Glutamate receptors potentiate single K-ATP channels through intracellular ATP changes. Implications to Rett Syndrome and mechanisms of epilepsy.

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I hereby declare that I prepared PhD thesis „ Glutamate receptors potentiate single K-ATP channels through intracellular ATP changes. Implications to Rett Syndrome and mechanisms of epilepsy.” on my own and no other sources and aids than quoted.

03. September 2013 Göttingen Rustam Mollajew

"Almost 10 years after the discovery of the first mutations in Mecp2 causing RS and 16 years after the discovery of the gene itself in the mouse genome, the exact function of the Mecp2 protein is still unknown and remains the subject of many debates and many research projects!"

(Medical Genetics & Functional Genomics Group)

"you who weep now, for you will laugh."

Luke 6:20–22

Contents

Abbreviations

$[Ca^{2+}]_i$ – cytoplasmic free Ca^{2+};

$[cAMP]_i$ – intracellular concentration of cAMP;

AAV – adeno-associated virus;

AC – adenylate cyclase;

ACSF – artificial cerebro-spinal fluid;

AMPA – a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate;

AP – action potential;

Ateam 1.03 - neuron-targeted ATP sensor;

ATP – adenosine triphosphate;

BAPTA – 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid);

BDNF – brain-derived neurotrophic factor;

BrOMecAMP – 8-bromo-2'-OMe-cAMP;

CaM – calmodulin;

CaMK – calmodulin dependent protein kinase;

cAMP – cyclic adenosine monophosphate;

CFP – cyan fluorescent protein;

CICR – Ca^{2+}-induced Ca^{2+} release;

CNS – central nervous system;

CREST – calcium-responsive transactivator;

CSF – cerebrospinal fluid;

DDA – 2′5′-dideoxyadenosine;

DHPG – (S)-3,5-dihydroxyphenylglycine;

DIV – days *in vitro*;

D-MEM – Dulbecco's/Vogt modified Eagle's minimal essential medium;

DMSO – dimethyl sulfoxide;

D3cpv - genetically encoded calcium indicators;

EDTA – ethylenediaminetetraacetic acid;

EGFP – enhanced green fluorescent protein;

EGTA – ethylene glycol tetraacetic acid;

Epac – cAMP-dependent exchange factor;

Epac1-camps – exchange protein directly activated by cAMP;

ER – endoplasmic reticulum;

Fig. – Figure;

FRET – fluorescence resonance energy transfer;

GABA – gamma-aminobutyric acid;

GFP – green fluorescent protein;

GPCR – G-protein-coupled receptor;

H-89 – *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinoline sulphonamide hydrochloride;

HDAC – histone deacetylases;

HEK 293 – human embryonic kidney 293 cells;

ICAN – Ca^{2+} -Activated Nonselective Cationic Current;

IGF-1 – iinsulin-like growth factor 1 (IGF-1), somatomedin C;

INaP – persistent sodium current;

Int – chimeric intron

IO – *inferior olive;*

IP(3)R – inositol triphosphate receptor;

IPSC – inhibitory postsynaptic current;

ITR – inverted terminal repeats of AAV-2;

K-ATP channels – ATP-sensitive potassium channels;

KO – *Mecp2*$^{-/y}$ (null) mice;

L-NMMA – N-monomethyl-L-arginine;

LED – light-emitting diode;

LSM – laser scanning microscopy;

Mecp2 – MeCP2 coding gene;

MeCP2 – methyl-cytosine binding protein;

NDD – neurodevelopmental disorder

NMDA – N-methyl-D-aspartate;

NMRI – inbred line of mice for 51 generations that transferred to the Naval Medical Research Institute;

NOS – nitric oxide synthase;

P3-P49 – postnatal days 3-49;

PBS – phosphate buffered saline;

PDE – phosphodiesterase;

PLC – phospholipase C;

PKA – protein kinase A;

PKC – proteinkinase C;

PreBötC – pre-Bötzinger complex;

PXX – postnatal day;

ro-GFP1 – mitochondrial redox sensor

RS – Rett Syndrome;

RT – room temperature;

SERCA – Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase;

