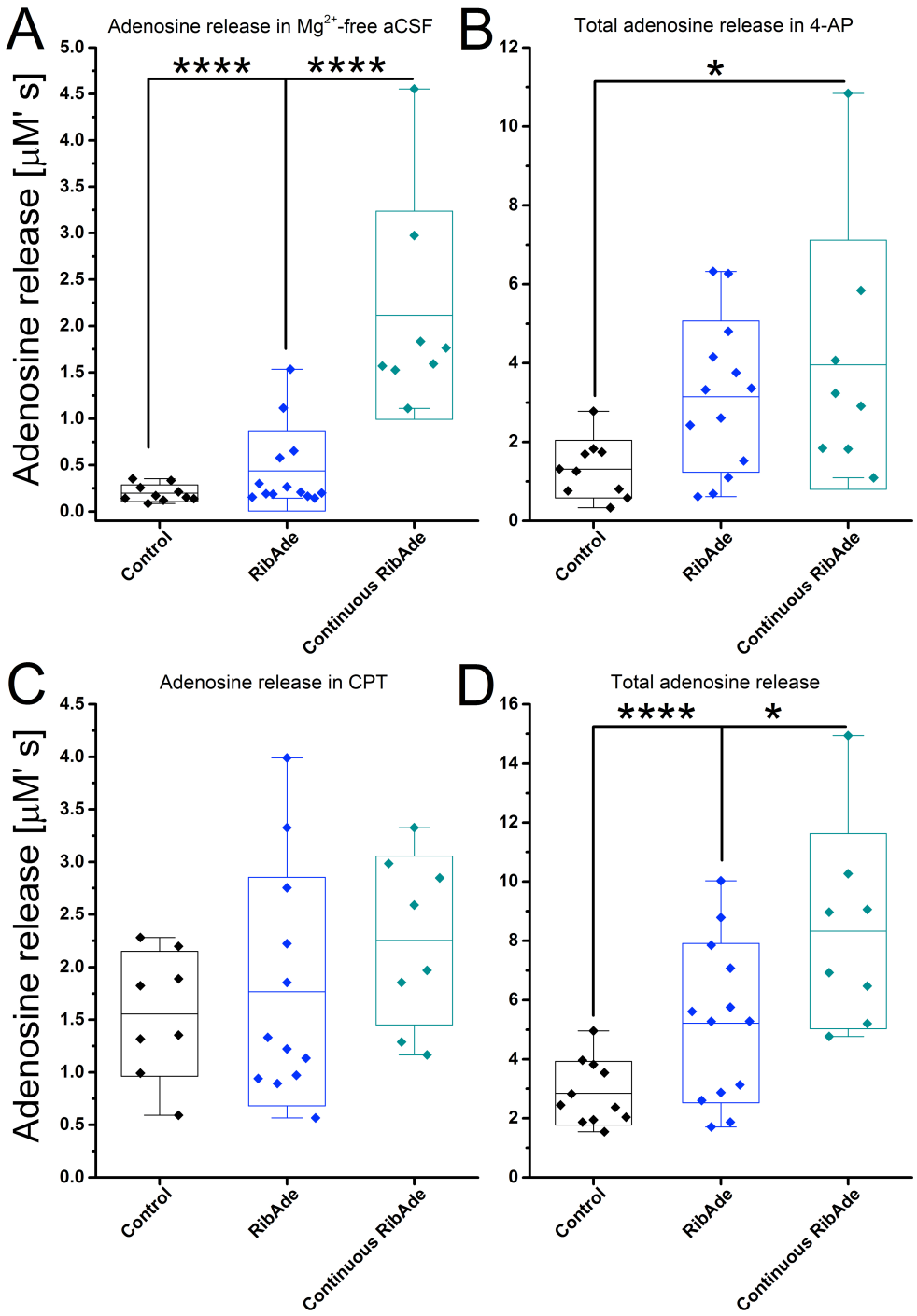

Figure 4.7. Integrated measurements of activity-dependent release of adenosine with Continuous RibAde slices. The integral of the area under the curve measurements of adenosine release following 15 minutes in magnesium (Mg^{2+})-free solution (A) and during a maximum of three 50 μM 4-aminopyridine (4-AP) induced seizures (B) was increased in Continuous RibAde slices (n = 8) compared to control (n = 11). The release of adenosine was only different between Continuous RibAde and RibAde slices during washout of Mg^{2+} from slices, but not during seizure activity. No differences (C) were found during application of the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (CPT; 1 μM). Total adenosine release was greatly increased in Continuous RibAde slices (D) compared to control and RibAde-treated slices. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by *$p < 0.05$ and ****$p < 0.001$.

4.2.1.3.2. Effect of continuous application of RibAde on burst-dependent adenosine release

The burst 1 associated release of adenosine was greater in Continuous RibAde-treated slices compared to control (Fig 4.8; One-way ANOVA: $p = 0.013$; $F_{(2,29)} = 5.105$; Post-hoc Bonferroni: $p = 0.025$).

No difference in adenosine release was found for the release of adenosine during burst 2 (One-way ANOVA: $p = 0.443$; $F_{(2,25)} = 0.842$; control = 10; RibAde, n = 12; Continuous RibAde = 6) and burst 3 (One-way ANOVA: $p = 0.335$; $F_{(2,19)} = 1.160$; control = 9; RibAde, n = 9; Continuous RibAde = 4).

These biosensor data indicate that overall, the amount of activity-dependent release of adenosine was greater in Continuous RibAde treated slices. However, during seizure activity, the amount of adenosine released in Continuous RibAde slices was not different to that in RibAde-treated slices.

Table 4.4. Summary of burst-dependent release of adenosine with Continuous RibAde slices. Measurements were made in control, RibAde-and Continuous RibAde-treated slices. One-way ANOVA: $^{**}p < 0.01$ compared to Continuous RibAde-treated slices, n = 6-13 slices. Data shown as mean ± SEM

Burst number	**Control (µM´ s)**	**RibAde (µM´ s)**	**Continuous RibAde (µM´ s)**
1	$0.66 \pm 0.18^{*}$ (n = 11)	2.43 ± 0.51 (n = 13)	2.54 ± 0.65 (n = 8)
2	0.79 ± 0.21 (n = 10)	1.12 ± 0.22 (n = 12)	1.19 ± 0.22 (n = 6)
3	0.56 ± 0.13 (n = 9)	0.40 ± 0.10 (n = 9)	0.69 ± 0.16 (n = 4)

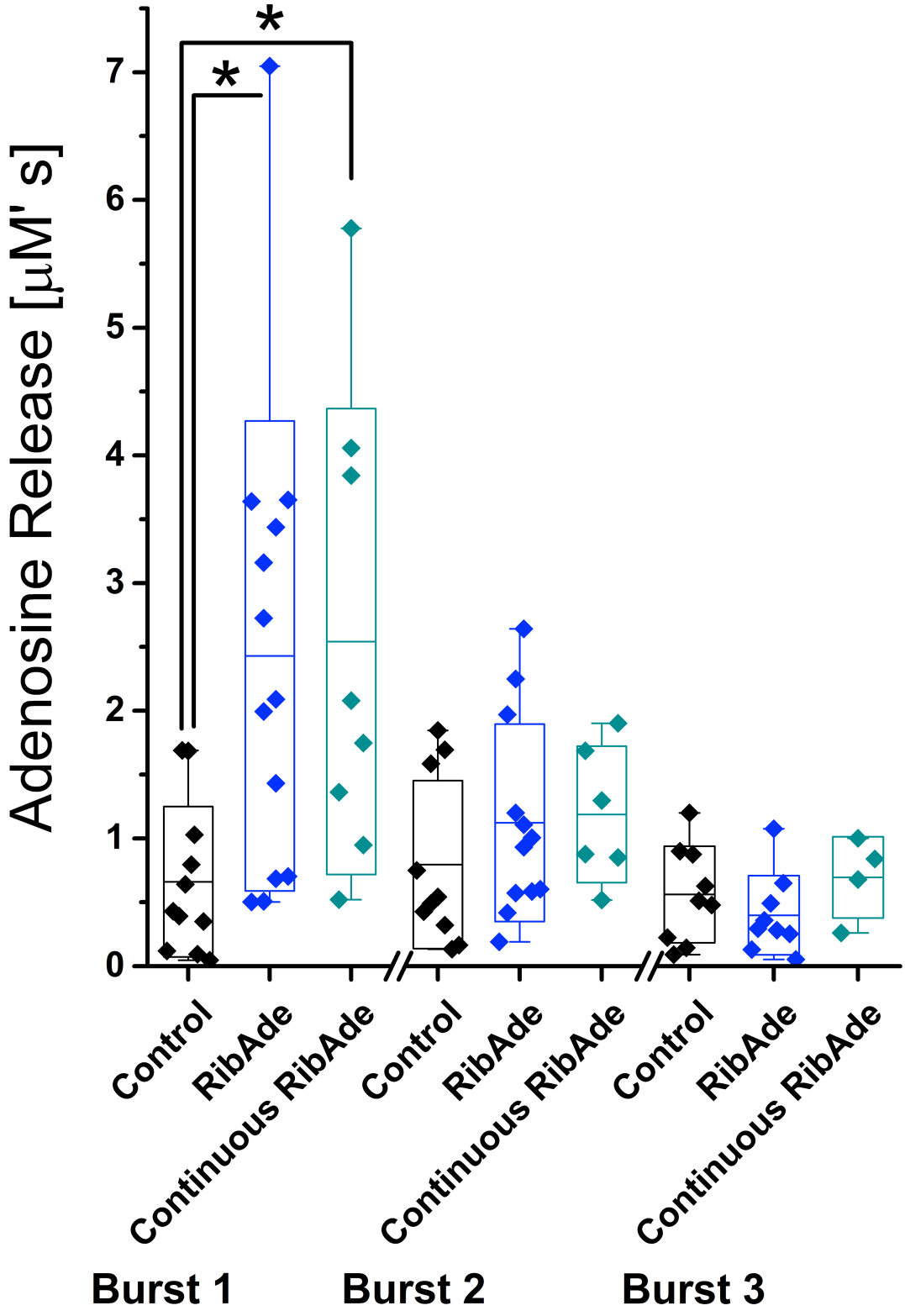

Figure 4.8. Burst-dependent release of adenosine in RibAde and Continuous RibAde slices. Burst 1 adenosine release was increased in slices pre-treated with RibAde (n = 13) and Continuous RibAde (n = 8) compared to control (n = 11). No difference in the release of adenosine was found for bursts 2 and 3. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by *$p < 0.05$.

4.3. Discussion

In summary these data show that pre-treating slices with RibAde and creatine has a measurable consequence on the activity-dependent release of adenosine.

4.3.1. Effect of RibAde and creatine on adenosine release

Previous research (Etherington*, et al.*, 2009; Frenguelli and Wall, 2016; Lopatář*, et al.*, 2011; Lopatář*, et al.*, 2015; Wall and Richardson, 2015) and my own results show that it is possible to measure real-time release of adenosine during brief seizures in hippocampal slices. My data suggests that adenosine release in RibAde-treated slices occurs in an NMDA-dependent manner where there was a trend for an increase in the release of adenosine during washout of Mg^{2+} from RibAde-treated slices compared to creatine-treated slices. This response was also observed during 4-AP-induced seizures where the release of adenosine during seizure activity was significantly increased in RibAde-treated slices compared to creatine-slices. Additionally, the release of adenosine in RibAde-treated slices was also increased compared to control slices. Surprisingly, no difference was found during challenge with CPT, suggesting that the role of the adenosine A_1 receptor in the different treatments was similar. Overall, these results show that the activity-dependent release of adenosine was greater in RibAde-treated slices compared to control slices and those pre-treated with creatine, which released a reduced amount of adenosine. These findings are similar to the activity-dependent release associated with brief oxygen/glucose deprivation (5 min), where the greatest release of adenosine occurred in RibAde-treated slices and pre-treatment with creatine resulted in the least amount of adenosine release (zur Nedden *et al*., 2014).

4.3.2. Effect of Continuous RibAde on adenosine release

To test whether it was possible to prevent depletion of adenosine after burst 1 adenosine release in RibAde-treated slices, continuous application of RibAde was used. Continuous application of RibAde only resulted in a larger release of adenosine compared to pre-incubation alone during washout of Mg^{2+} from slices compared to RibAde-treated slices. This had a profound effect on total adenosine release, where Continuous RibAde-treated slices had a greater total release compared to pre-incubated RibAde slices. No effect of Continuous RibAde was found on the release of adenosine during 4-AP-induced seizures compared to RibAde-treated slices. No difference in the release of adenosine between treatments during challenge with CPT was found. Additionally, there was no effect on burst-dependent adenosine release in Continuous RibAde-treated slices compared to RibAde-treated slices. This is likely due to the inability of these slices to make more ATP quickly enough during the preceding intermediate periods, i.e. during the Inter Burst Interval. Although the release of adenosine was not different to RibAde-treated slices during 4-AP induced seizures, the release of adenosine in Continuous RibAde slices was greater than that of control slices during seizure activity and washout of Mg^{2+} from slices.

These data suggest that continuous application of RibAde does not prevent the depletion of adenosine after burst 1 in RibAde-treated slices. Additionally, the release of adenosine during seizure activity during continuous application of RibAde was not different to that with pre-incubation only. This suggests that pre-incubation of slices for 3 hours with RibAde is sufficient to produce robust changes to the activity-dependent release of adenosine.

In summary, these data suggest that pre-incubating slices for 3 hours with RibAde followed by washout out of RibAde from slices in normal aCSF was sufficient to influence extracellular release of adenosine.

These experiments show that pre-treating slices with RibAde and creatine had differential effects on the activity-dependent release of adenosine. In the next chapter I will further investigate whether the activity-dependent release of adenosine measured in RibAde- and creatine-treated slices influenced seizure activity itself.

5. Modulation of intracellular ATP in *in vitro* models of seizure activity: effect on basal synaptic transmission and seizure parameters

5.1. Introduction

The findings from chapter 4 show that pre-treating slices with RibAde resulted in the greater activity-dependent release of adenosine. The present chapter will investigate the effects of RibAde and creatine on basal synaptic transmission and seizure parameters.

Adenosine was initially observed as a naturally occurring anticonvulsant in 1984 (Barraco*, et al.*, 1984), in which it was found that the anticonvulsant properties were mediated by the adenosine A_1Rs. The A_1Rs function by inhibiting excessive neuronal activity in the epileptic brain. The importance of these receptors types is also supported with genetic mouse models where A_1Rs knock (A_1Rs KO) out mice showed an increased susceptibility to developing seizures and developed lethal status epilepticus (SE) after experimentally induced brain injury (Kochanek*, et al.*, 2006). These data provide strong evidence for the adenosine A_1Rs as potential therapeutic targets for the treatment of epilepsy.

Adenosine plays an important role in the regulation and coordination of synaptic strength and synaptic networks and any dysfunction in the adenosinergic pathway can have a major impact on the synaptic and network activity. In the present chapter I will investigate the effects of pre-treating slices with creatine or RibAde on basal synaptic transmission by measuring their effects on a number of parameters (discussed below). One of the parameters measure is paired-pulse facilitation. Paired-pulse facilitation is the ability of synapses to increase neurotransmitter release on the second of two closely spaced afferent stimulations (50 ms in these experiments). Paired-pulse facilitation can be viewed as a pre-synaptic form of short-term plasticity, as it depends on the residual Ca^{2+} concentrations in the pre-synaptic terminal. If a pulse is given for example 50 ms after an initial stimulation, this does

not allow for the clearance of residual Ca^{2+} from the pre-synaptic. The second stimulation leads to an increase in pre-synaptic Ca^{2+} concentrations, which is then associated with a greater release of neurotransmitters and a larger second pulse. If the second pulse slope is larger than the first then the paired-pulse ratio will be high. The paired-pulse ratio is inversely related to paired-pulse facilitation whereby a high ratio would indicate reduced paired-pulse facilitation.

In addition to measures of basal synaptic transmission, I will also investigate the effects of creatine on seizure activity by measuring the inter spike interval, inter burst interval and burst duration.

5.2. Results

The results from the microelectrode biosensors indicate that slices pre-treated with RibAde release a greater amount of seizure-dependent adenosine from slices compared to creatine-treated and control slices. To determine whether this modulation of adenosine release had a bearing on basal neuronal activity and on seizure parameters, input-output curves, paired-pulse facilitation, burst duration, inter spike interval and inter burst interval were measured.

Two separate experiments were performed: 1) where microelectrode biosensors were inserted into the slices to measure the release of adenosine and 2) those without biosensors. Kolmogorov-Smirnov tests were performed to determine if the populations of both groups of experiments were different for each seizure parameter and measurement of basal neuronal activity. No overall differences were found for the seizure parameters and the decision was made to combine the data.

5.2.1. Assessment of the effects of RibAde and creatine pre-treatment on basal neuronal transmission

No difference in field excitatory postsynaptic potential (fEPSP) slope during the input/output curve (Fig 5.1A) was found (One-way ANOVA repeated measures: $p = 0.110$; $F_{(2,14)} = 8.477$; creatine, n = 29; control, n = 53; RibAde, n = 47 slices). Assessment of the fibre-volley amplitude at a stimulus intensity of 300 μA was also not different (Fig 5.1 B) between treatment groups (One-way ANOVA: $p = 0.400$; $F_{(2,33)} = 0.942$; creatine, n = 9; control, n = 17; RibAde, n = 10). Similarly, paired-pulse ratios were found not to be different (Fig 5.1 C; One-way ANOVA: $p = 0.0878$; $F_{(2,107)} = 2.4903$; creatine, n = 22; control, n = 47; RibAde, n = 41).

No difference in the maximum percentage change in fEPSP slope from baseline during washout of Mg^{2+} from the slices (15 minutes; Fig 5.1 D) was found (One-way ANOVA: $p = 0.927$; $F_{(2,99)} = 0.76$; creatine, n = 19; control, n = 50; RibAde, n = 32). These data suggest that pre-treating slices with RibAde or creatine did not influence basal synaptic transmission.