SNpc – *substantia nigra pars compacta;*

Tg – thapsigargin;

TRD – transcription repression domain;

TRH – thyrotropin-releasing hormone;

TRPM4 – calcium-activated nonselective cation channels;

UV – ultraviolet;

VGCC – voltage-dependent calcium channel;

VLM – ventrolateral medulla;

XII – *nucleus hypoglossus;*

YFP – yellow fluorescent protein

Summary

Rett Syndrome (RTT) is a neurodevelopmental disorder caused primarily by mutations in a *Mecp2* gene and exclusively affects females. RTT is one of the prevalent developmental disorders with a high frequency of occurrence (about 1:15,000 to 1:10,000) and patients are prone to gastro-intestinal disorders and about 80% have seizures. Although the genetic defect in the *Mecp2* gene defines RTT, the insights from recent studies have highlighted the complex pathophysiological mechanisms that point to developmental disorder. A better understanding of the interplay of these pathophysiological mechanisms is one of the challenges in establishing of effective therapeutic strategies. In the present study, we used *Mecp2*-null mice as a model to examine the physiological and/or pathophysiological cellular mechanism pathways that can play important role in the development of Rett Syndrome.

The main aspect of this study was to reveal the role of glutamate receptors (GluR) /K-ATP channels crosstalk in the development of RTT. Accordingly, the activities of these channels were examined in neurons from wild type (WT) and *Mecp2*-null mice that represent physiological and pathophysiological examples, respectively. Excessive stimulation of glutamatergic receptors can overexcite neurons. This can be dampened by K-ATP channels linking metabolic and neuronal activities. The crosstalk has not yet been examined on the single channel level. I aimed to examine how K-ATP channel activity changes after stimulation of neuronal glutamate receptors and whether those effects may be mediated through variations in the intracellular ATP levels.

In the hippocampal neurons, GluR agonists augmented the open state probability (P_{open}) of K-ATP channels with relative efficacy: Kainate = AMPA > NMDA > t-ACPD. Inhibition of calcium influx and chelation of intracellular calcium did not modify the GluR effects. Kainate did not augment production of reactive oxygen species measured with roGFP1. The oxidant H_2O_2 increased P_{open} about 2-fold within 1 min but the subsequent application of AMPA/Kainate

produced further potentiation; in the same proportion as in the control. The data indicate that changes in the activity of K-ATP channels due to ROS and GluR stimulation have different mechanisms. GluR actions were abolished in Na^+-free solutions and after blockade of Na^+-K^+-ATPase. GluR stimulation enhanced ATP consumption that decreased submembrane ATP levels, whereas metabolic poisoning diminished bulk ATP.

Increase of the K-ATP channel open state probabilities after GluR stimulation less in Mecp2 $^{-/y}$ hippocampal neurons. It allows to speculate whether protective role attributed commonly to K-ATP channels in RTT hippocampal neurons may be less effective.

I examined the role of GluR and K-ATP channels cross-talk in relation to mechanisms of epilepsy. In hippocampal organotypic slices using well established model, a seizure-like activity was evoked by removal of magnesium from bath. This was accompanied by calcium and ATP changes. The ATP responses were distinctly different in specific hippocampal regions: in dentate gyrus (DG) the neurons possessed higher ATP resting levels and showed smaller changes to epileptogenic stimuli in comparison with CA1 and CA3 areas.

ATP levels in RTT neurons were significantly higher than those measured in WT cells. This corroborates well with observations of diminished K-ATP channel open state probability. We propose that K-ATP channels and GluRs are functionally coupled and can regulate long-lasting changes of neuronal activity in the CNS neurons.

The obtained data indicate that RTT symptom as propensity to seizures can be essentially contributed by improper K-ATP channel functioning.

1. Introduction to Rett Syndrome and underlying mechanisms.

1.1 Definition, causes, clinical picture and epicrisis.

According to the Dorland's Pocket Medical Dictionary Rett Syndrome (RTT), originally termed as cerebroatrophic hyperammonemia is a neurodevelopmental disorder (NDD) of the grey matter of the brain. In the Diagnostic and Statistical Manual of Mental Disorders (DSM) Rett Syndrome is listed under the category of pervasive developmental disorders.