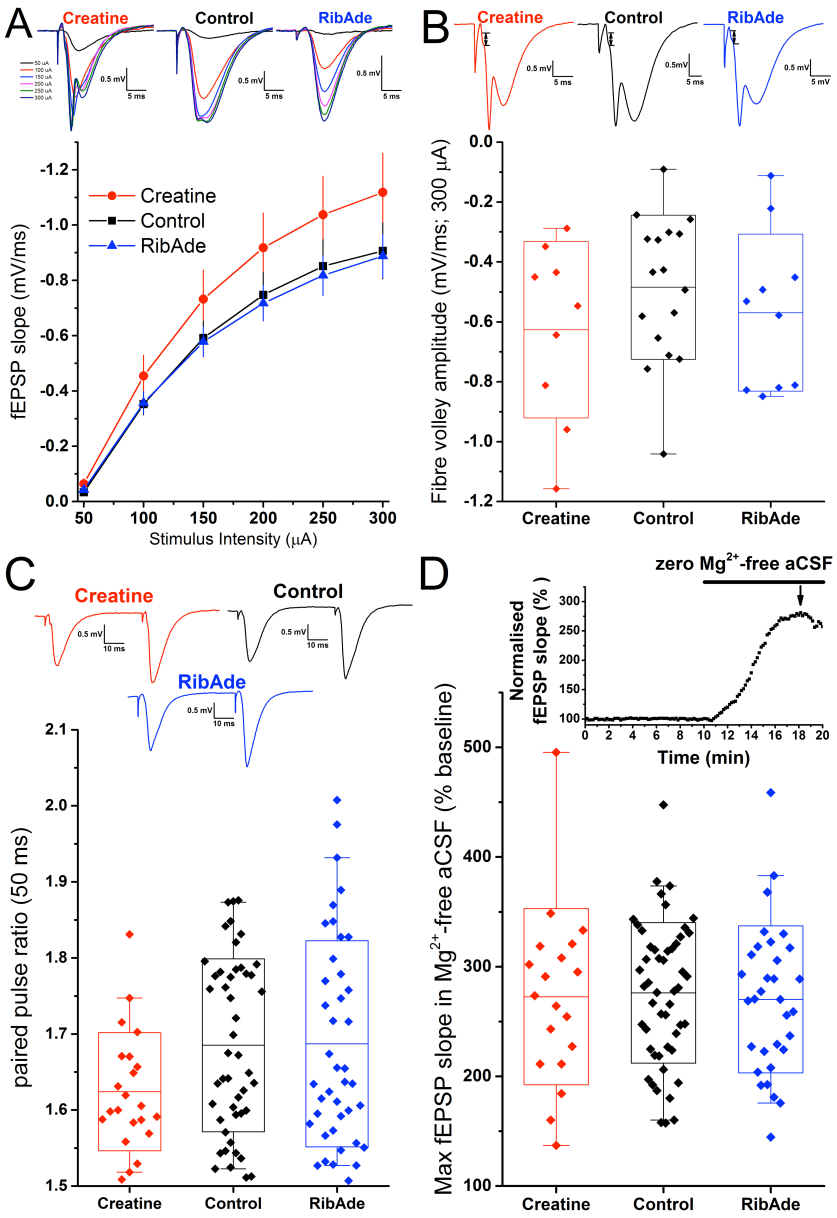

Figure 5.1. **Basal neuronal activity was unaffected by manipulations of intracellular ATP.** Stepwise stimulation current increases were applied to slices to generate an input/output curve (A; top panel shows example potentials generated during the input/output curve) to test for basal synaptic transmission. No difference in input/out curves was found between the three treatments (creatine, n = 28; control, n = 53; RibAde, n = 47). Similarly, the peak amplitude of the fibre volley at 300 µA (B; (⇳) in top panel) was not different (creatine, n = 9; control, n = 17; RibAde, n = 10). The probability of release where paired-pulses were given 50 milliseconds apart (C; example fEPSPs are shown in the top panel) at stimulus intensities between 50-60% of the maximum intensity used during the input/output curve was also not different (creatine, n = 22; control, n = 47; RibAde, n = 41). Slices were left to equilibrate and a baseline of 10 minutes was recorded, after which nominally magnesium-free artificial cerebrospinal fluid (zero Mg^{2+}free aCSF; black bar in inset graph) was used as a means of increasing the excitability of the tissue (15 mins). The maximum field excitatory postsynaptic potential (fEPSP) slope during Mg^{2+}-free aCSF (D; arrow head in inset graph) was also not different (creatine, n = 19; control, n = 50; RibAde, n = 32). A) Data shown as mean ± SEM; B-D) Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits.

5.2.2. RibAde and creatine pre-treatment does not influence burst duration

The duration of 0 mM Mg^{2+}/50 μM 4-aminopyridine-induced bursts (defined as a seizure followed a period of quiescence; see chapter 2.9) was not different between the three treatments. Burst durations (Burst 1 - 3) were not different (Fig 5.2) between control, creatine- and RibAde-treated slices (Table 5.1; Burst 1: One-way ANOVA, $p = 0.848$; $F_{(2,83)} = 0.166$; creatine, n = 24; control, n = 32; RibAde, n = 30 slices); (Burst 2: One-way ANOVA, $p = 0.150$; $F_{(2,65)} = 1.954$; creatine, n = 19; control, n = 22; RibAde, n = 27 slices); (Burst 3: One-way ANOVA, $p = 0.883$; $F_{(2,51)} = 0.124$; creatine, n = 15; control, n = 17; RibAde, n = 22 slices).

Combined with the adenosine release data in chapter 4, the data burst duration data suggests that there may not be a relationship between burst-dependent release of adenosine and the length of a seizure. It may be possible that an increase in the release of adenosine observed during burst 1 might change the other seizure parameters such as the intensity and/or the frequency of seizures.

Table 5.1. Summary of burst 1-3 durations. Measurements made in creatine, control and RibAde-treated slices. Data shown as mean ± SEM; n = 15-32 slices.

Burst number	Burst duration		
	Creatine (s)	Control (s)	RibAde (s)
1	45.8 ± 5.5 (n = 24)	45.1 ± 5.5 (n = 32)	49.0 ± 4.5 (n = 30)
2	29.0 ± 3.6 (n = 19)	30.8 ± 3.8 (n = 22)	38.6 ± 3.8 (n = 27)
3	20.6 ± 3.4 (n = 15)	23.0 ± 3.9 (n =17)	49.0 ± 2.8 (n = 22)

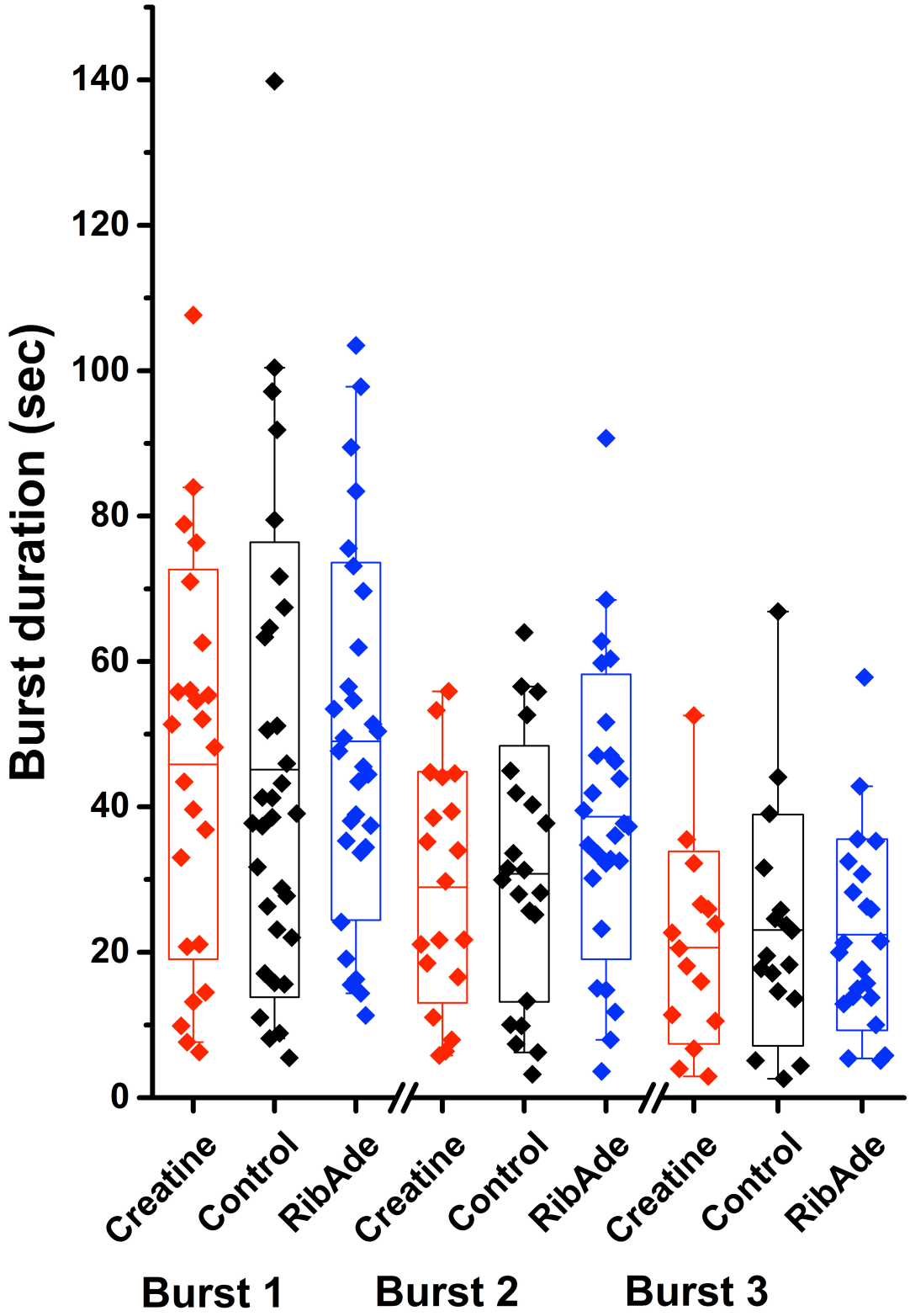

Figure 5.2. Modulation of intracellular ATP does not influence burst duration. The length of bursting activity induced with 50 µM 4-aminopyridine was not different across groups. Note the burst-dependent decreases in the length of seizures, which is common amongst all treatments. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Burst 1: creatine, n = 24; control, n = 32, RibAde, n = 30. Burst 2: creatine, n = 19; control, n = 22; RibAde, n = 27. Burst 3: creatine, n = 15; control, n = 17; RibAde, n = 22.

5.2.3. RibAde pre-treatment reduced seizure spiking intensity

To test whether there is a relationship between adenosine release and seizure intensity the inter spike interval (ISI) was measured. Although the durations of the seizure bursts were not different between treatments (Fig 5.2), seizure spiking intensity was significantly reduced (increased ISI) in RibAde-treated slices (Fig 5.3; Table 5.2) compared to creatine slices during burst 1 (Fig 5.3 A-B; Kolmogorov-Smirnov test: Burst 1: $p < 0.0001$; $D = 0.53$; $Z = 1.95$; creatine, n = 24; RibAde, n = 30 slices) and burst 2; (Fig 5.3 C-D; Kolmogorov- Smirnov test: Burst 2: $p = 0.003$; $D = 0.52$; $Z = 1.72$; creatine, n = 19; RibAde, n = 27 slices). The Inter Spike Interval of control slices was broadly intermediate to the ISI of creatine- and RibAde-treated slices and was not significantly different compared to creatine- and RibAde-treated slices during burst 1 (Kolmogorov-Smirnov test: creatine vs. control, $p = 0.36$; $D = 0.24$; $Z = 0.89$; RibAde vs. control, $p = 0.11$; $D = 0.29$; $Z = 1.16$; control, n = 32 slices) and burst 2 (Kolmogorov-Smirnov test: (creatine vs. control, $p = 0.16$; $D = 0.33$; $Z = 1.07$; RibAde vs. control, $p = 0.16$; $D = 0.31$; $Z = 1.07$; control, n = 22 slices).

In addition, no difference in Inter Spike Interval was observed during burst 3 (creatine, n = 15; control, n = 7; RibAde, n = 22 slices) between treatments (Fig 5.3 E-F; Kolmogorov-Smirnov test: creatine vs. control, $p = 0.82$; $D = 0.20$; $Z = 0.58$; RibAde vs. control, $p = 0.86$; $D = 0.18$; $Z = 0.55$; creatine vs. RibAde, $p = 0.45$; $D = 0.27$; $Z = 0.80$).

These data suggest that the differences in the release of adenosine found in RibAde- and creatine-treated slices produce differential effects on the intensity of seizures. In contrast to RibAde-treated slices, when adenosine levels are reduced as measured in

creatine slices, there is less inhibition of neuronal excitability resulting in an increase in spiking intensity.

Table 5.2. Summary of Kolmogorov-Smirnov test for the inter spike interval of burst 1-3. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to RibAde; n = 9-32.

Kolmogorov-Smirnov test	**Burst number**		
	1	**2**	**3**
Creatine vs. Control	p = 0.36 D = 0.24 Z = 0.89	p = 0.16 D = 0.33 Z = 1.07	p = 0.82 D = 0.20 Z = 0.58
Creatine vs. RibAde	p < 0.0001*** D = 0.53 Z = 1.95	p = 0.003** D = 0.52 Z = 1.72	p = 0.45 D = 0.27 Z = 0.80
RibAde vs. Control	p = 0.11 D = 0.29 Z = 1.16	p = 0.16 D = 0.31 Z = 1.07	p = 0.86 D = 0.18 Z = 0.55

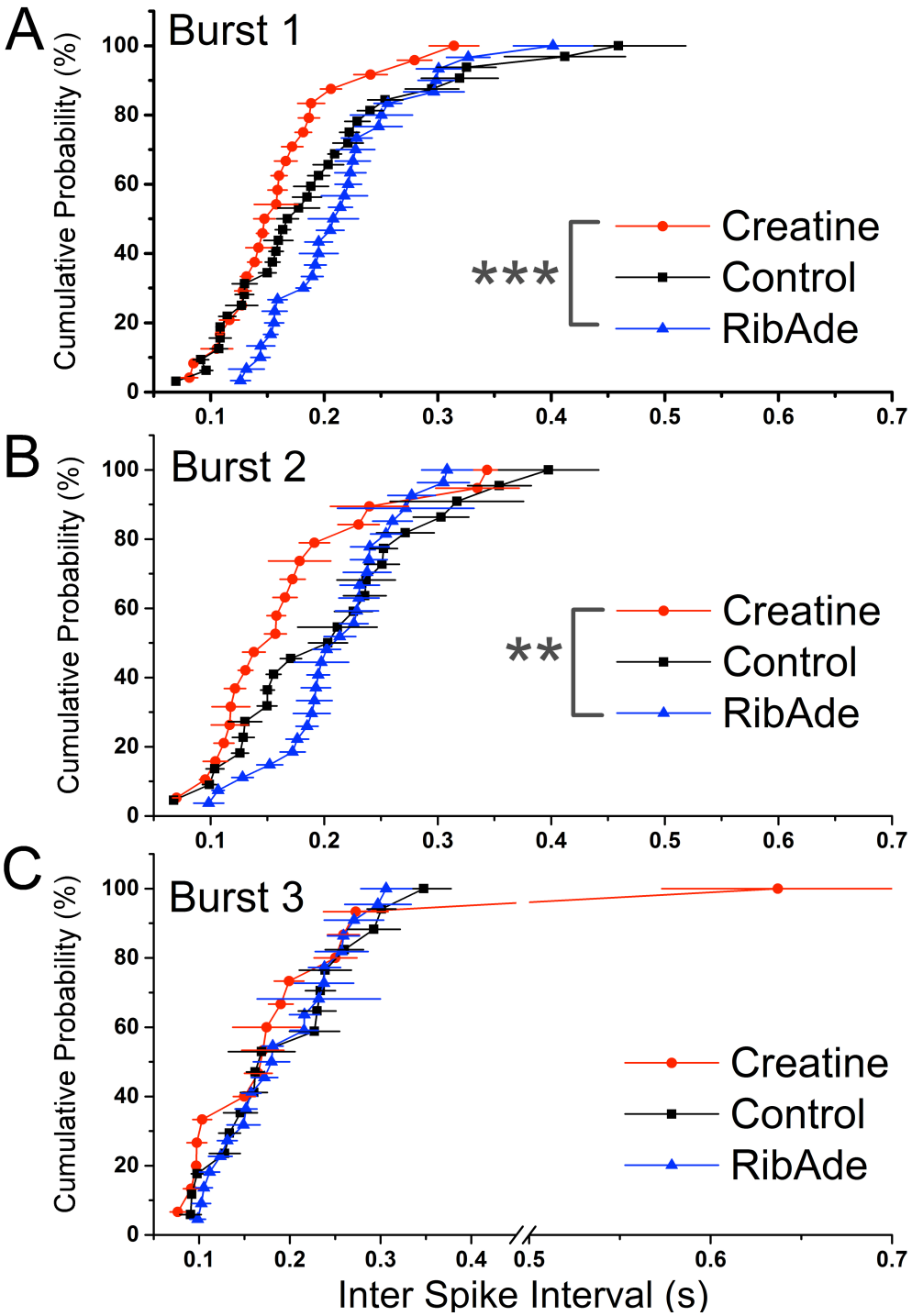

Figure 5.3. Modulation of adenosine release influences seizure intensity. Burst 1 (A-B) cumulative probability (A) and histogram of the frequency counts (B) of the Inter Spike Interval was reduced (increased spiking frequency) in slices pre-treated with RibAde compared to creatine-treated slices (increased spiking frequency). Similarly, during burst 2 (C-D), slices treated with creatine showed increased spiking frequency compared to RibAde-treated slices. No difference in the Inter Spike Interval during burst 3 (E-F) was found. Control slices were not found to be significantly different in spiking frequency to creatine or RibAde-treated slices. For cumulative probability plots, individual symbols represent a slice. Burst 1: creatine, n = 24; control, n = 32, RibAde, n = 30. Burst 2: creatine, n = 19; control, n = 22; RibAde, n = 27. Burst 3: creatine, n = 15; control, n = 17; RibAde, n = 22. Data shown as the mean ± SEM. Significances indicated by ***$p < 0.001$ and **$p < 0.01$.

5.2.4. Influence of adenosine A_1 receptor antagonism on spiking intensity

The adenosine A_1 receptor antagonist CPT (1 μM) was added to slices after 3 bursts, which disrupted synchronous bursting and produced continuous spiking activity at a slower rate (Fig 4.3A). Here, analysis of ISI was restricted to slices showing 3 bursts and the effect of CPT on ISI was measured over durations identical to that of the preceding Burst 3. Challenge with CPT reduced (Fig 5.4) spiking intensity in control slices (0.19 ± 0.02 sec in burst 3 and 0.98 ± 0.22 sec in CPT; paired t-test: $p = 0.001$; $t_{(22)} = -3.63$; Burst 3 = 12 slices; CPT = 12 slices) and RibAde-treated slices (0.23 ± 0.03 sec in burst 3 and 1.64 ± 0.52 sec in CPT for RibAde slices; paired t-test: $p = 0.013$; $t_{(20)} = -2.71$; Burst 3 = 11; CPT = 11 slices).

Surprisingly, application of CPT in creatine slices (Fig 5.4 top panel) did not result in any significant change in ISI compared to burst 3 (0.21 ± 0.02 sec in burst 3 and 1.21 ± 0.53 in CPT; t-test: $p = 0.080$; $t_{(12)} = -1.91$; Burst 3 = 7; CPT = 7 slices).

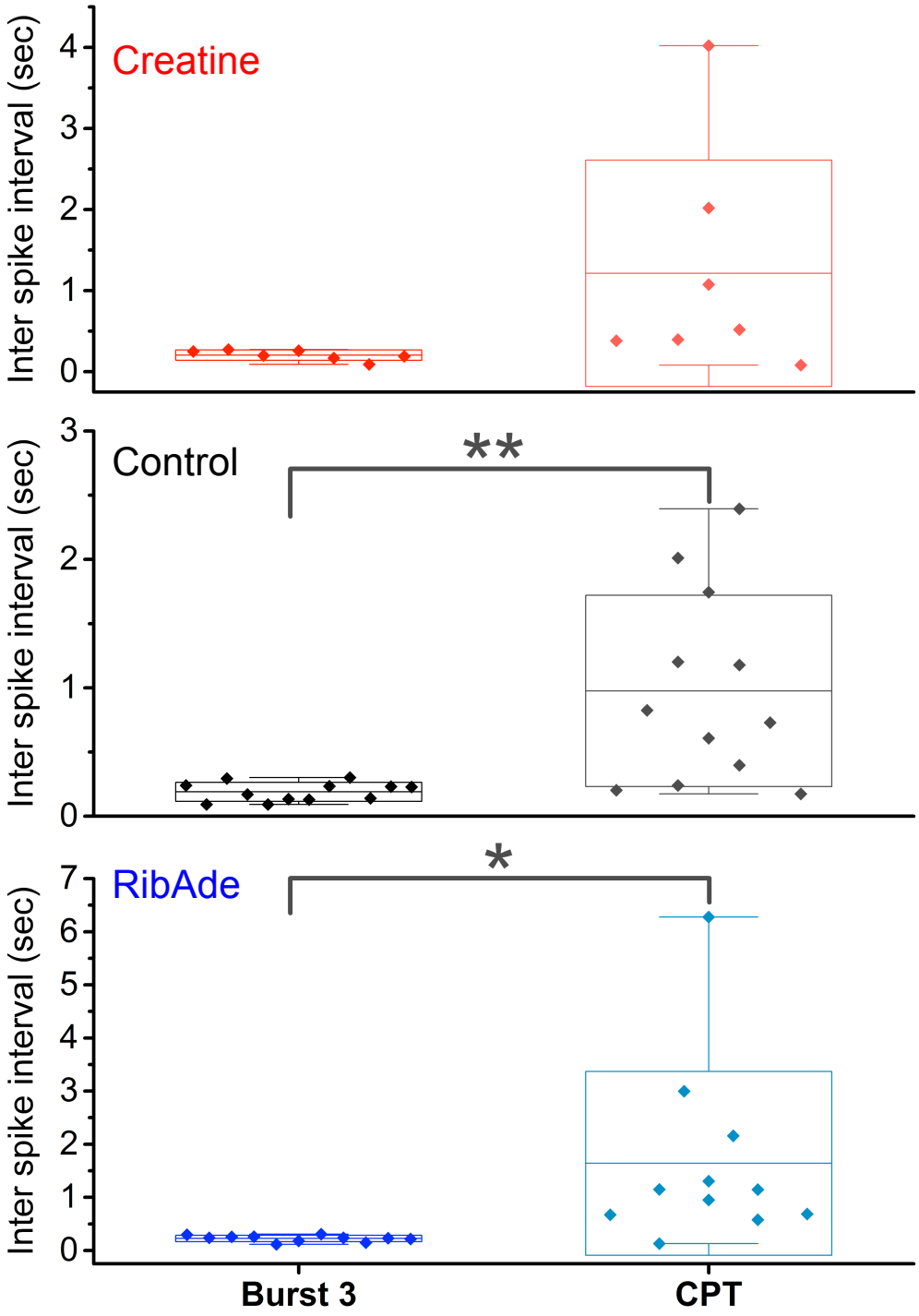

Figure 5.4. Effect of antagonism of the adenosine A_1 receptor on inter spike interval. During continuous spiking activity induced by 1 μM 8-cyclopentyltheophylline (CPT) the Inter Spike Interval in control (middle panel) and RibAde-treated slices (bottom panel) was increased. Only slices showing 3 bursts were used for these comparisons and the duration of activity in CPT over which measurements of ISI were made was identical to that of the preceding burst 3. The Inter Spike Interval in CPT was not different in creatine-treated slices (top panel). Burst 3: creatine, n = 7; control, n = 12; RibAde, n = 11. CPT: creatine, n = 7; control, n = 12; RibAde, n = 11. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by $**p < 0.01$ and $*p < 0.05$.

5.2.5. Assessment of the effect RibAde and creatine pre-treatment on seizure frequency

To further test whether creatine and RibAde had an effect on the rate at which bursting occurred, the Inter Burst Interval was measured (Fig 5.5). The time of occurrence of burst 2 (Inter Burst Interval 1) in RibAde slices was delayed (107.8 ± 9.3 sec) compared to creatine-treated (61.19 ± 7.38 sec; Post-hoc Bonferroni: $p < 0.0001$) and control slices (76.5 ± 5.8 sec; Post-hoc Bonferroni: $p = 0.018$; creatine, n = 19; control, n = 22; RibAde, n = 27 slices; One-way ANOVA: $p < 0.0001$; $F_{(2,65)} = 8.983$)

Similarly, the time to burst 3 (Inter Burst Interval 2) was increased (One-way ANOVA: $p = 0.008$; $F_{(2,50)} = 5.398$; creatine, n = 15; control, n = 17; RibAde, n = 22 slices) in RibAde slices (93.2 ± 8.4 s) compared to creatine (63.88 ± 7.59 sec; Post-hoc Bonferroni: $p = 0.031$) and control slices (63.5 ± 5.8 sec; Post-hoc Bonferroni: $p = 0.019$). These data are consistent with the idea that there is a role for adenosine in regulating the timing of seizure activity.

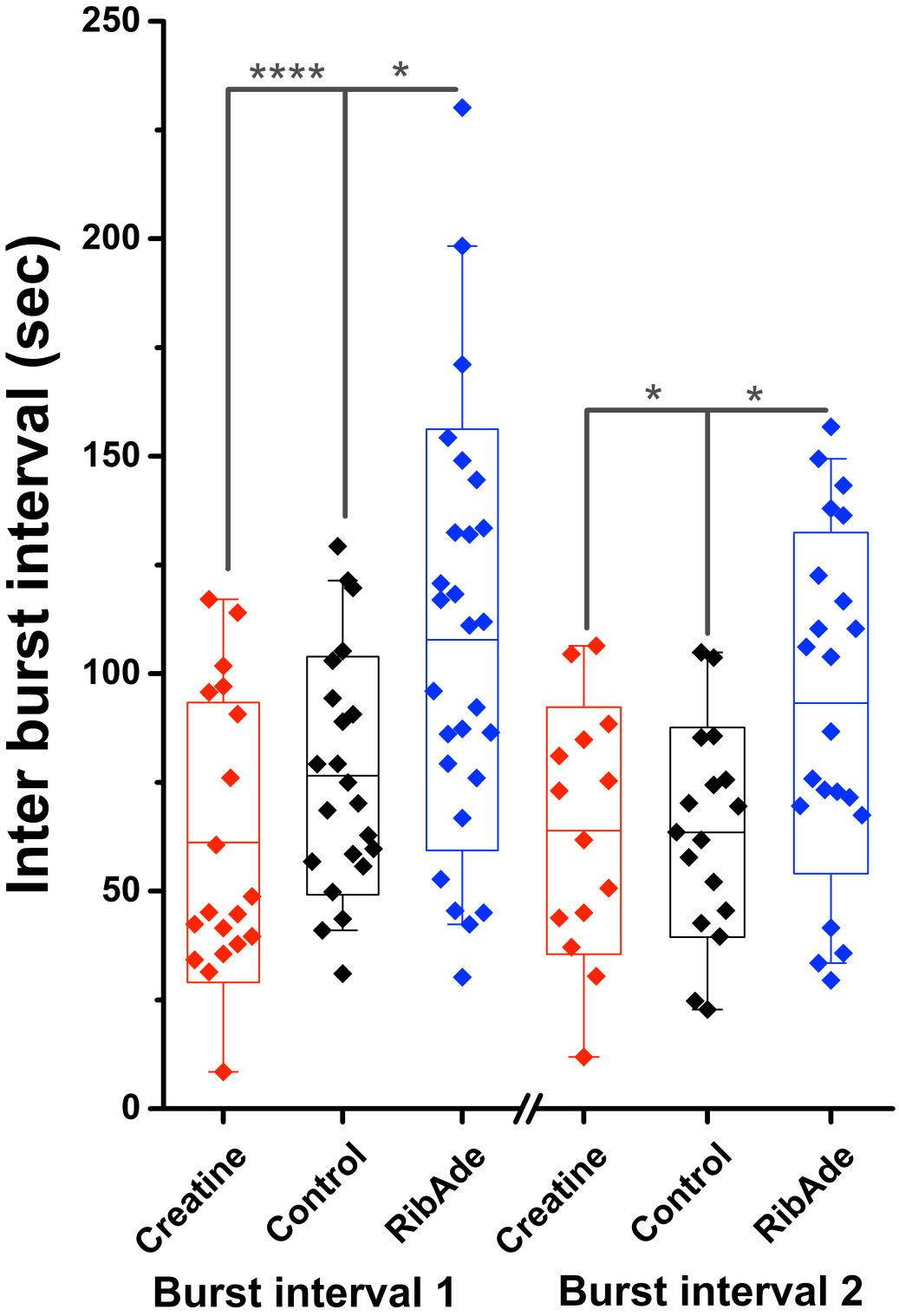

Figure 5.5. Modulation of intracellular ATP and adenosine release influences the onset of seizure activity. The time to burst 2 (Burst Interval 1) was greatly reduced in creatine-treated (n = 19) and control (n = 22) slices compared to RibAde-treated slices (n = 27). Similarly, the time to burst 3 (Burst Interval 2) was reduced in creatine-treated (n = 14) and control (n = 17) slices compared to RibAde-treated slices (n = 22). Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by ****$p < 0.001$ and *$p < 0.05$.

5.2.3. Assessment of the role of the adenosine A_{2A} receptor in regulating seizure parameters

In RibAde-treated slices during initial washout of Mg^{2+} from slices an increase in the number of spontaneous seizures (25%) compared to (19%) in creatine and (6%) in control slices. It is possible that these observations are due to the activation of the adenosine A_{2A} receptors, as studies have shown that these receptor subtypes show mild pro-convulsive behaviour in nominally Mg^{2+}-free aCSF when seizure activity was induced electrically (Etherington and Frenguelli, 2004). To further investigate if this increase in excitation was due to an adenosine A_{2A} receptor component the selective antagonist 50 nM SCH 58261 (SCH) was used. SCH is a potent and selective non-xanthine A_{2A} adenosine receptor antagonist (K_i = 1.3 nM) in in vitro slice preparations and a concentration at 50 nM has been shown to effectively block the A_{2A}R in a number of preparations (Marcoli*, et al.*, 2003; Melani*, et al.*, 2003; Stone, 2002; Zocchi*, et al.*, 1996). RibAde-treated and control slices were pre-incubated for 10 minutes in 50 nM SCH. After 10 minutes, 50 μM 4-AP + SCH was perfused to induce seizures.

5.2.3.1. Assessment of the role adenosine A_{2A} receptors play in the regulation of seizure length and frequency

Pre-incubation of slices with 50 nM SCH (Table 5.3), had no effect on burst duration (Fig 5.6A; One-way ANOVA: p = 0.387; $F_{(3,83)}$ = 1.021; control, n = 32; control + 50 nM SCH, n = 14; RibAde, n = 30; RibAde + 50 nM SCH, n = 11 slices); (Burst 2: One-way ANOVA: p = 0.378; $F_{(3,64)}$ = 1.047; control, n = 22; control + 50 nM SCH, n = 11; RibAde, n = 27; RibAde + 50 nM SCH, n = 8 slices); (Burst 3: One-

way ANOVA p = 0.611; $F_{(3,46)}$ = 0.612; control, n = 17; control + 50 nM SCH, n = 6; RibAde, n = 22; RibAde + 50 nM SCH, n = 5 slices).

Table 5.3. Summary of the effect of SCH 58261 on burst duration. Measurements made in control and RibAde-treated slices; n = 11-32 slices; Data shown as mean ± SEM.

Burst duration	Control (s)	Control + SCH (s)	RibAde (s)	RibAde + SCH (s)
1	45.12 ± 5.53 (n = 32)	39.08 ± 6.75 (n = 14)	49.01 ± 4.49 (n = 30)	34.21 ± 5.18 (n = 11)
2	30.79 ± 3.75 (n = 22)	28.84 ± 5.30 (n = 11)	38.64 ± 3.77 (n = 27)	26.56 ± 6.19 (n = 8)
3	23.05 ± 3.86 (n = 17)	26.56 ± 6.19 (n = 6)	22.42 ± 2.80 (n = 22)	15.19 ± 4.908 (n = 5)

Antagonism of slices with 50 nM SCH did not affect the timing of bursting seizure activity (Fig 5.6 B; Table 5.4). The time to Burst Interval 1 was longer in RibAde-treated slices (Post-hoc Bonferroni, p = 0.043) compared to control slices (One-way ANOVA: p = 0.021; $F_{(3,64)}$ = 3.489; control, n = 22; control + 50 nM SCH, n = 11; RibAde, n = 27; RibAde + 50 nM SCH, n = 8 slices).

Burst Interval 2 was not significantly different (One-way ANOVA: p = 0.051; $F_{(3,46)}$ = 2.794; control, n = 17; control + 50 nM SCH, n = 11; RibAde, n = 22; RibAde + 50 nM SCH, n = 8 slices) although a trend for a increase in the time to burst 3 was observed for RibAde-treated slices compared to control slices (Post-hoc Bonferroni: p = 0.053).

Table 5.4. Summary of the effects of SCH 58261 on inter burst interval. Measurements made in control and RibAde-treated slices. One-way ANOVA: $^{*}p < 0.05$ compared to RibAde-treated slices; n = 8-27 slices. Data shown as mean ± SEM.

Burst Interval	Control (s)	Control + SCH (s)	RibAde (s)	RibAde + SCH (s)
1	76.53 ± 5.84* (n = 22)	86.14 ± 11.2 (n = 11)	107.79 ± 9.32 (n = 27)	115.25 ± 11.67 (n = 8)
2	63.50 ± 5.85 (n = 17)	82.15 ± 16.40 (n = 6)	93.23 ± 8.36 (n = 22)	65.42 ± 11.39 (n = 5)

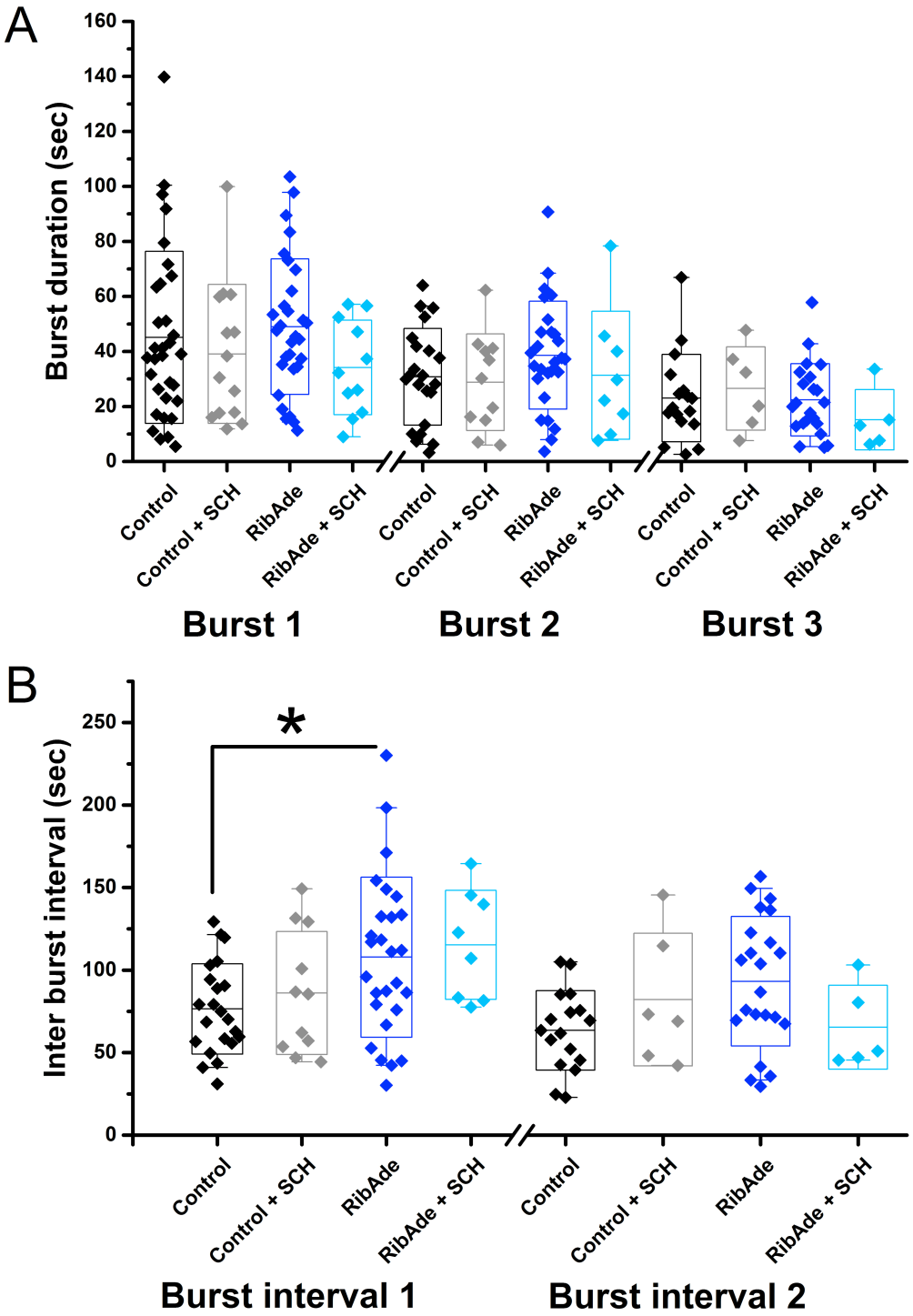

Figure 5.6. Assessment of the effects of the adenosine A_{2A} receptor on burst duration and frequency. Pre-incubating slices with the adenosine A_{2A} receptor antagonist SCH 58261 (SCH; 50 nM) did not influence the length of bursts (A) (Burst 1: control, n = 32, control + SCH, n = 14; RibAde, n = 30; RibAde + SCH, n = 11. Burst 2: control, n = 22; control + SCH, n = 11; RibAde, n = 27, RibAde + SCH, n = 8. Burst 3: control, n = 17; control + SCH, n = 6; RibAde, n = 22; RibAde + SCH, n = 5). Burst 2 frequency (B) in control slices (n = 22) was higher compared to RibAde-treated (n = 27) slices. Control + SCH (n= 11) was not different in the frequency of seizures compared to control slices. Similarly, frequency of seizures in RibAde + SCH (n = 8) slices was not different to RibAde-treated slices. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicted by *$p < 0.05$.

5.2.3.2. Assessment of the role adenosine A_{2A} receptors play in the regulation of seizure intensity

The Inter Spike Interval of control + SCH slices during burst 1 was found to be increased (Fig 5.7 A-B; Table 5.5) compared to control slices (Kolmogorov-Smirnov test: p = 0.039; D = 0.43; Z = 1.34; control, n = 32; control + 50 nM SCH, n = 14 slices). RibAde + SCH slices were not different to RibAde slices (Kolmogorov-Smirnov test: p = 0.424; D = 0.29; Z = 0.82; RibAde, n = 30; RibAde + 50 nM SCH, n = 11 slices). Similarly, Control + SCH vs. RibAde + SCH was not different (Kolmogorov-Smirnov test: p = 0.579; D = 0.23; Z = 0.72; control + 50 nM SCH, n = 14; RibAde + 50 nM SCH, n = 11 slices). No differences were found during burst 2 (Fig 5.7 C-D; Kolmogorov-Smirnov test: control vs. control + SCH, p = 0.271; D = 0.36; Z = 0.99; RibAde vs. RibAde + SCH, p = 0.575; D = 0.29; Z = 0.73; control + SCH vs. RibAde + SCH, p = 0.87; D = 0.25; Z = 0.54; control, n = 22; control + 50 nM SCH, n = 11; RibAde, n = 27; RibAde + 50 nM SCH, n = 8 slices) or burst 3 (Fig 5.7 E-F; Kolmogorov-Smirnov test: control vs. control + SCH, p = 0.126; D = 0.53; Z = 1.15; RibAde vs. RibAde + SCH, p = 0.500; D = 0.37; Z = 0.75; control + SCH vs. RibAde + SCH, p = 0.591; D = 0.43; Z = 0.72; control, n = 17; control + 50 nM SCH, n = 6; RibAde, n = 22; RibAde + 50 nM SCH, n = 5 slices).

In summary, the SCH data shows that pre-incubating slices with the adenosine A_{2A} receptor antagonist SCH 28561 for 10 minutes did not influence seizure activity in RibAde-treated slices. However, in control slices SCH was found to reduce spiking intensity in a time-dependent manner, whereby only the first burst was influenced by A_{2A} antagonism. These data suggest that under normal conditions, the adenosine A_{2A} receptors may play an excitatory role in the sense of promoting increased spiking intensity.

Table 5.5. Summary of Kolmogorov-Smirnov test for the inter spike interval of burst 1-3 with SCH 58261. $^{*}p < 0.05$ compared to control; n = 5-32.