The nosological diagnosis was described and characterized *in tote* by Austrian children's doctor, Andreas Rett in 1966 *(Rett, 1966)*. Even in 1954 he noticed the symptoms in two female teenagers making the same characteristic repetitive hand-washing movements *(Rett, 1966; Hagberg et al., 1983).* Rett publications appeared only in German medical literature and did not attract an attention of other colleagues and for a long time the disorder was examined episodically. Only in 1983 Dr. Hagberg in his clinical examination of striking progressive encephalopathy raised again the problem of RTT and revived interest to that *(Hagberg et al., 1983).* Hagberg and colleagues examined and systematized RTT symptoms in details described clinical picture and possible development and presented RTT as separate nosological unit.

In 1999, it was discovered, that RTT is caused primarily by a genetic mutation in the gene that encodes MeCP2 - methyl-cytosine binding protein *(Amir et al., 1999)* and can arise sporadically or from germline mutations. This mutation has now been found in more than 95% cases for typical RTT *(Neul et al., 2008*) and in 50–70% of atypical cases *(Guerrini and Parrini, 2012).*

The disorder frequency varies from 1:15.000 to 1:8.000 by age of 12 years *(Francke, 2006)*. According to the *Rett Syndrom Deutschland e.V.* the frequency of RTT in Germany fluctuates from 1:15.000 to 1:10.000 and every year about 50 children with Rett Syndrome appear i.e. RTT is one of the prevalent NDD in the Federal Republic. Statistical data 1:15.000 -

1:10.000 are generally accepted for present day. NND mainly does not depend on locality, race, climate and other factors and has the same clinical manifestations and picture world over.

1.1.1 Clinical picture.

Diagnostic. In practice, RTT usually is diagnosed on the base of the clinical symptoms and rarely using electrophysiological or genetic examinations. Parents resort to help when the clinical manifestations are evident, *viz.* or on *Rapid Developmental Regression Period of RTT (Stage II),* or less on *Early Onset Period (Stage I) (Smeets et al., 2012; Dolce et al., 2013).*

Hagberg and Witt-Engerström suggested 4 clinical stages for RTT development and clinical course *(Hagberg and Witt-Engerström, 1986).* Briefly, the stage I is from 6 to 18 months and lasts without any regression. The stage II is from 18 months to 3-4 years, it is the stage of regression. The stage III and IV (from 7 till adult age) is term after regression. This division is active for present day.

Prophylaxis examination which is used normally to identify of some genetic deseases such as Gaucher's disease *(Zimran, 2011),* Huntington's disease (*Tang and Feigin, 2012),* Edwards syndrome *(Cereda and Carey, 2012)* and many other for RTT is problematic due to that the parents are genotypically normal and the pregnancy usually is not defective *(Zoghbi et al., 1989; Trappe et al., 2001).*

According to Rett Syndrome Diagnostic Criteria Working Group (1988), there are the following typical clinical manifestations: seizures, scoliosis, spasticity, apnea, hyperventilation and other. It is nothing else but convenient diagnostic cliché and basically in many cases the diagnostic is tractable problem without necessity to make genetic or electrophysiological examinations *(Hagberg et al., 2005; Neul et al., 2010*). In some case however the diagnostic can be difficult due to symptomatic resemblance of many common features of RTT and Angelman syndrome *(Williams et al., 2010)* and the similarity between RTT and autism *(Castro et al.,*

2013).