Kolmogorov-Smirnov test	Burst number		
	1	**2**	**3**
Control + SCH vs. Control	p = 0.04* D = 0.43 Z = 1.34	p = 0.27 D = 0.36 Z = 0.99	p = 0.13 D = 0.53 Z = 1.15
RibAde + SCH vs. RibAde	p = 0.42 D = 0.29 Z = 0.82	p = 0.58 D = 0.29 Z = 0.73	p = 0.5 D = 0.37 Z = 0.75
Control + SCH vs. RibAde + SCH	p = 0.58 D = 0.23 Z = 0.72	p = 0.87 D = 0.25 Z = 0.54	p = 0.59 D = 0.43 Z = 0.72

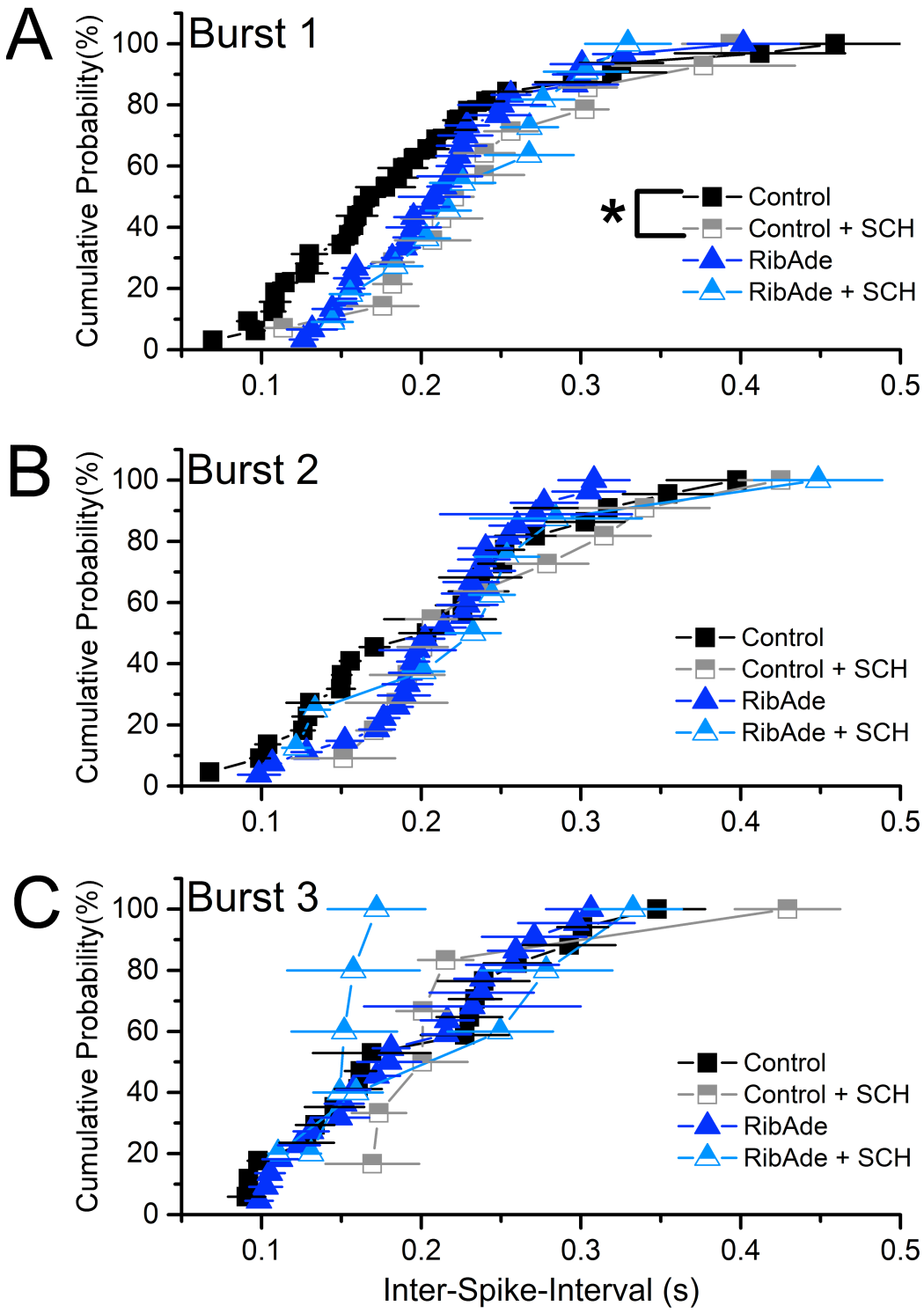

Figure 5.7. Assessment of the effects of partial inhibition of the adenosine A_{2A} receptor on seizure intensity. Pre-incubating slices with 50 nM SCH 59261 (SCH), an adenosine A_{2A} receptor antagonist, reduced (A-B) the inter-spike-interval in control+ SCH (grey line and symbols) compared to control slices (black lines and symbols) during burst 1 cumulative probability (A) and histogram of the frequency counts (B) of the Inter Spike Interval. In contrast, during burst 2 (C-D) and burst 3 (E-F) no difference was found. (Burst 1: control, n = 32, control + SCH, n = 14; RibAde, n = 30; RibAde + SCH, n = 11. Burst 2: control, n = 22; control + SCH, n = 11; RibAde, n = 27, RibAde + SCH, n = 8. Burst 3: control, n = 17; control + SCH, n = 6; RibAde, n = 22; RibAde + SCH, n = 5). Data shown as mean ± SEM and significances indicted by $*p < 0.05$.

5.3. Discussion

In summary these data show that pre-treating slices with RibAde and creatine has a measurable consequence on seizure frequency and intensity.

5.3.1. Effect of RibAde and creatine on basal synaptic transmission

In support of what has been shown (zur Nedden*, et al.*, 2014; zur Nedden*, et al.*, 2011) RibAde and creatine pre-treatment does not affect basal neuronal transmission. These data suggest that under conditions where intracellular ATP are recovered close to *in vivo* levels with RibAde or where the degradation of ATP is buffered with creatine, there is no increase in basal adenosine tone, and low-frequency stimulation of afferent fibres does not result in the release of adenosine into the extracellular space. This would be expected to cause activation of presynaptic adenosine A_1Rs, inhibition of glutamate release and hence result in a decrease in the probability of release and an increase in paired-pulse facilitation. Similarly, in slices incubated for 2 hours with RibAde although adenosine release was increased during theta burst stimulation no difference in long-term potentiation was found (zur Nedden*, et al.*, 2011). It may also be possible that other non-neuronal regulatory stimuli such as astrocytic adenosine kinase (ADK) may play a role in modulating adenosine release and synaptic transmission. Studies have shown that mutant ADK mice showed larger facilitation when ADK was under expressed compared to wildtype mice and mutant ADK with an over expression of ADK (Diógenes*, et al.*, 2014). These researchers also showed that mice under expressing ADK showed greater adenosine release using biosensors and longer post- theta burst stimulation depression of synaptic

transmission and weaker potentiation of the fEPSP compared to wildtype mice. In contrast, mice over expressing ADK showed brief transient depression of the fEPSP and lower levels of adenosine release. This research provides evidence for the role of ADK to regulate the degree of tonic adenosine- dependent synaptic inhibition.

5.3.2. Pre-treatment of slices with RibAde reduces the intensity and frequency of seizures

Pre-treating slices with RibAde, as opposed to creatine, resulted in the reduction in the intensity and frequency of seizures. In contrast, creatine pre-treatment increased the frequency and intensity of seizures. Similar to what has been previously shown my data suggest that adenosine may play a role in the temporal regulation of seizures (Lopatář*, et al.*, 2015).

In the literature, creatine has been shown to have both anticonvulsant and pro-convulsant properties. Although creatine administration has been shown to be well tolerated in both humans (Gualano*, et al.*, 2011c) and rodents (Rebaudo*, et al.*, 2000; Zhu, 2004) continuous dosing for prolonged periods of time (weeks) is needed (Dechent*, et al.*, 1999; Lyoo*, et al.*, 2003). This is because, uptake of creatine occurs against a large concentration gradient across the blood-brain barrier where an inverse relationship exists between intracellular creatine concentration and creatine uptake (Dodd*, et al.*, 1999). Following administration of creatine, extracellular concentrations increases, this leads to an initial intracellular rise followed by a down regulation in creatine concentration. In order to main high levels of intracellular

pools of creatine continuous administration of creatine is needed to raise brain creatine levels.

Acute application and creatine supplementation has been shown to be protective against pentylenetetrazol-induced (Rambo*, et al.*, 2013; Rambo*, et al.*, 2009) and L-methylmalonic acid- induced seizures (Royes*, et al.*, 2003). In comparison, creatine supplementation failed to protect against seizure susceptibility after traumatic brain injury (Saraiva*, et al.*, 2012) and seizure activity was blocked in creatine kinase knockout mice (CK-/-) suggesting that creatine may play a convulsive role (Streijger*, et al.*, 2010). The authors in the Streijger (2010) study suggest that creatine causes seizures. However, given my results I believe that it is the reduced availability of adenosine in the (CK-/-) mice that is at fault in creatine causing seizures.

In contrast to the evidence, which supports the use of creatine as an anticonvulsant my experiments shows that creatine has convulsant tendencies. Of the three treatments the tendency towards convulsant activity during 4-AP-induced epileptiform activity is creatine > control > RibAde. This is likely due to the reduced release of adenosine observed in creatine slices, which would result in less activation and resultant inhibitory effects mediated by the adenosine A_1Rs. The differences reported here and in experiments where creatine was found to be protective might be due to the length of pre-treatment tested in this thesis (3 hours) and those that report anticonvulsive effects after acute challenge with creatine (30 and 45 minutes) and creatine supplementation (45 minutes, 5 times a week for 5 weeks). With regards to the acute *in vivo* studies it might be possible that during 30-45 minutes there is a rise in intracellular creatine concentration capable of producing the short-term effects reported (Rambo*, et al.*, 2013; Royes*, et al.*, 2003). It can be argued that after 3 hours incubation in creatine the intracellular creatine concentrations may have returned to

baseline. Similarly, the continuous oral administration used in (Rambo*, et al.*, 2009) is similar to dosage regime used in humans (Gualano*, et al.*, 2011a; Gualano*, et al.*, 2011b; Gualano*, et al.*, 2011c) that is successfully raises creatine concentration high enough to see appreciable effects.

5.3.3. Influence of A_1 and A_{2A} receptors on seizure activity

The activation of the adenosine A_1Rs is associated with the inhibition of the release of many neurotransmitters where the most prominent inhibitory actions are generally on excitatory glutamatergic systems. In regards to epilepsy, increases in the levels of glutamate in both humans and rodents has been found been reported (During and Spencer, 1993; Ronne-Engström*, et al.*, 1992; Wilson*, et al.*, 1996), therefore inhibition of the glutamatergic systems may be a useful tool in the treatment of epilepsy.

Antagonism of the A_1Rs receptors would result in a loss of the inhibitory effects of the A_1Rs. Here we found that application of the adenosine A_1R antagonist 8-CPT (1 μM) disrupted the synchronous bursting seizure activity observed in 4-AP, suggesting that the adenosine A_1Rs maybe involved in the timing of seizure activity, possibly through its presynaptic inhibition of excitatory neurotransmitter release. Bursting, and the energy use associated with this neuronal spiking activity, results in breakdown of ATP and release of adenosine into the extracellular space. Adenosine can reach the extracellular space either through exocytosis of ATP, and extracellular breakdown of ATP by ectonucleotidases to adenosine, or directly, along its concentration gradient via ENTs. Under standard conditions extracellular adenosine binds the A_1R and exerts negative feedback on network activity by hyperpolarising cells, and reducing release probability pre-synaptically, as described above.

However, extracellular adenosine concentrations are tightly regulated; importantly, the extracellular enzyme ecto-adenosine deaminase rapidly metabolises adenosine to inosine, and adenosine also diffuses through ENTs back into the intracellular space. Following clearance of extracellular adenosine, the network can return to the bursting state and thus the feedback loop continues. However, following application of the A_1R antagonist CPT, this adenosine negative feedback loop is disrupted, and adenosine only influences network activity when extracellular adenosine concentrations are sufficient to out-compete this competitive antagonist, which contributes to the continuous spiking observed in CPT.

To further assess the difference in the intensity of seizures in CPT and 4-AP the ISI during burst 3 and CPT (duration adjusted to burst 3) were calculated. The results showed that the ISI during CPT in control and RibAde was increased (reduced intensity) whereas no difference in ISI was found for creatine. These results are in contrast to what is expected for the actions of CPT on seizure intensity. It would be expected that an inhibition of the adenosine A_1R with CPT should result in an increase in the intensity of seizures as measured by a reduction in ISI measurements as well as a reduction in the number of spikes per unit time as reported in (Lopatář*, et al.*, 2015). Here surprisingly, the opposite was found where the intensity of seizures as measured by the ISI was reduced in CPT, similar to the actions of CPT on seizures induced by the Group I metabotropic glutamate receptor agonist DHPG (Lopatář*, et al.*, 2015). CPT produced continuous spiking activity, which might be expected to be associated with additional load on neurons and glial cell. CPT also resulted in an increase in the release of adenosine compared to that measured directly before (Table 4.1 and 4.2). Given that the amounts of adenosine present in slices during CPT was greater than 1 μM´ s this might be sufficient to compete with the actions of 1 μM

CPT, given that CPT has a greater affinity for the A_1R, and thereby reduce the antagonism effects of CPT on ISI. In creatine treated slices where adenosine release was reduced this provided less completion with CPT, which did not result in any difference in CPT, related ISI.

In the hippocampus the A_1Rs are the most abundant subtypes and adenosine acts with high affinity at these receptor (Burnstock, 2013; Fredholm, 2011b; Masino and Boison, 2013; Sebastião and Ribeiro, 2009). The A_2 and the A_3Rs are also found in the hippocampus but are sparsely distributed. A_2Rs are expressed in GABAergic neurons and activation of these in particular the $A_{2A}Rs$, is generally associated with the increase in excitatory effects (Burnstock, 2013; Fredholm, 2011b; Masino and Boison, 2013; Sebastião and Ribeiro, 2009). An increase in spontaneous seizures during initial washout of Mg^{2+} from RibAde-treated slices was observed. To investigate whether this response was due to an excitatory $A_{2A}R$ response slices were pre-treated for 10 minutes with the competitive A_{2A} antagonist SCH 58261. In my data adenosine $A_{2A}Rs$ do not appear to play a significant role in the regulation of seizure activity in RibAde-treated slices. It may be possible that the adenosine effects in these slices were predominantly on A_1Rs and that minor effects on the $A_{2A}Rs$ could have been obscured. In control slices however, the adenosine $A_{2A}Rs$ appear to play a role in the regulation of the intensity of seizures (Fig 4.12) suggesting that the adenosine $A_{2A}Rs$ may play an excitatory role in the sense of promoting increased spiking intensity under normal conditions. These data are similar to studies where the $A_{2A}Rs$ were found to be midly pro-convulsive in electrically evoked seizure activity in Mg^{2+}-free aCSF (Etherington and Frenguelli, 2004).

In summary, these data suggest that pre-incubating slices for 3 hours with RibAde followed by washout out of RibAde from slices in normal aCSF was sufficient to influence extracellular release of adenosine and thus influence brief seizure activity (20-50s). The appearance of even brief seizures is enough to deplete intracellular energy stores needed to generate seizures and contributes to the release of adenosine. The release of adenosine greatly influenced seizure activity, whereby an increase in adenosine as seen in RibAde-treated slices resulted in a reduction in both seizure intensity and frequency. In comparison, pre-treating slices with creatine resulted in a reduction in adenosine release, which contributed to an increase in seizure intensity and frequency.

These experiments make the assumption that in RibAde-treated slices intracellular ATP is broken down intracellularly to form adenosine, which is then released into the extracellular space where adenosine carries out its inhibitory effects. Therefore, in the next chapter I will further investigate whether the activity-dependent release of adenosine measured in RibAde-treated slices arises from intracellular pools of adenosine.

6. Influence of ‘trapping’ intracellular adenosine with homocysteine thiolactone on the release of adenosine and seizure activity

6.1. Introduction

In chapter 4 I showed that it is possible to increase the activity-dependent release of adenosine and reduce seizure intensity and frequency by pre-treating slices with RibAde. In the present chapter, I will further investigate whether extracellular adenosine, as measured with biosensors, is released as adenosine per se. Additionally, I will test whether 'trapping' intracellular adenosine reverses the effect of RibAde on seizure intensity, frequency and duration. This will be achieved by using homocysteine thiolactone (HTL; 100 μM) to 'trap' intracellular adenosine in the form of S-adenosylhomocysteine thiolactone (SAHTL).

Adenosine is not only a breakdown product of ATP, but can also be made via the transmethylation pathway (chapter 1.8.2.). In this pathway the methyl donor S-adenosylmethio nine (SAM) is synthesized from methionine and ATP by methionine adenosyltransferase (MAT). SAM is further converted to S-adenosylhomocysteine (SAH) by the actions of themethyltransferases. SAH can be further hydrolysed to adenosine and homocysteine by the cytosolic enzyme SAH hydrolase (Cantoni*, et al.*, 1989; Lloyd*, et al.*, 1988; Lloyd*, et al.*, 1993; Mandaviya*, et al.*, 2014; McIlwain and Poll, 1985; McIlwain and Poll, 1986; Schatz*, et al.*, 1983). When adenosine and homocysteine levels are high this reaction is reversed, promoting SAH synthesis. Perfusing slices with HTL increases intracellular HTL levels, which drives the SAH hydrolase reaction towards the production of SAHTL. This means that intracellularly formed adenosine is synthesised to SAHTL and 'trapped' within the cell, effectively reducing intracellular levels of adenosine (Lloyd*, et al.*, 1993; McIlwain and Poll, 1986). L-HTL (0.1- 1.0 mM) was found to significantly (75-80%) reduce both basal and electrically evoked release of adenosine in rat hippocampal slices (Lloyd*, et al.*, 1993; McIlwain and Poll, 1985), as well as reducing the release of adenosine during

ischemia (Frenguelli*, et al.*, 2007). HTL (0.3 mM) not only diminishes tissue content of adenosine but also its breakdown products inosine and hypoxanthine (McIlwain and Poll, 1986).

The application of HTL can have pro-convulsive activity in cortical tissue (Folbergrová, 1974; Folbergrová, 1981). HTL has been used *in vivo* to induce differing types of seizures in rat models (Stanojlović*, et al.*, 2008). The increase in convulsive behaviour is not well understood but it is believed that it may be due to a number of reasons including: (i) changes in adenosine concentration that is diminished through the formation of SAH (McIlwain and Poll, 1986), (ii) activation of NMDA receptor activity (Rašić-Marković*, et al.*, 2011), or (iii) inhibition of Na^+/K^+-ATPase activity (Rašić-Marković*, et al.*, 2009).

In this chapter I will show that 'trapping' intracellular adenosine with HTL does not result in significant changes in activity-dependent release of adenosine. Additionally, although adenosine release was not different, HTL did influence the intensity and frequency of seizures.

6.2. Results

6.2.1.1. Effect of homocysteine thiolactone on activity-dependent release of adenosine

In this chapter I will test whether application of HTL will reduce the release of adenosine in RibAde-treated and control slices. Figure 6.1 shows the mean adenosine release during initial washout of Mg^{2+} from the slice (0 mM Mg^{2+} aCSF; control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices), during bursting seizures in 50 μM 4-aminopyridine (4-AP; control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices) and during challenge with the adenosine A_1 receptor antagonist CPT (1 μM; control, n = 8; control/HTL, n = 6; RibAde, n = 12; RibAde/HTL, n = 9 slices). CPT was added after the presentation of three bursts or after 10 minutes in 4-AP, in Fig 5.2 a break in the traces is used to represent this. HTL treatment was found to be pro-convulsive in these experiments. Here, spontaneous seizures in 0 mM Mg^{2+} aCSF occurred in 50% of control/HTL (n = 20), 24% in RibAde/HTL (n = 32), 6% in control slices (n = 33) and 25% (n = 29) in RibAde-treated slices.

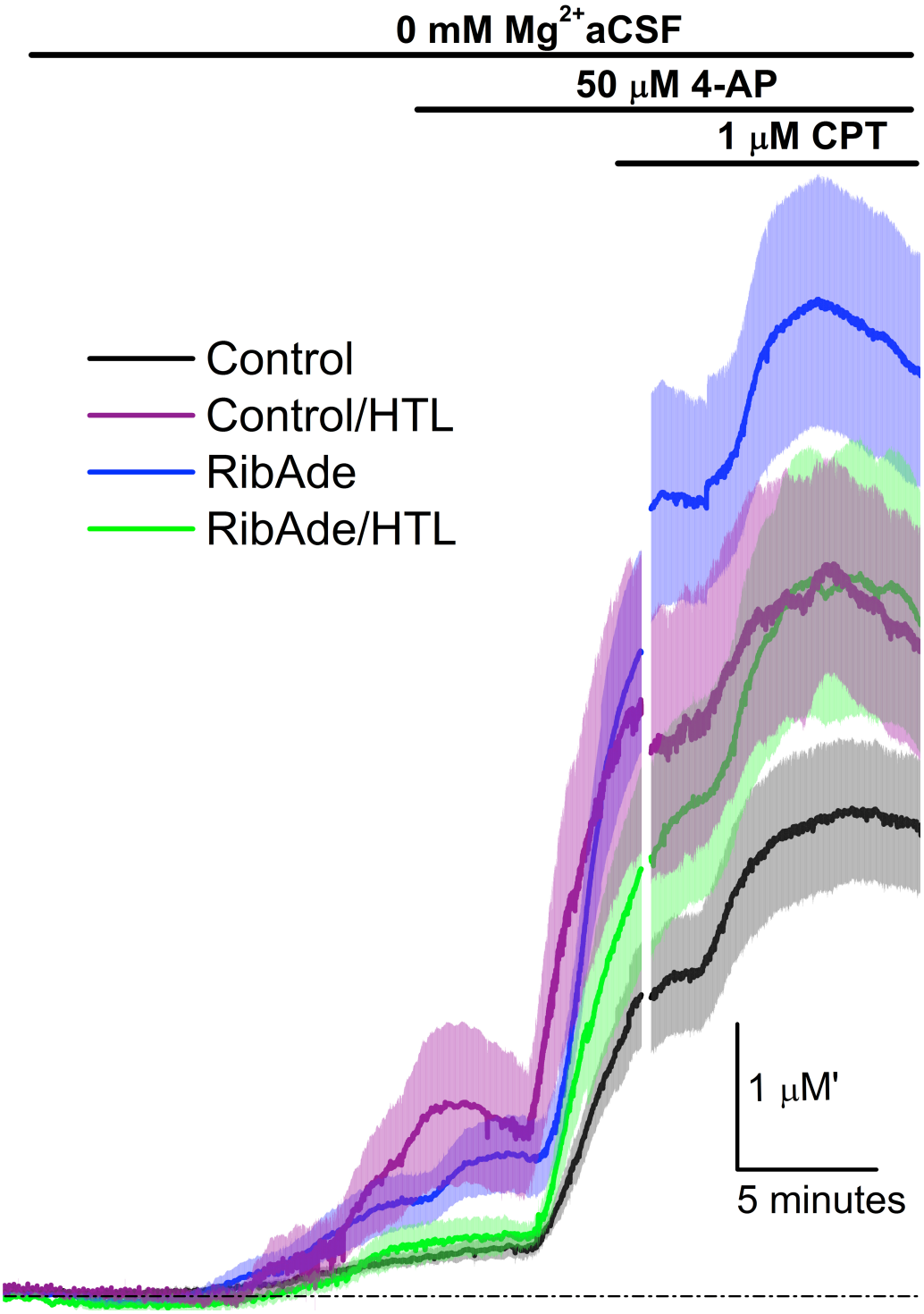

Figure 6.1. Effect of homocysteine thiolactone activity-dependent release of adenosine in rat hippocampal slices. Adenosine release in 0 mM magnesium (Mg^{2+}) artificial cerebrospinal fluid (aCSF), during 50 μM 4-aminopyridine (4-AP) induced seizures and challenge with 1 μM 8-cylopentyltheophylline (CPT), the adenosine A_1 receptor antagonist. Note the non-significant increase adenosine release in control/HTL (-) during Mg^{2+}-free aCSF, which was associated with spontaneous seizures (visible on the sensor trace). This increase in spontaneous seizures was absent in RibAde/HTL (-) slices. Dotted line represents baseline prior to washout of Mg^{2+} from slices. Control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices. Data shown is average of recordings made from these 6-13 slices shown as mean ± SEM.

6.2.1.2. Assessment of the influence of homocysteine thiolactone on activity-dependent release of adenosine

Area under the curve measurements showed no significant differences (Fig 6.2A; Table 6.1) in adenosine release were found in Mg^{2+}-free aCSF (One-way ANOVA, $p = 0.154$; $F_{(3,34)} = 1.863$; control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices).

No significant difference during 4-AP-induced seizures was found (Fig 5.3B) (One-way ANOVA, $p = 0.060$; $F_{(3,34)} = 2.717$; control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices).

In addition, no difference (Fig 5.3C) was also found in CPT (One-way ANOVA, $p = 0.586$; $F_{(3,31)} = 0.654$; control, n = 8; control/HTL, n = 6; RibAde, n = 12; RibAde/HTL, n = 9 slices) and for total release (One-way ANOVA, $p = 0.071$; $F_{(3,35)} = 2.556$; control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices).

Table 6.1. Integrated measurements of adenosine release for control, RibAde and HTL-treated slices. n = 6-13 slices. Data shown as mean ± SEM

	Control (µM´ s)	**Control/HTL (µM´ s)**	**RibAde (µM´ s)**	**RibAde/HTL (µM´ s)**
0 mM Mg^{2+} aCSF	0.20 ± 0.03 (n = 11)	0.54 ± 0.19 (n = 6)	0.44± 0.12 (n = 13)	0.29 ±0.05 (n = 9)
50 µM 4-AP	1.31 ± 0.30 (n = 11)	2.50 ± 0.64 (n = 6)	3.15 ± 0.53 (n = 13)	2.17 ± 0.53 (n = 9)
1 µM CPT	1.56 ± 0.21 (n = 8)	1.30 ± 0.19 (n = 6)	1.77 ± 0.31 (n = 12)	1.99 ± 0.44 (n = 9)
Total release	2.85 ± 0.33 (n =11)	4.34 ± 0.77 (n = 6)	5.22 ± 0.75 (n = 13)	4.45 ± 0.74 (n = 13)

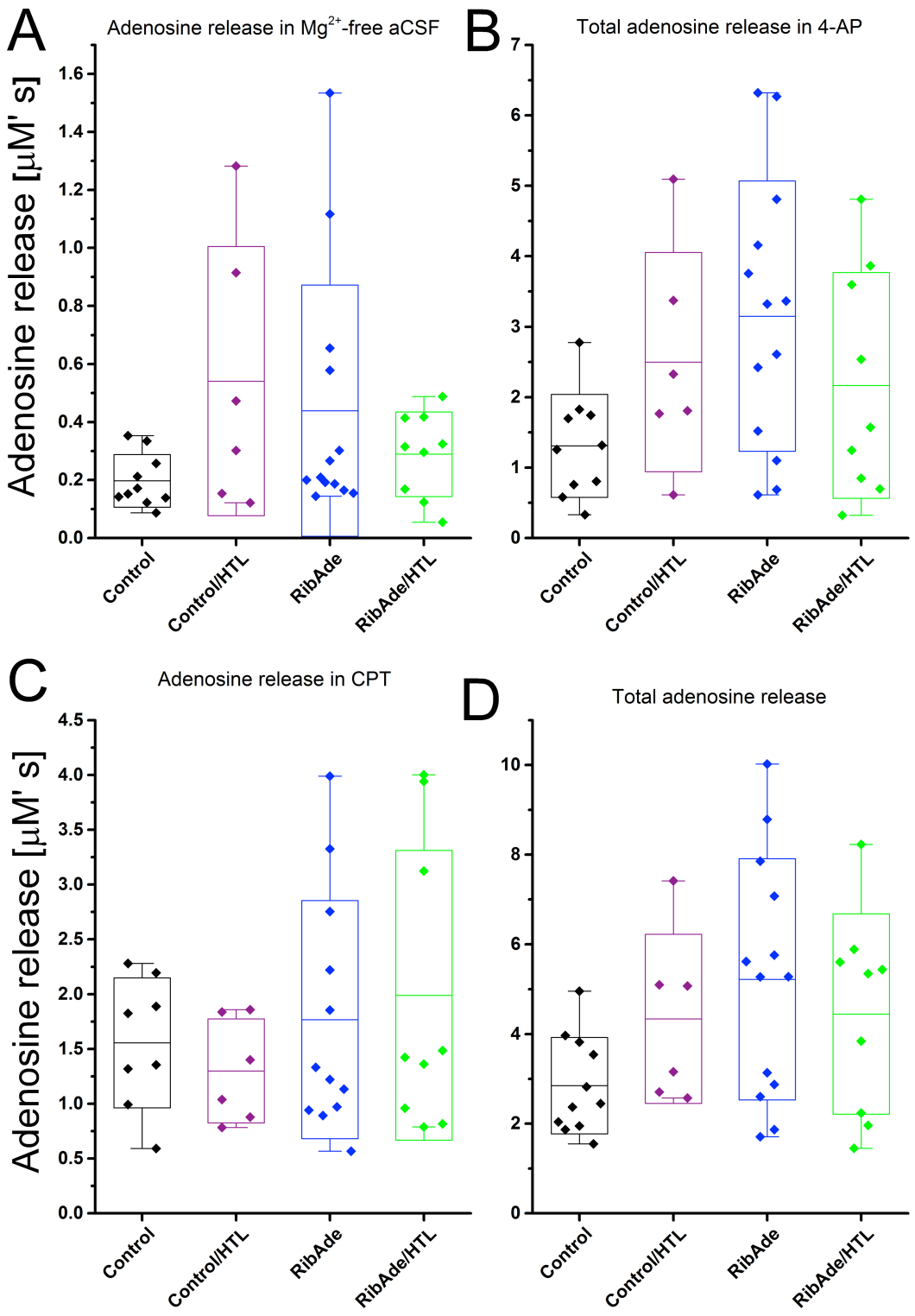

Figure 6.2. Integrated measurements of activity-dependent release of adenosine with homocysteine thiolactone. The integral of the area under the curve measurements of adenosine release in 15 minutes in magnesium (Mg^{2+})-free solutions (A), during a maximum of three 50 µM 4-aminopyridine (4-AP) induced seizures (B), application of the adenosine A_1 receptor antagonist (C) 8-cyclopentyltheophylline (CPT; 1 µM) and total adenosine release (D). There was no significant difference in the release of adenosine between control (n = 11), control/HTL (n = 6), RibAde-treated slices (n = 13) and RibAde/HTL (n = 9) slices. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits.

6.2.1.3. Assessment of the effects of homocysteine thiolactone on burst-dependent release of adenosine

In these experiments, during burst 1 the release of adenosine was increased in RibAde-treated slices (Fig 6.3; Table 6.2; One-way ANOVA, $p = 0.0005$; $F_{(3,35)} = 7.497$) compared to control (Post-hoc Bonferroni, $p = 0.004$) and RibAde/HTL-treated slices (Post-hoc Bonferroni, $p = 0.001$; control, n = 11; control/HTL, n = 6; RibAde, n = 13; RibAde/HTL, n = 9 slices). A trend for an increase in the amount of adenosine released during burst 1 in RibAde-treated slices was also observed compared to control/HTL-treated slices (Post-hoc Bonferroni, $p = 0.051$).

No differences in adenosine release during burst 2 (One-way ANOVA, $p = 0.106$; $F_{(3,30)} = 2.222$; control, n = 10; control/HTL, n = 5; RibAde, n = 12; RibAde/HTL, n = 7) and burst 3 (One-way ANOVA, $p = 0.167$; $F_{(3,21)} = 1.861$; control, n = 9; control/HTL, n = 3; RibAde, n = 9; RibAde/HTL, n = 4) were found.

The biosensor data indicate that application of HTL to RibAde-treated slices does reduce burst 1-dependent release of adenosine close to levels measured in control slices.

Table 6.2. Burst-dependent release of adenosine for control, RibAde and HTL-treated slices. One-way ANOVA: $^{**}p < 0.01$ compared to RibAde-treated slices; n = 6-13 slices. Data shown as mean ± SEM

Burst number	Control (µM´ s)	Control/HTL (µM´ s)	RibAde (µM´ s)	RibAde/HTL (µM´ s)
1	0.66 ± 0.18** (n = 11)	0.83 ± 0.32 (n = 6)	2.43 ± 0.51 (n = 13)	1.10 ± 0.24** (n = 9)
2	0.79 ± 0.21 (n = 10)	1.00 ± 0.34 (n = 5)	1.12 ± 0.22 (n = 12)	1.04 ± 0.27 (n = 7)
3	0.56 ± 0.13 (n = 9)	0.54 ± 0.05 (n = 3)	0.40 ± 0.10 (n = 9)	0.49 ± 0.12 (n = 4)

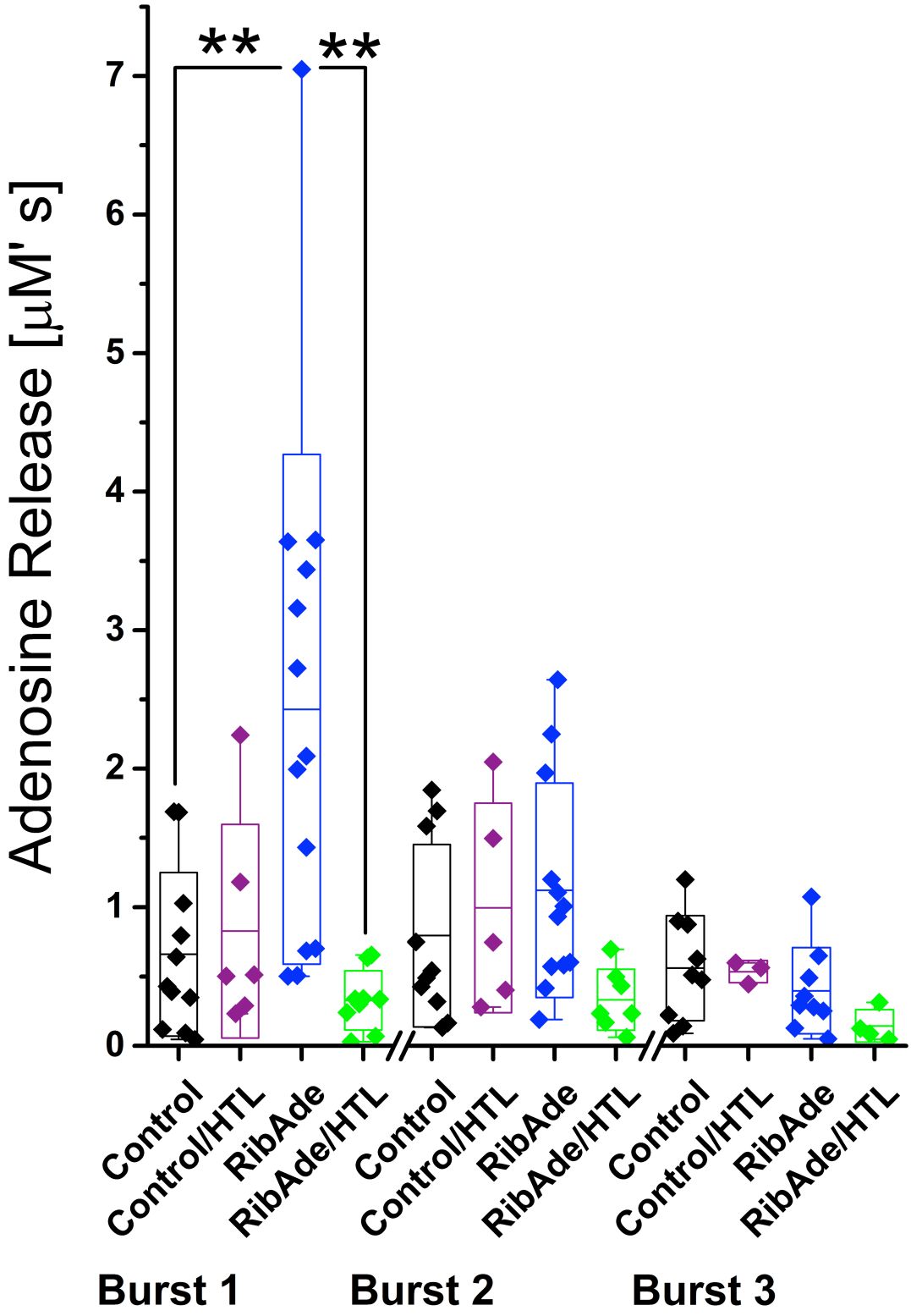

Figure 6.3. Burst-dependent release of adenosine in rat hippocampal slices treated with homocysteine thiolactone. Release of adenosine was greatest during burst 1 in RibAde-treated slices (-) compared to control (-) and RibAde/HTL-treated slices (-). No significant differences were found between RibAde and control/HTL-treated slices (-). Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by $^{**}p < 0.01$.

6.2.2. Effect of homocysteine thiolactone on basic neuronal excitability

6.2.2.1 Influence of homocysteine thiolactone on basal synaptic transmission

In the above section the 'trapping' of intracellular adenosine with HTL was investigated. In this section, I will describe the effect of HTL on basal synaptic transmission.

One-way repeated measures analysis was performed on input/output curves (Fig 6.4A) and a trend was observed (Multivariate test: $p = 0.62$; $F_{(3,23)} = 7.363$; control, n = 56; control/HTL, n = 26; RibAde, n = 47; RibAde/HTL, n = 26). Pairwise comparison Bonferroni tests showed that at stimulus intensities of 250 and 300 μA the fEPSP slope of RibAde/HTL was increased (-1.50 ± 0.19 and -1.61 ± 0.21 mV/ms; $p < 0.05$) compared to RibAde (-0.82 ± 0.07 and -0.89 ± 0.08 mV/ms). There was no difference in the peak amplitude of the fibre-volley at 300 μA (Fig 6.4B; One-way ANOVA: $p = 0.684$; $F_{(3,54)} = 0.500$; control, n = 17; control/HTL, n = 17; RibAde, n = 10; RibAde/HTL, n = 14).

To test whether HTL had any effect on presynaptic probability of release I compared paired-pulse ratios of slices treated with HTL (control/HTL and RibAde/HTL) and with control and RibAde-treated slices. Surprisingly, paired-pulse facilitation was increased as measure by a reduction in the probability of release in RibAde/HTL slices (Fig 6.4C) compared to control (Post-hoc Bonferroni, $p = 0.002$) and RibAde-treated slices (Post-hoc Bonferroni, $p = 0.004$).

To further test the effect of HTL on synaptic transmission, the maximum fEPSP slope during washout of Mg^{2+} from slices was calculated and no difference was

found between the four treatments (Fig 6.4D; One-way ANOVA: $p = 0.431$; $F_{(3,116)} = 0.926$; control, n = 50; control/HTL, n = 17; RibAde, n = 32; RibAde/HTL, n = 20).

Overall, treating slices with HTL has ambiguous effects on basal synaptic transmission. These data suggest that at lower stimulation intensities, the release at presynaptic terminals in RibAde/HTL is reduced. However, at higher stimulation intensities, an increase in outputs from afferent fibres was observed.