Recent RTT studies show some physiological dysfunctions such as EEG abnormalities *(Moser et al., 2007)* and elevation of glutamate level in cerebrospinal fluid *(Hamberger et al., 1992; Lappalainen and Riikonen, 1996)* and several minor other in-patient variables *(see Smeets et al., 2009).*

Symptoms. Clinically, the affected girls have a normal pre-natal development and normal postnatal period extending up to 18 months of age. Typical features of clinical manifestation are small hands and feet, a delay of the rate of head growth, scoliosis and growth failure *(Rett, 1966)*, also people with Rett Syndrome are predisposed to breathing and cardiac arrests, gastrointestinal disorders and about 80% of all patients develop seizures *(Hagberg et al., 1983; Greene and Greenamyre, 1996 (a, b); Kim et al., 2012; Jian et al., 2006)*. Other signs of Rett Syndrome belong to a group of motor disorders. These signs such as chorea, dystonia, and ataxia are mainly concomitant symptoms of RTT *(FitzGerald et al., 1990; Percy, 2011).*

The most illustrative examples of clinical manifestation are breathing arrest and seizures. Breathing arrest occurs in the following sequence: short time apnea (3-5 sec) -- active hyperventilation (10-20 sec) -- normal breathing *(Viemari et al., 2005; Roux et al., 2007; Stettner et al., 2007 (a, b); personal author observations*). Breathing arrests are accidental and begin without any apparent reasons.

Almost invariably, seizures accompany patients with Rett Syndrome *(Jian et al., 2007; Kim et al., 2012).* The seizures have not only the negative influence on child life quality but are very often the cause of disease morbidity. In connection with topicality of epilepsy manifestation in RTT patients, this symptom will be discussed in a separate chapter.

Epileptic seizures and Rett Syndrome

One of the most disputable features of Rett Syndrome is the manifestation of seizures.

From 70% to 90% of the patients with RTT develop seizures (*Hagberg et al., 2002; Jian et al., 2007; Glaze et al., 2010; Kim et al., 2012).* Epileptic seizures at RTT usually begin from the second-third years of life, increase in frequency with age, reach the maximal rate at 7 -8 years and significantly decrease at the age after 40 *(Jian et al., 2006; Glaze et al., 2005; Glaze et al., 2010; Nissenkorn et al., 210; Kim et al., 2012*). The epileptic forms can vary significantly, from more or less controlled epilepsy to tonic-clonic seizures (Table 1.) About 13% of sudden death in Rett Syndrome occurs in the frame of epileptic paroxysm *(Kerr, 1997; Kerr, 2006).*

Table 1.4 Prevalence of seizure types and epilepsy features *(after Cardoza et al., 2011)*.

	Number (*n* = 60)	**Percentage (%)**
Seizure type		
Generalised tonic–clonic	37	62
Secondarily generalised tonic–clonic	15	25
Complex partial	16	27
Tonic	2	3.3
Atonic	1	1.6
Absence seizures	1	1.6
Myoclonic	1	1.6
Epilepsy type		
Symptomatic generalized	23	38.3
Symptomatic focal	35	58.3
Diagnosis not possible	2	3.3

Genotype-phenotype correlation of the epileptic seizures with the type of* MECP2 *mutation in RTT.

The data concerning genotype-phenotype correlation are more or less consilient. Using the combination of genetic examination, physiological study and survey Glaze showed that the frequency of epileptic seizure correlated with *Mecp2* mutations (*Glaze et.al, 2010*). The epileptic seizures occur more frequently at mutation type such as T158M (74% of all examined cases), R106W (78%), and less frequently at R255X (49%) and R306C (49%) mutation *(Glaze et.al, 2010).* At the same time Jian and colleagues showed that seizure rates were reduced at p.R294X, p.R255X mutations and C terminal deletions (*(Jian et al., 2006; Jian et al., 2007).* Most other examinations mainly corroborate Juan and Glaze studies with small nationality ***(sic!)*** deviations *(Pan et al., 2002; Fukuda et al., 2005; Kim et al., 2012).*

In all cases howerever, the mutations influence only on the frequency but not on the character of seizures or age dependence *(Glaze et al., 2009).* Thus, this problem needs further thorough examination.

Possible epileptogenic causes in RTT. Epileptic seizure *per se* it is an excessive or synchronous neuronal activity of neuronal cell *(Fisher et al., 2005).* The causes for seizure onset can be emotional stresses, alcohol, brain traumas, CNS infections and many others *(Frucht et al., 2000).* For example children with pyknolepsy may be susceptible to hyperventilation *(Hirsch et al., 2007).* In the case of RTT it may represent an epileptogenic stimulus. Epilepsy in RTT can be caused by hypoxic ischemic encephalopathy *(Malhotra R. et al., 2001)* or sleepless *(Holmes et al., 2008; Derry and Duncan, 2013).*

Thus, the link between epileptic seizures and some definite cause has not been proved *(see Helbig and Lowenstein, 2013).*

Therapy. The therapy is mainly symptomatic and depends on prevalent clinical manifestations. For the most part, ill children stay at home under parents care.