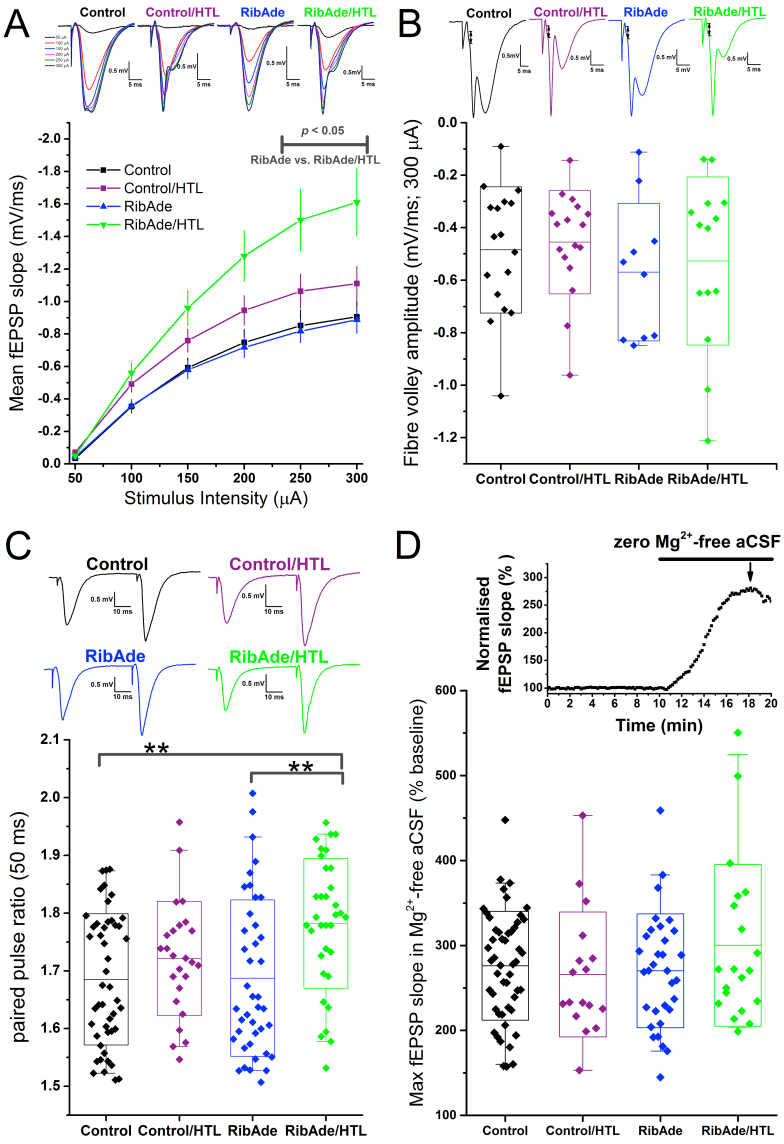

Figure 6.4. Basal synaptic transmission in slices treated with homocysteine thiolactone. Stepwise stimulation current increases were applied to slices to generate an input/output curve (A; top panel shows example potentials generated during the input/output curve). At intensities of 250 and 300 µA the field excitatory postsynaptic potential (fEPSP) was increased in RibAde/HTL ($p < 0.05$; n = 26) compared to RibAde-treated slices (n = 47). The peak amplitude of the fibre volley at 300 µA (B; (⇳) in top panel) was not different (control, n = 17; control/HTL, n = 17; RibAde, n = 10; RibAde/HTL, n = 14). The probability of release, where paired-pulses were given 50 milliseconds apart (C; example fEPSPs are shown in the top panel) at stimulus intensities between 50-60% of the maximum intensity used during the input/output curve, was increased in RibAde/HTL slices compared to control and RibAde-treated slices (control, n = 47; control/HTL, n = 25; RibAde, n = 41; RibAde/HTL, n = 34). Slices were left to equilibrate and a baseline of 10 minutes was recorded, after which nominally magnesium-free artificial cerebrospinal fluid (zero Mg^{2+}free aCSF; black bar in inset graph), was used as a means of increasing the excitability of the tissue (15 mins). The maximum field excitatory postsynaptic potential (fEPSP) slope during Mg^{2+}-free aCSF (D; arrow head in inset graph) was also not different (control, n = 50; control/HTL, n = 17; RibAde, n = 32; RibAde/HTL, n = 20). A) Data shown as mean ± SEM; B-D) Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by $**p < 0.01$ and $***p < 0.001$.

6.2.3. Effect of homocysteine thiolactone on seizure parameters

6.2.3.1 Assessment of the effects of homocysteine thiolactone on burst duration

The 'trapping' of intracellular adenosine in the form of SAH with HTL did not affect burst 1 duration (Fig 6.5; Table 6.3; One-way ANOVA: $p = 0.919$; $F_{(3,94)} = 0.178$; control, n = 32; control/HTL, n = 16; RibAde, n = 30; RibAde/HTL, n = 20 slices). In addition, the length of seizures during burst 2 was also not different between the four treatments (One-way ANOVA: $p = 0.087$; $F_{(3,74)} = 2.275$; control, n = 22; control/HTL, n = 13; RibAde, n = 27; RibAde/HTL, n = 16 slices).

Similarly, no significance difference in the length of seizures was found during burst 3 (One-way ANOVA: $p = 0.065$; $F_{(3,56)} = 2.547$; control, n = 17; control/HTL, n = 9; RibAde, n = 22; RibAde/HTL, n = 12 slices).

Table 6.3. Burst duration of control, RibAde and HTL-treated slices. n = 20-32 slices. Data shown as mean ± SEM

Burst number	Control (s)	Control/HTL (s)	RibAde (s)	RibAde/HTL (s)
1	**45.12 ± 5.53** (n = 32)	**50.56 ± 10.10** (n = 16)	**49.01 ± 4.49** (n = 30)	**45.16 ± 6.38** (n = 20)
2	**30.80 ± 3.75** (n = 22)	**26.96 ± 5.45** (n = 13)	**38.64 ± 3.77** (n = 27)	**25.27 ± 3.81** (n = 16)
3	**23.05 ± 3.86** (n = 17)	**11.01 ± 1.74** (n = 9)	**22.42 ± 2.80** (n = 22)	**15.28 ± 3.22** (n = 12)

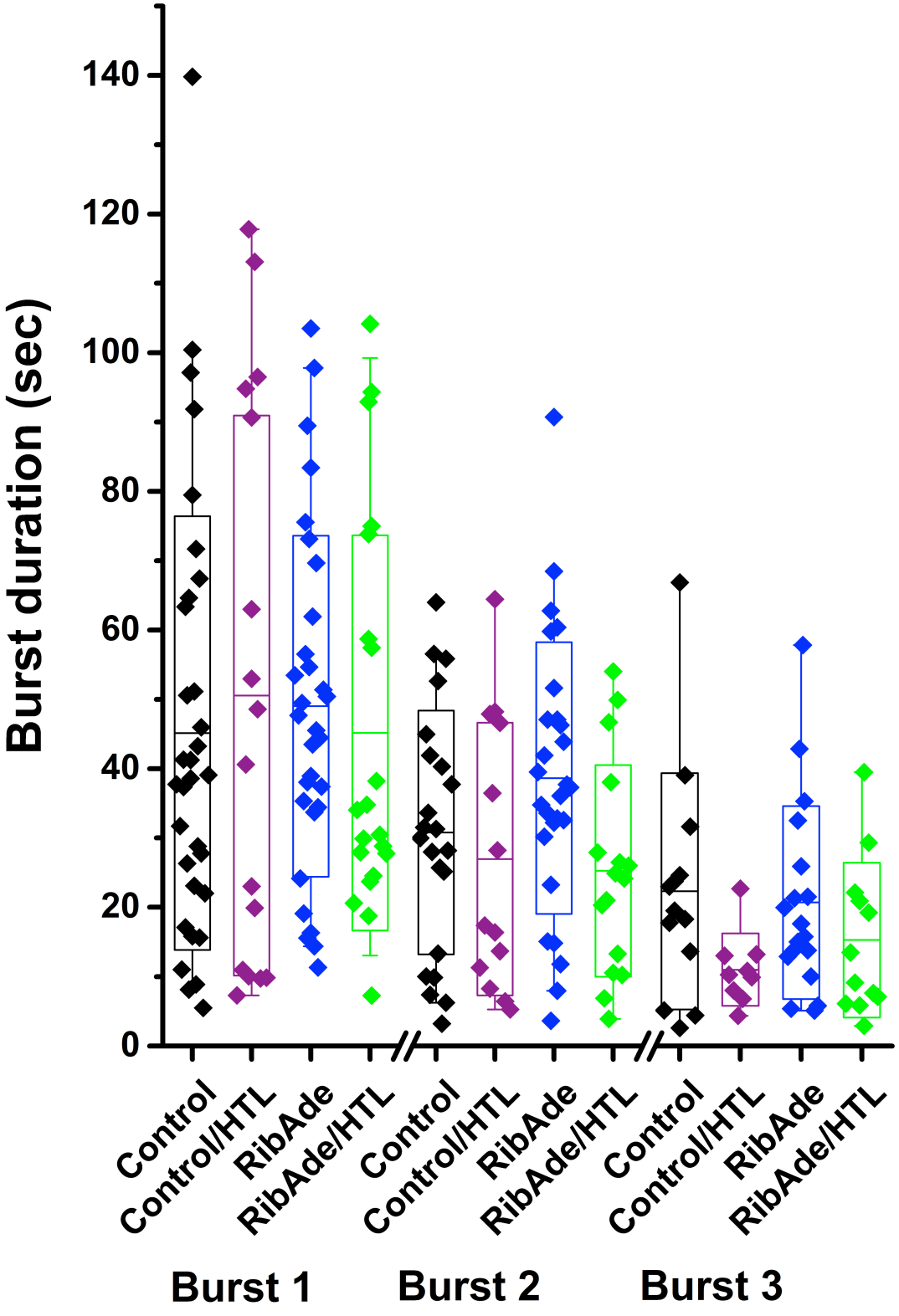

Figure 6.5. 'Trapping' intracellular adenosine with homocysteine thiolactone does not influence burst duration. The length of bursting seizure activity induced with 50 µM 4-aminopyridine was not different across groups. Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Burst 1: control, n = 32; control/HTL, n = 16; RibAde, n = 30; RibAde/HTL, n = 20; Burst 2: control, n = 22; control/HTL, n = 13; RibAde, n = 27; RibAde/HTL, n = 16; Burst 3: control, n = 17; control/HTL, n = 9; RibAde, n = 22; RibAde/HTL, n = 12.

6.2.3.2. 'Trapping' intracellular adenosine with homocysteine thiolactone influences spiking intensity

The effect of HTL on seizure intensity was measured by comparing the inter spike interval (ISI) of HTL-treated slices and those of control and RibAde-treated slices.

No significant difference in ISI was observed between treatments during burst 1 (Fig 6.6A-B; Table 6.4; control, n = 32; control/HTL, n = 16; RibAde, n = 30; RibAde/HTL, n = 20 slices).

During burst 2 control/HTL (n = 13) spiking intensity was increased (Fig 6.6C-D; Table 6.4) compared to RibAde/HTL (n = 16 slices) and also compared to RibAde-treated (n = 27 slices). No other difference in ISI was found during burst 2 (Fig 6.6E-F; control, n = 22 slices).

No significant difference in ISI was found during burst 3 (control, n = 17; control/HTL, n = 9; RibAde, n = 22; RibAde/HTL, n = 12 slices).

These data indicate that HTL alone was sufficient to increase the spiking intensity only during burst 2. In addition, the presence of ATP precursors RibAde was enough to reduce the increased spiking intensity induced by HTL.

Table 6.4. Summary of Kolmogorov-Smirnov test for the inter spike interval of burst 1-3. *$p < 0.05$ compared to RibAde and RibAde/HTL; n = 9-32.			
Kolmogorov-Smirnov test	**Burst number**		
	1	**2**	**3**
Control/HTL vs. Control	p = 0.95 D = 0.16 Z = 0.51	p = 0.53 D = 0.26 Z = 0.75	p = 0.54 D = 0.31 Z = 0.75
RibAde/HTL vs. Control	p = 0.31 D = 0.26 Z = 0.92	p = 0.21 D = 0.33 Z = 1.00	p = 0.53 D = 0.28 Z = 0.74
Control/HTL vs. RibAde	p = 0.06 D = 0.39 Z = 1.25	p = 0.03 * D = 0.47 Z = 1.38	p = 0.42 D = 0.32 Z = 0.82
RibAde/HTL vs. RibAde	p = 0.32 D = 0.27 Z = 0.92	p = 0.46 D = 0.25 Z = 0.80	p = 0.82 D = 0.21 Z = 0.57
Control/HTL vs. RibAde/HTL	p = 0.25 D = 0.33 Z = 0.97	p = 0.04 * D = 0.49 Z = 1.31	p = 0.73 D = 0.28 Z = 0.63

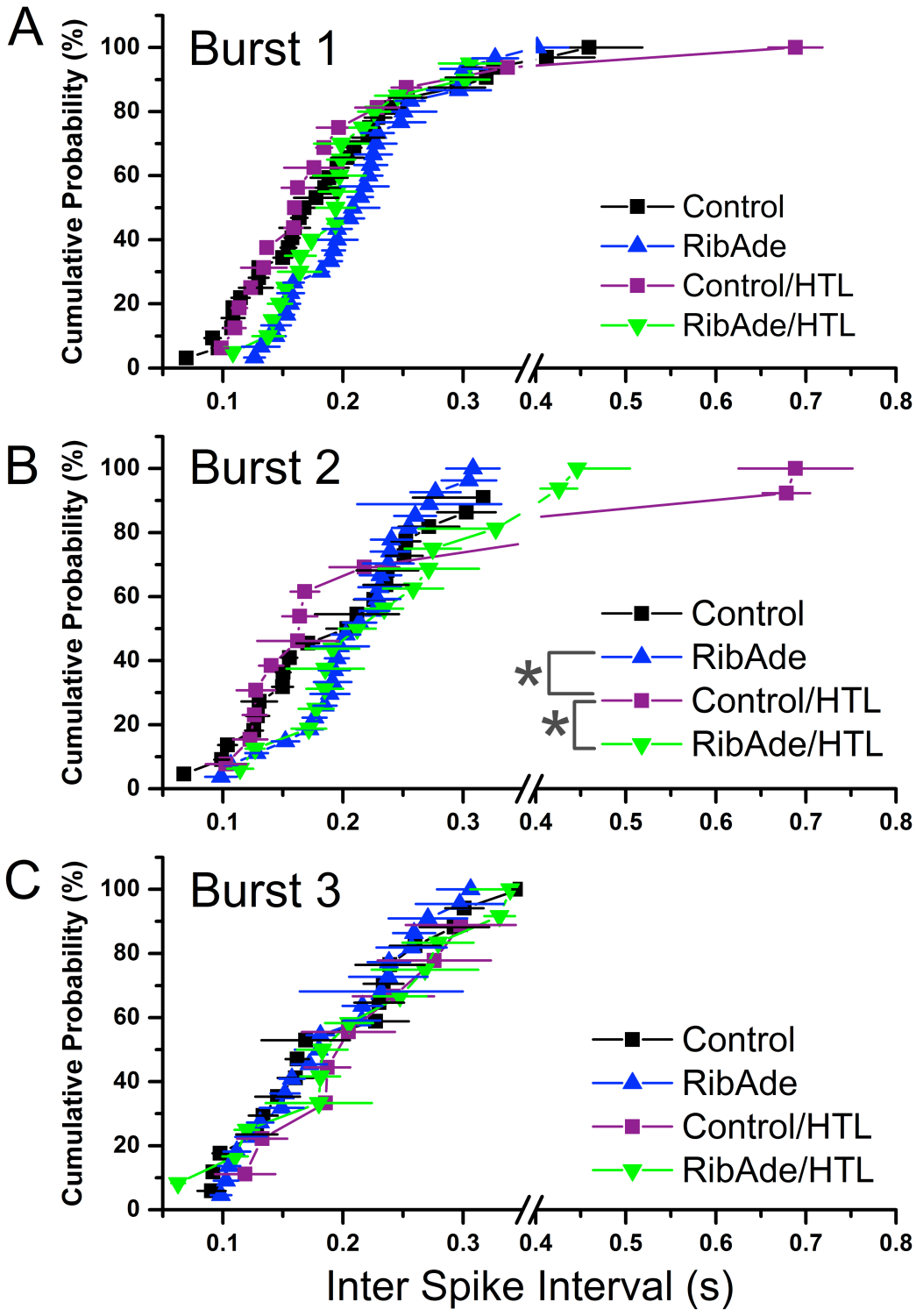

Figure 6.6. Influence of homocysteine thiolactone on seizure intensity. Burst 1 (A-B) cumulative probability (A) and histogram of the frequency counts (B) of the Inter Spike Interval was not different between treatments. During burst 2 (C-D), the spiking frequency was reduced (increased spiking frequency) in slices pre-treated with RibAde and RibAde/HTL-treated slices compared to control/HTL slices (increased spiking frequency). No difference in the Inter Spike Interval during burst 3 (E-F) was found. For cumulative probability plots, individual symbols represent a slice. Burst 1: control, n = 32; control/HTL, n = 16; RibAde, n = 30; RibAde/HTL, n = 20; Burst 2: control, n = 22; control/HTL, n = 13; RibAde, n = 27; RibAde/HTL, n = 16; Burst 3: control, n = 17; control/HTL, n = 9; RibAde, n = 22; RibAde/HTL, n = 12. Data shown as mean ± SEM. Significances indicated by $*p < 0.05$.

6.2.3.3. Assessment of the effects of homocysteine thiolactone on seizure frequency

To further test the effect of HTL on the rate at which bursting occurred the Inter Burst Interval (IBI) was calculated (Fig 6.7). The time to burst 2 (Burst Interval 1) was reduced in slices treated with HTL (control/HTL and RibAde/HTL; Post-hoc Bonferroni, $p < 0.0001$ and $p = 0.0004$ respectively) compared to RibAde-treated slices (One-way ANOVA: $p < 0.001$; $F_{(3,74)} = 12.179$; control, n = 22; control/HTL, n = 13; RibAde, n = 27; RibAde/HTL, n = 16 slices).

Interestingly, in control/HTL slices the time to burst 2 was also reduced compared to control slices (Post-hoc Bonferroni, $p = 0.039$). The time to burst 2 was increased in RibAde-treated slices (Post-hoc Bonferroni, $p = 0.019$) compared to control slices.

A similar increase in frequency of seizures for HTL-treated slices was observed for the time to burst 3 (Burst Interval 2- One-way ANOVA: $p = 0.0006$; $F_{(3,56)} = 6.597$; control, n = 17; control/HTL, n = 9; RibAde, n = 22; RibAde/HTL, n = 12 slices). In control/HTL (Post-hoc Bonferroni, $p = 0.012$) and RibAde/HTL-treated slices (Post-hoc Bonferroni, $p = 0.002$) the time to burst 3 was shorter than that of RibAde-treated slices.

These data suggest that treatment with HTL increases the frequency of bursting seizures.

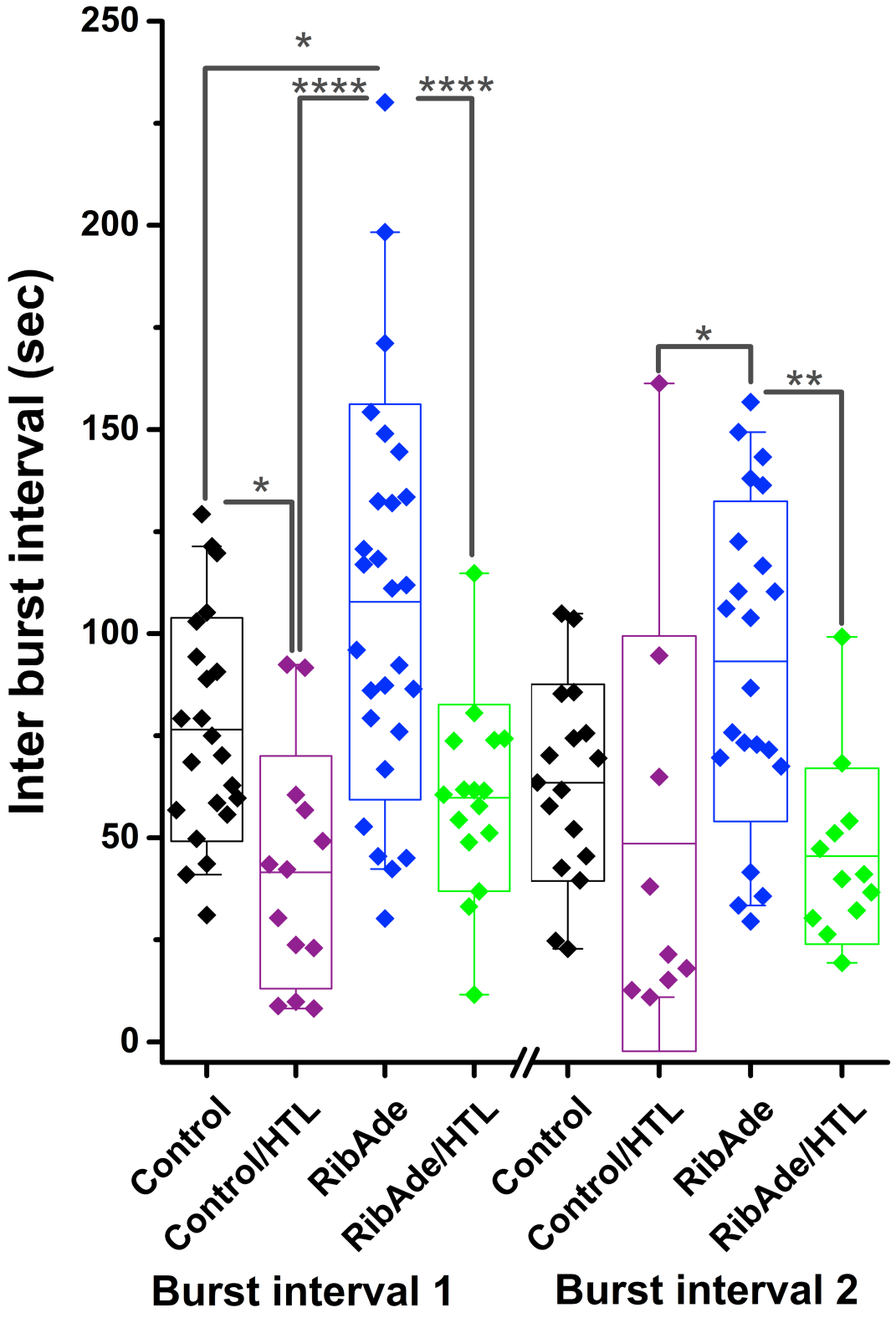

Figure 6.7. Homocysteine thiolactone influences the onset to seizure activity. The time to burst 2 (Burst Interval 1) was greatly reduced in control (n = 22), control/HTL,(n = 13) and RibAde/HTL (n = 16) slices compared to RibAde-treated slices (n = 27). Similarly, the time to burst 3 (Burst Interval 2) was reduced in control/HTL (n = 9) and RibAde/HTL (n = 12) slices compared to RibAde-treated slices (n = 22). Individual symbols represent slices; mean is shown as the central line, box as ± SD and whiskers 5-95% confidence limits. Significances indicated by ****$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

6.3. Discussion

In the present chapter I have shown that unexpectedly, HTL did reduce the burst 1-dependent release of adenosine but had inconsistent effects on basal synaptic transmission. HTL was also found to increase the intensity of seizures, which was reversed with RibAde and increased the frequency of seizures.

6.3.1. Adenosine release in homocysteine thiolactone influenced adenosine release in burst-dependent manner

In this thesis I have shown that the application of HTL to RibAde-treated slices significantly reduced burst 1-dependent release but not subsequent bursts compared to RibAde-treated slices. The release of adenosine in RibAde/HTL slices during burst 1 was reduced close to that of control slices, suggesting that HTL treatment may be reducing the intracellular release of adenosine into the extracellular space. Although, HTL did influence adenosine release in a burst-dependent manner, it was found not to significantly affect total adenosine release and combined release in 4-AP. The possibility cannot be eliminated that HTL experiments were underpowered ($p = 0.071$ and $p = 0.060$ respectively, RibAde/HTL, $n = 9$ and control/HTL, $n = 6$) and an increase in sample size may have revealed a difference.

My results show that using HTL as a means of reducing intracellular adenosine and thus reducing the release of adenosine into the extracellular space was successful in slices with an increase in adenosine tone and was restricted to burst 1. These data similar to what has been shown where L-homocysteine thiolactone reduced both basal and evoked release of adenosine in brain slices (Lloyd*, et al.*, 1993; McIlwain

and Poll, 1985) as well as adenosine release during ischemia (Frenguelli*, et al.*, 2007). HTL was also found to reduce the tissue content of adenosine, inosine and hypoxanthine (McIlwain and Poll, 1986). In the work conducted by Lloyd*, et al.*, 1993, where HTL was found to reduce adenosine release to electrical pulses given for 5 or 15 minutes and biosensors were used to measure adenosine release are similar to the studies conducted in this work in regards to the use of biosensors to measure real-time adenosine release during increased neuronal excitability.

Surprisingly, on its own HTL failed to reduce the release of adenosine. HTL itself was found to result in the release of adenosine following spontaneous seizures (control/HTL slices were more excitable in Mg^{2+} free aCSF) and this complicates the interpretation of its potential role for inhibiting intracellular adenosine release into the extracellular space. This suggests that HTL might have off target effects on the purinergic system and may also be influencing basal tone in some way. Therefore, any interpretation needs to take this into account.

The increase in tissue excitability in control/HTL slices maybe due to the combination of HTL with the washout of Mg^{2+} from the slice where it might be possible that this additional increase in tissue excitability from the removal of Mg^{2+} block may exaggerate the pro-convulsive actions of HTL. Thus, my results can be interpreted in a number of ways (1) HTL may not be a suitable method for detecting adenosine release that arises from intracellular sources due to its proconvulsive effects or (2) the activity-dependent release measured in my seizure model may partially arise from extracellular pools of adenosine.

Studies conducted by (Wall and Dale, 2013) showed that at least 40% of stimulated-adenosine release was carried out via neuronal equilibrative nucleoside transporters

(ENTs) and that there is a separate component of adenosine release, which arises from the extracellular metabolism of ATP released from astrocytes. Given these data it would be important to address whether the release of adenosine during seizures arise from extracellular breakdown of ATP and also whether the measured adenosine release arises either from neurons or astrocytes, as discussed below.

One way of addressing whether the adenosine measured in these studies arises from extracellular sources of ATP is to use an ectonucleotidase inhibitor. Extracellular ATP and its nucleotides undergo rapid degradation by ectonucleotidases to provide a readily available pool for the production of adenosine. Ecto-5'-nucleotidase inhibitors such as α, β-Methylene-ADP derivatives and analogues have been characterised (Bhattarai*, et al.*, 2015), which are effective at inhibiting the breakdown of extracellular AMP to adenosine.

Adenosine is not only regulated and released from neurons, but astrocytes also play an important role in the regulation of adenosine levels in the brain (Aronica*, et al.*, 2011; Boison, 2010; Boison*, et al.*, 2010; Boison*, et al.*, 2013; Li*, et al.*, 2008). Therefore, in order to gain a better understanding of the site of cell specific release of adenosine a number of approaches could be made. To accomplish this neuronal and/or astrocytic specific release or regulation of adenosine could be further investigated by use of genetic mouse models such as the use of dn-SNARE mice, which lack the ability to release transmitters by exocytosis in glial cells (Pascual*, et al.*, 2005). Additionally, pharmacological methods such as the gliotoxin fluoroacetate, which would block glial specific release of adenosine, could also be employed.

6.3.2. Effect of 'trapping' intracellular adenosine with homocysteine thiolactone on basal synaptic transmission and seizure parameters

6.3.2.1 Basal synaptic transmission

HTL application to slices had inconsistent effects on basal synaptic transmission measures. The effects observed for HTL during the input/output curves were found at higher stimulations where there is a greater recruitment of fibres compared to the stimulus intensities used during paired-pulse stimulations. Although a greater recruitment in the number of synapses at higher stimulations is not expected to influence the paired-pulse ratio if the population of synapses are homogenous, to investigate the differences in basal synaptic transmission observed paired-pulse stimulations should be repeated at 250-300 μA. Additionally, to account for variability in baseline response and stimulus intensity used, it is important to repeat these experiments whilst using a stimulus intensity that would generate a fixed peak fEPSP amplitude.

6.3.2.2. Increased seizure intensity with HTL is reversed with RibAde

In the previous chapter I showed that RibAde, as opposed to creatine, reduced the intensity and frequency of seizures. In the present chapter I tested the effect of HTL on seizure intensity and whether RibAde would help to mitigate its effect.

My data shows that HTL treatment increased the intensity of bursting seizures in control/HTL slices but had no effect on seizure intensity in RibAde-treated slices

only during burst 2. The effects of increased spiking intensity observed during burst 2 could be related to the burst 1 associated release where release in control/HTL was lower than that of both RibAde and RibAde/HTL-treated slices. This lowered adenosine release could exert a reduced inhibitory effect of spiking intensity that may account for the differences reported in this thesis. Other off target effects of HTL can also be responsible for the effects highlighted here. What these data do importantly indicate is that the pre-treatment of slices with RibAde helps to reduce reverse the HTL-induced hyperexcitability.

6.3.2.3 HTL increased seizure frequency

Although the combination of RibAde and HTL was effective at reversing the HTL-induced increases in seizure intensity, the frequency of seizures associated with this treatment was not dissimilar to that in Control/HTL slices. However, the time to the occurrence of burst 2 was reduced in control/HTL slices compared to control, suggesting that on its own HTL does increase the likelihood of seizures. This is also supported from the observation that in Mg^{2+}-free aCSF there was an increase in the occurrence of spontaneous seizures in control/HTL-treated slices that was not present in the RibAde/HTL slices.

My data on the effect of HTL on seizure parameters consistently shows that on its own HTL increased both the intensity and frequency of seizures compared to RibAde-treated slices. However, in comparison to control slices, HTL only increased the frequency, but not the intensity of seizures. The combination of both HTL and RibAde produced inconsistent effects in relation to reversing the pro-convulsive effect of HTL on seizure parameters.

In summary, these data do not support the idea of using HTL to 'trap' intracellular adenosine in the form of SAH, and HTL was not successful in reducing the release of intracellularly formed adenosine. These studies may indicate that the adenosine measured by the microelectrode biosensors may arise from sources other than intracellular breakdown of ATP such as the extracellular breakdown of ATP to adenosine or the release of adenosine from astrocytes.

7. General Discussion

7.1. Summary of findings

It has previously been shown that the decreased ATP content in brain slices compared to the situation *in vivo* is due to a loss of ATP precursors, since treating slices with a combination of D-ribose (1 mM) and adenine (50 μM; RibAde) is sufficient to recover tissue ATP levels to *in vivo* values (zur Nedden*, et al.*, 2011). This improvement of ATP levels is also associated with greater activity-dependent release of adenosine and, via activation of adenosine A_1Rs, the threshold for the induction of long-term potentiation is raised (zur Nedden*, et al.*, 2011). It has also been shown that RibAde and the phosphate buffer creatine are capable of modulating the decline and recovery of the intracellular ATP associated with metabolic stress, with corresponding effects on the depression and recovery of synaptic transmission (zur Nedden*, et al.*, 2014). These studies provide evidence for the use of RibAde and creatine as a means of modulating the availability of both intracellular ATP, and, importantly, its breakdown product adenosine during various physiological or pathophysiological conditions in which ATP and adenosine play a major role. In the current thesis I utilised these strategies for manipulating ATP and adenosine levels during epileptiform seizure activity in hippocampal slices.

The aim of my thesis was to investigate how the modulation of intracellular ATP with creatine or RibAde pre-treatment influenced seizure activity via the activity-dependent release of adenosine. I used a model of chemically-induced bursting seizure activity to address the following questions:

1. Do creatine or RibAde influence the activity-dependent release of adenosine during seizure activity?
2. If so, can I measure the release of adenosine under these conditions?

3. Does the modulation of adenosine release influence seizure activity?
4. Does this adenosine originate from intracellular sources?

With regard to these questions, our model was indeed sensitive to changes in the release of adenosine (Chapter 3). RibAde influenced activity-dependent release of adenosine and this was measurable in real-time with enzyme-based biosensors and had an appreciable effect on seizure activity. I also measured activity-dependent adenosine release in creatine-treated slices (Chapter 4). I found that high micromolar concentrations of adenosine were released per burst, which is consistent with previous work in electrically-evoked and spontaneous seizures (Etherington*, et al.*, 2009; Lopatář*, et al.*, 2011; Lopatář*, et al.*, 2015) and in humans with complex partial seizures (During and Spencer, 1992). This increased release of adenosine in RibAde-treated slices was associated with a reduction in seizure frequency and intensity compared to creatine-treated slices (chapter 5).

In addition, I have demonstrated that pre-treating slices with creatine results in an increase in seizure intensity and frequency compared to pre-treatment with RibAde in a chemically-induced, model of bursting seizures (Chapter 5). My results are in contrast to previous studies where creatine was found to be anticonvulsive (Rambo*, et al.*, 2013; Rambo*, et al.*, 2009). However the viability of creatine as an anticonvulsant remains doubtful as, similar to the present results, creatine has also been shown to promote seizure-like activity (Streijger*, et al.*, 2010) and creatine was not found to be protective against seizure susceptibility after traumatic brain injury (Saraiva*, et al.*, 2012).

During cellular metabolism the enzyme adenylate kinase breaks down ATP to ADP and ADP to AMP. AMP is then further broken down to adenosine by the action of 5'-nucleotidase. In slices treated with creatine, CK catalyzes the reversible transfer of the N-phosphoryl group from PCr to ADP to generate ATP and stabilizes cellular ATP. Due to the ability of CK to help manufacture more ADP and ultimately ATP, ATP metabolism to adenosine is reduced. In comparison, under RibAde conditions, the brain uses the purine salvage pathway a means of making adenine nucleotides and this leads to an increase in the concentration of AMP, which can be made to make further ADP, ATP and adenosine.

In summary of the data presented on the effects of creatine and RibAde on adenosine concentration and epileptiform activity, the following model of rhythmic epileptiform bursting is proposed (Fig 7.1). A rhythm generator controls internal cellular mechanisms that includes but is not limited to the levels of adenosine in the slice that determines a rhythm for epileptiform bursting activity. The rhythm determines the duration, interval and intensity of a burst. In turn the duration, intensity and interval of epileptiform bursting activity then go on to influence the release of adenosine in the slice. This release of adenosine further impacts the rhythm generator and bursts. When creatine is added to the system this result in a reduction in adenosine, which acts to reduce the inhibitory effects of adenosine on bursts and the rhythm generator. Under RibAde conditions, an increase adenosine levels result in an increase in the inhibitory effects of adenosine bursts and the rhythm generator. The effects of creatine and RibAde on adenosine and adenosine subsequent modulation of the rhythm generator and burst account for the difference in reported in this thesis for these treatments on adenosine release and epileptiform activity.

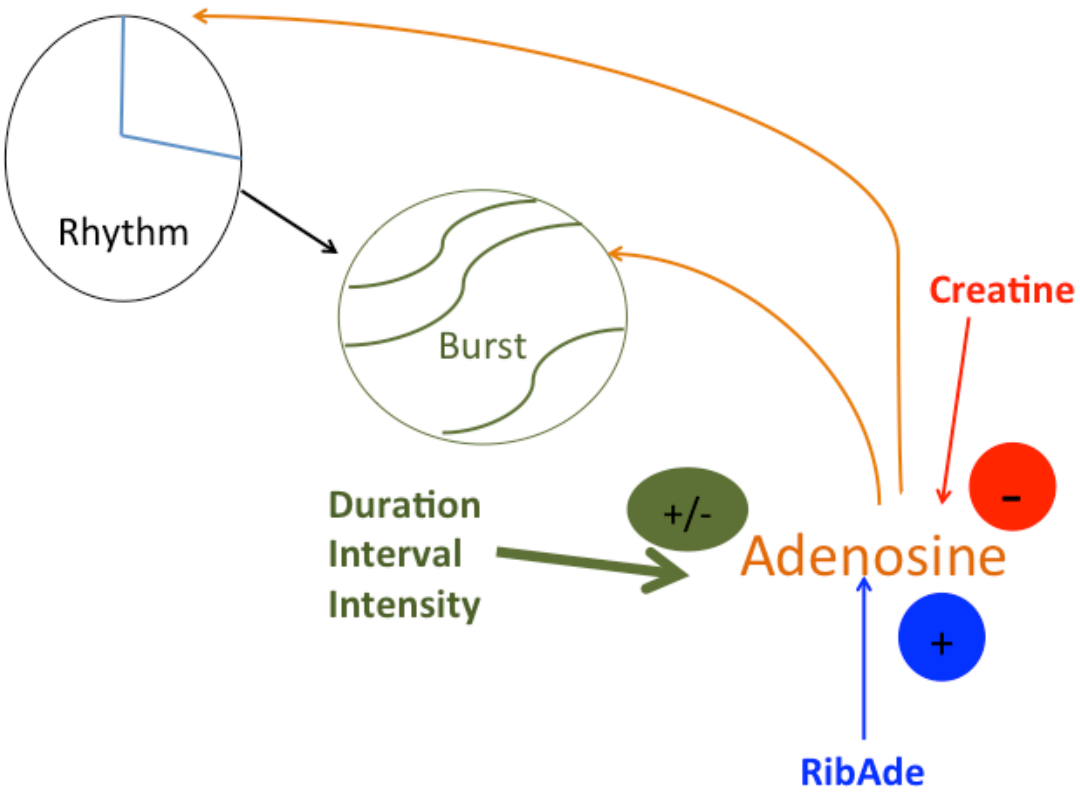

Figure 7.1. Model of rhythmic epileptiform bursting. A rhythm generator determines a rhythm for epileptiform bursting activity. The rhythm determines the duration, interval and intensity of a burst. In turn the duration, intensity and interval of epileptiform bursting activity then go on to influence the release of adenosine in the slice. This release of adenosine further impacts the rhythm generator and bursts. Creatine reduces the release of adenosine, which acts to reduce the inhibitory effects of adenosine on bursts and the rhythm generator. Under RibAde conditions, an increase adenosine levels result in an increase in the inhibitory effects of adenosine bursts and the rhythm generator.

My results also show that antagonism of the adenosine A_1 receptor led to an aggravation of seizure duration, which is in line with previous studies using pharmacological tools in *in vitro* models of seizure activity (Dragunow and Robertson, 1987; Dunwiddie, 1980; Eldridge*, et al.*, 1989; Etherington and Frenguelli, 2004; Lopatář*, et al.*, 2011), *in vivo* seizure models (Ault*, et al.*, 1987; Gouder*, et al.*, 2003; Weiss*, et al.*, 1985), and using A_1 knockout mice (Kochanek*, et al.*, 2006). Additionally, adenosine A_{2A}Rs were found to play a role in promoting

increased spiking intensity under normal conditions, which is similar to previously reported findings where A_{2A}Rs were found to be mildly pro-convulsive in electrically evoked seizure activity in Mg^{2+}-free aCSF (De Sarro*, et al.*, 1999; Etherington and Frenguelli, 2004; O'Kane and Stone, 1998).

Extracellular sources of adenosine can arise from two main routes: (1) the release of intracellular adenosine into the extracellular space via nucleoside transporters; or (2) the extracellular breakdown of ATP to adenosine. Given this, I set out to investigate whether the measured adenosine originates from intracellular adenosine pools, by treating slices with homocysteine thiolactone. My results show that burst 1-associated adenosine release may originate from intracellular sources (Chapter 6) since HTL reduced burst-1 adenosine release in RibAde-treated slices. HTL was also found to increase the intensity and frequency of seizures, and the effect on intensity was reversed with RibAde. These data are similar to observations where L-homocysteine thiolactone reduced both basal and evoked release of adenosine in brain slices (Lloyd*, et al.*, 1993; McIlwain and Poll, 1985), as well as adenosine release during oxygen/glucose deprivation (Frenguelli*, et al.*, 2007). The results from chapter 6 also suggests that HTL might have off target effects on the purinergic system and may be influencing basal adenosine tone as is suggested by the increase in excitability in control/HTL slices and associated increased release of adenosine as well as the ambiguous effects of HTL on electrophysiological responses to evoked stimulation observed in RibAde/HTL-treated slices. Further elucidation of these off target effects is necessary to get a better understanding of the effects of HTL on intracellular adenosine release.

In summary, the work in this thesis provides evidence to support the idea of modulation of the adenosinergic system as a potential therapeutic target for the treatment of epilepsy (Boison, 2009).

7.2. Possible therapeutic use of RibAde for the treatment of epilepsy

This thesis provides evidence for the use of RibAde as a means of reducing the occurrence of seizure activity and seizure intensity via enhanced release of the anticonvulsant adenosine.

Brain slices are a useful model system for the study of novel epileptic seizure treatments and to establish their mechanism of action. However, in order to study their effects on the system as a whole brain slices are not suited since they are reduced biological systems and are not representative of the whole brain. Although epileptic seizures can originate from site-specific regions, they often spread and involve the whole brain. It is therefore important to investigate possible treatments of epilepsy in *in vivo* models. Ribose and adenine have been shown to be well tolerated in humans and have been used previously to treat different disorders as discussed in Chapters 1 and 4. Given the promising effects of RibAde on adenosine-dependent decreases in seizure parameters it is possible that RibAde treatment could be adapted in the clinic. This thesis provides evidence for the use of RibAde in *in vivo* studies as well as early small scale, low risk pre-clinical trials in people with epilepsy. Due to the greater ATP pool in RibAde-treated slices (zur Nedden*, et al.*, 2014; zur Nedden*, et al.*, 2011) it would be expected that there might be a greater activity-dependent release of adenosine that might raise the threshold for, or reduce seizure activity.

As discussed in Chapter 4 ribose and adenine are able to pass easily through cell membranes (Cornford*, et al.*, 1982; Cornford and Oldendorf, 1975; McCall*, et al.*, 1982; Prather and Wright, 1970; Sacerdote and Szostak, 2005). Ribose is well tolerated in rodents (Griffiths*, et al.*, 2007b; Griffiths*, et al.*, 2007a). In addition, both compounds are well tolerated in humans. Oral-administration of ribose has been successfully trialled in clinical studies for the treatment of a variety of disorders of the heart (Brookman and St Cyr, 2010; MacCarter*, et al.*, 2009; Omran*, et al.*, 2003; Pauly and Pepine, 2000; Perkowski*, et al.*, 2011; Pliml*, et al.*, 1992). Ribose can also be administered safely via the intravenous route (Goodman and Goetz, 1970; Gross*, et al.*, 1989; Gross and Zöllner, 1991; Gunning*, et al.*, 1996) where intravenous ribose is often given as part of an imaging procedure used to measure the extent of damaged heart muscle in patients with coronary heart disease (Hegewald*, et al.*, 1991; Perlmutter*, et al.*, 1991; Wilson*, et al.*, 1991). Adenine has also been administered in humans. However, because adenine can be metabolised by xanthine oxidase (Bendich*, et al.*, 1950), and the by-product 2,8-dihydroxy-adenine can then lead to the development of kidney stones (Van Acker*, et al.*, 1977), adenine is given with the xanthine oxidase inhibitor allopurinol (Edvardsson*, et al.*, 1993; Greenwood*, et al.*, 1982; Simmonds, 1986) to prevent the formation of kidney stones. Adenine and allopurinol have been given for the treatment of metabolic disorders (Balasubramaniam*, et al.*, 2014; Jinnah*, et al.*, 2013). Adenine, when given in combination with clozapine as a treatment for schizophrenia, was found to reduce the frequency of treatment discontinuation due to clozapine-induced neutropenia (Takeuchi*, et al.*, 2015). Adenine has also been used to treat metabolic disorders such as adenylosuccinate lyase deficiency disorder and Lesch-Nyhan disease. No adverse effects of adenine other than kidney damage mentioned above has been reported,

however no beneficial effects in the treatment of metabolic disorders has yet been found (Jaeken*, et al.*, 1988; Jinnah*, et al.*, 2013; Jurecka*, et al.*, 2014). To date, there are no reported studies that have used RibAde as a treatment for epilepsy. Given that the heart and the brain show similarities in the use of the salvage pathway as a means of regulating energy homeostasis, and the beneficial effects of ribose/adenine on ischemic stress response (Schneider*, et al.*, 2008), post-ischemic ATP recovery and cell viability (Watanabe*, et al.*, 2003; zur Nedden*, et al.*, 2012; zur Nedden*, et al.*, 2014), it is reasonable to assume that the epileptic brain might benefit from Rib/Ade or RibAde/allopurinol given the disruption in energy homeostasis found in the epileptic brain (Fredholm, 2007; Masino*, et al.*, 2009). There is little cost associated with the production of RibAde/allopurinol as ribose is commercially available and is widely used a nutritional supplement and allopurinol is used as a treatment for gout whereby it reduces the build up of uric acid crystals which leads to inflammatory arthritis. In addition, adenine is a common constituent of blood products to support red blood cell metabolism during storage used since the 1960s (Akerblom*, et al.*, 1967; Akerblom and Kreuger, 1975; Cancelas*, et al.*, 2015; Deverdier*, et al.*, 1964a; Deverdier*, et al.*, 1964b; Dumont*, et al.*, 2015; Kreuger*, et al.*, 1975) and has been successfully used in humans at doses ranging from 10 mg/kg/24 hr to 75 mg/kg/24 hr (Benke*, et al.*, 1973; Schulman*, et al.*, 1971).

In vivo studies in either humans or rodent models would be useful for the clarification as to whether orally administered RibAde/allopurinol is neuroprotective with regard to reducing the abnormal excessive synchronised neuronal discharges associated with an epileptic seizure and improving behavioural outcomes, and may provide additional support for the initiation of clinical epilepsy trials.

7.3. RibAde as a potential therapy vs. anaplerotic reactions

In the brain the purine salvage pathway is a major pathway for making adenine nucleotides. Adenine and hypoxanthine are combined with the sugar donor 5-phosphoribosyl-1-pyrophosphate (PRPP) to form AMP and inosine monophosphate (IMP) in a reaction catalysed by the enzymes adenine phosphoribosyltransferase (APRT) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT), respectively (Barsotti*, et al.*, 2002; Mascia*, et al.*, 2000).

Another method of modulating cellular ATP levels is via the use of citric acid cycle substrates (Fig 7.2.). Mitochondria are a main source of ATP in the cell, and ATP levels are maintained by the potential gradient across the mitochondrial membrane (Kovac*, et al.*, 2012). The glycolysis product pyruvate is transported across the mitochondrial membrane into the matrix where it undergoes further oxidation to produce the citric acid substrate acetyl-CoA by pyruvate dehydrogenase complex. The citric acid cycle produces carriers such as $NADH/H^+$ equivalents, which helps to maintain the potential gradient. NADH is then fed into the transmembrane electron transport pathway to make hydrogen ions. Hydrogen ions then move across the inner membrane through the large ATP synthase protein complex helping to synthesis ATP from ADP and free phosphate ions.

Anaplerosis is the process of replenishment of depleted metabolic cycle or pathway intermediates of the citric acid cycle. Substrates such as pyruvate and succinate have been shown to have good potential for suppressing seizure activity, as reviewed in (Kovac*, et al.*, 2013). *In vitro* preparations with pyruvate (5 mM) found that it helped

to reduce cell death in rat hippocampal cell cultures (Kovac, *et al.*, 2012). Both compounds have been used *in vivo* to study their effects on seizure activity. Oral administration of pyruvate (0.5 g/kg/day) was found to improve seizure control in a patient with epileptic encephalopathy (Koga, *et al.*, 2012) whilst, succinate (0.5 microl of 1.5 mM intrastriatal injection) in rats was found to reduce both the number of seizures and seizure duration (Royes, *et al.*, 2003).

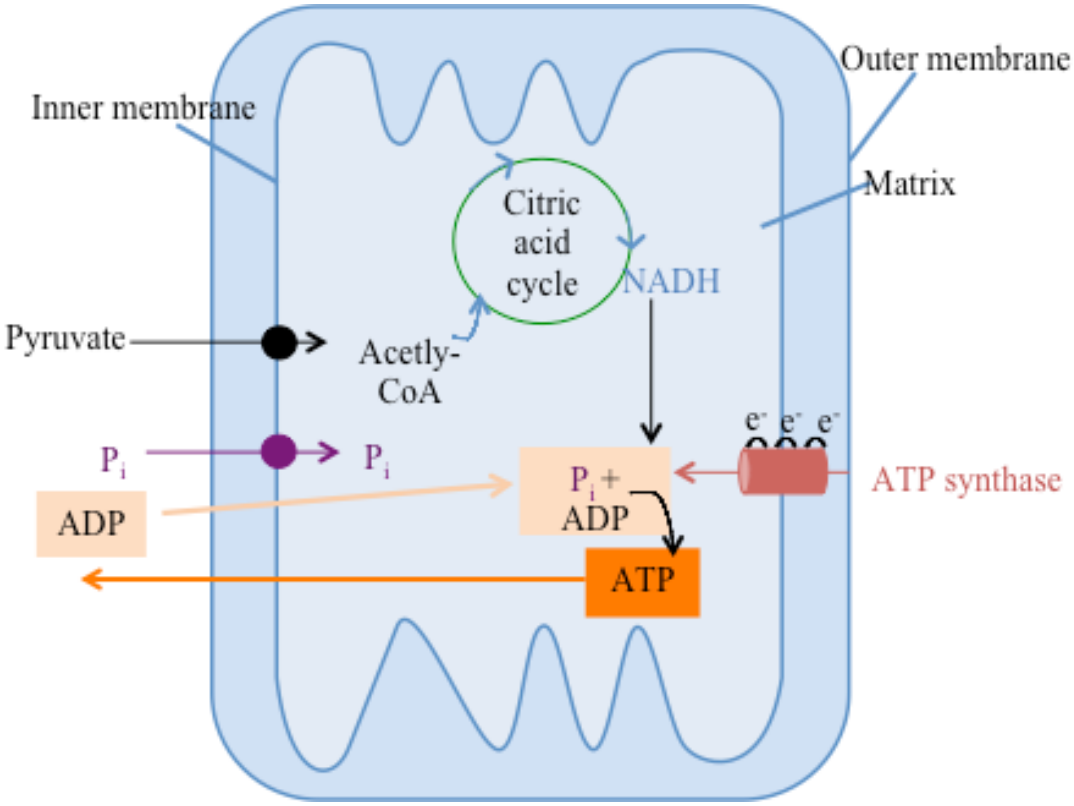

Figure 7.2. Schematic representation of mitochondrial ATP synthesis. Pyruvate is transported across the mitochondrial membrane via specific tranporters into the matrix where it undergoes further oxidation to produce the citric acid substrate acetyl-CoA by pyruvate dehydrogenase complex. The citric acid cycle produces carriers such as $NADH/H^+$ equivalents. NADH is then fed into the transmembrane electron transport pathway to make hydrogen ions. Hydrogen ions then move across the inner membrane through the large ATP synthase protein complex. The ATP synthase protein complex helps to synthesise ATP from ADP and free phosphate ions. ATP is then transported to sites of energy consumption. Taken from (Dahout-Gonzalez, *et al.*, 2006).

However, there are some disadvantages associated with pyruvate or succinate as a potential therapeutic supplementation for the treatment of epilepsy such as their poor permeability at cell membranes and the blood brain barrier (Cremer*, et al.*, 1979; Kovac*, et al.*, 2013; Pardridge and Oldendorf, 1977). In addition, although both compounds have been shown to have anticonvulsant properties, undesirably, proconvulsive properties have also been shown (Gonzalez*, et al.*, 2005; Roehrs*, et al.*, 2004). A rapid intravenous injection of 9 mmoles/kg pyruvate to mice *in vivo* was enough to lead to the development of seizures (Gonzalez*, et al.*, 2005) whereas, injections of succinate (0.8, 2.5 and 7.5 micromoles/microllitres, intra-cerebro-ventricular) caused dose-dependent convulsive behaviour in mice (Roehrs*, et al.*, 2004). In comparison to supplementation of citric acid cycle substrates, supplementation with metabolites of the purine salvage pathway, such as ribose/adenine, co-administered with the xanthine oxidase inhibitor allopurinol to prevent the development of kidney stones caused by the degradation of adenine by xanthine oxidase to an insoluble metabolite 2,8-dihydroxy-adenine (Greenwood*, et al.*, 1982). Given this RibAde/allopurinol given together are safe to use, and has been shown to be well tolerated in humans. I therefore believe that the use of RibAde supplementation as opposed to citric acid cycle substrates, such as pyruvate or succinate, may be a more promising approach for the treatment of epilepsy. Unlike AEDs, the use of RibAde/allopurinol does not target a specific receptor or pathway but instead it addresses the energy state of tissues such as those seen during epileptic seizures. Therefore, RibAde/allopurinol may be given either shortly after an insult in order to aid in the restoration of physiological function or potentially given to those with established epilepsy. Patients with traumatic brain injury may also develop epileptic seizures. Traumatic brain injury patients have impaired

mitochondria function and associated decrease in ATP production that might add to the development of epileptogenesis (Giza and Hovda, 2001; Verweij*, et al.*, 2000). It is plausible to suggest that treating patients with traumatic brain with RibAde/allopurinol as a preventative measure for the stabilisation of metabolic function in order to prevent the occurrence of recurrent epileptic seizures may be a potential option for those patients with infrequent seizures or those with less severe epileptic seizures. Also, it might be useful to also investigate the therapeutic effect of RibAde/allopurinol in those patients with severe epilepsy used in combination with low doses of AEDs. Other patients for which the use of RibAde/allopurinol might be of benefit are those with drug-resistant seizures and those where surgery or alternative therapies such as deep brain stimulation is not an option and in children. I believe that RibAde/allopurinol supplementation might prove beneficial for the treatment of different types of epilepsy.

8. Appendix 1

8.1. Seizure parameters for 6 and 9 mM K^+

Analysis was carried on those slices showing bursting epileptiform activity when challenged with either 6 mM or 9 mM K^+. One-way ANOVA was calculated for seizure parameters burst duration and inter burst interval and Kolmogorov-Smirnov test for the inter spike interval. The results from these analysis is summarised in the tables below.

Summary of One-way ANOVA test for the inter burst interval 1 to 2 for slices treated with 6 mM K^+.						
	Treatment	N	Mean	SD	SE of mean	P value
IBI 1	Creatine	6	184.25888	52.24511	21.32898	0.80812
	Control	11	172.59004	51.15371	15.42342	
	RibAde	4	189.77408	42.15488	21.07744	
IBI 2	Creatine	4	160.4078	50.31396	25.15698	0.21367
	Control	8	116.3801	42.33742	14.96854	
	RibAde	3	165.76783	60.57875	34.97516	

Summary of One-way ANOVA test for the inter burst interval 1 to 2 for slices treated with 9 mM K^+.						
	Treatment	N	Mean	SD	SE of mean	P value
IBI 1	Creatine	7	100.33286	39.57068	14.95631	0.99273
	Control	4	98.465	23.31502	11.65751	
	RibAde	7	98.43286	27.86224	10.53094	
IBI 2	Creatine	6	86.38333	26.50858	10.82208	0.51942
	Control	4	76.645	16.3154	8.1577	
	RibAde	5	95.62	25.70986	11.4978	

Summary of One-way ANOVA test for the burst durations 1 to 3 for slices treated with 6 mM K^+.						
Burst number	Treatment	N	Mean	SD	SE of mean	P value
1	Creatine	6	36.57333	13.52944	5.52337	0.67406
	Control	13	36.81077	20.6161	5.71788	
	RibAde	5	28.562	14.99312	6.70513	
2	Creatine	6	30.46167	10.94021	4.46632	0.92658
	Control	11	32.38545	20.05968	6.04822	
	RibAde	4	28.695	13.13755	6.56877	
3	Creatine	4	35.1075	5.86649	2.93324	0.46089
	Control	8	34.6625	14.82897	5.24283	
	RibAde	3	24.05667	13.70268	7.91124	

Summary of One-way ANOVA test for the burst durations 1 to 3 for slices treated with 9 mM K^+.						
Burst number	Treatment	N	Mean	SD	SE of mean	P value
1	Creatine	12	58.29833	57.73376	16.6663	0.37166
	Control	12	44.66167	21.91577	6.32654	
	RibAde	8	32.82076	22.09077	7.81027	
2	Creatine	7	49.64286	34.2014	12.92691	0.24874
	Control	4	64.0725	18.78102	9.39051	
	RibAde	7	34.72248	22.58033	8.53456	
3	Creatine	6	49.915	32.43494	13.24151	0.10863
	Control	4	65.505	20.65476	10.32738	
	RibAde	6	31.2274	11.09339	4.52886	

8.2. Kolmogorov-Smirnov test of seizure parameters for 6 and 9 mM K^+

Summary of Kolmogorov-Smirnov test for the inter spike interval of burst 1-3 for slices treated with 6 mM K^+.			
Burst number	**Kolmogorov-Smirnov test**		
		Control	**RibAde**
1	**Creatine (n = 6) vs.**	n = 13 p = 0.07091 D = 0.60256 Z = 1.22088	n = 5 p = 0.33333 D = 0.81818 Z = 0.55048
	Control (n = 13) vs.	—	n = 5 p = 0.17647 D = 0.53846 Z = 1.02323
2	**Creatine (n = 6) vs.**	n = 11 p = 0.15562 D = 0.54545 Z = 1.07475	n = 4 p = 0.69524 D = 0.41667 Z = 0.6455
	Control (n = 13) vs.	—	n = 4 p = 0.66374 D = 0.38636 Z = 0.66172
3	**Creatine (n = 4) vs.**	n = 8 p = 0.08485 D = 0.75 Z = 1.22474	n = 3 p = 0.65714 D = 0.5 Z = 0.65465
	Control (n = 8) vs.	—	n = 3 p = 0.01212 D = 1 Z = 1.4771

Summary of Kolmogorov-Smirnov test for the inter spike interval of burst 1-3 for slices treated with 9 mM K^+.			
Burst number	**Kolmogorov-Smirnov test**		
		Control	**RibAde**
1	**Creatine (n = 12) vs.**	n = 12 p = 0.0015 D = 0.75 Z = 1.83712	n = 8 p = 0.33333 D = 0.81818 Z = 0.55048
	Control (n = 12) vs.	—	n = 8 p = 0.32405 D = 0.41667 Z = 0.91287
2	**Creatine (n = 7) vs.**	n = 4 p = 0.98788 D = 0.25 Z = 0.39886	n = 7 p = 0.57517 D = 0.42857 Z = 0.80178
	Control (n = 4) vs.	—	n = 7 p = 0.89091 D = 0.32143 Z = 0.51282
3	**Creatine (n = 6) vs.**	n = 4 p = 0.92381 D = 0.33333 Z = 0.5164	n = 5 p = 0.59091 D = 0.43333 Z = 0.71563
	Control (n = 4) vs.	—	n = 5 p = 0.87302 D = 0.35 Z = 0.52175

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