The current therapy of RTT includes constant physician consultation, self-care, use of drugs for the treatment of depression and insomnia, speech therapy and other *(Matsuishi et al., 2011; Ricceri et al., 2013; Castro et al., 2013)***.**

At present time there is no effective therapy for Rett Syndrome. It was supposed that probably, restoring MecP2 function may bring the convalescence *(Tropea et al., 2009; Neul and Zoghbi 2004; Castro et al., 2013).*

Prognosis. For RTT the general prognosis is unfavourable. Males die within first months of life. Females can live under the medical care for 40 years and sometimes even longer *(see Berg and Hagberg, 2001)*

Nevertheless the physicians continue the search of therapy methods for RTT *(Hampson et al., 2012; Smeets et al., 2012).* They e.g. organised International Rett Syndrome Association, several associations in different countries, and some regional groups. These groups distribute appropriate information for patients; provide moral, financial, jurisdictional and medical support to patients with RTT and their families and maintain close contact with medical organisations *(Fife et al., 2003).*

Mortality cases. The data concerning the causes of sudden death are enough contradictive.

For females with RTT Dr. Gillberg (1999) suggested three main reasons for sudden death: spontaneous brainstem dysfunction, cardiac arrest and epileptic seizures. Kerr *(Kerr, 1999)* proposed that an infant mortality is caused by: infections or asthenia (48%), other reasons

or accident (13%), due to epileptic paroxysm (13%), sudden death (26%) without explanation. Dr. Wilken (Kassel) proposes that apnea during the night sleep may represent a dominant cause for sudden death. According to *Rett Syndrome Deutschland e.V.* statistic, the causes such as infections, accidents and asthenia at least in Germany are not prevalent.

Autopsy examinations showed the reduction in weight and size of the brain *(Armstrong, 2005)*. In this study the death cause was not taken into account and morphological and cytological autopsy study of the brain was preferred.

1.2 Genetic basis of Rett Syndrome.

Structure and function. MeCP2 is mammalian single polypeptide which belongs to a family of methyl-CpG-binding domain proteins. It locates at the q28 locus on the X chromosome *(Amir et al., 1999; Aber et al., 2003)* and consists of two domains: a methyl-cytosine-binding domain (MBD) and a transcriptional repression domain (TRD) *(Wakefield, 1999; Free et al., 2001)*. The MBD fastens to the methylated CpG sites on the DNA (5′ CpG islands) and is accountable for chromatin localization of the protein. The TRD region reacts with SIN3A regulator to decreases the ability of the histones to bind to DNA *(Amir et al., 1999; Wakefield et al., 1999; Wade, 2004; Bowen et al., 2005*). MeCP2 protein *per se* is responsible for recruiting chromatin-modifying activities that causes the deacetylation and condensation of chromatin (*Bowen et al., 2005).* As a result the regions which bind MeCP2 in DNA became no more accessible to the transcription machinery and the corresponding genes cannot be expressed *(Amir et al., 1999)*. Nevertheless, MeCP2 protein is able to modify chromatin structure without methylation *(Georgel et al., 2003; Adkins and Georgel, 2011)*.

In spite of multiple mechanisms of regulation it is not possible today to link the deregulation of gene transcription to deficiency of the *Mecp2* gene *(Neul et al., 2008*). The target genes of MeCP2 are yet unknown. Probably that *Mecp2* mutation does not directly responsible

for neurodegeneration or any other disorders (*Schanen et al., 1998).* It seems that mutation leads to the MeCP2-regulated changes in transcription patterns of signalling moieties required for proper development *(Guy, 2011; Cohen et al., 2011; Banerjee et al., 2012).*

Morphology and cytology. It is firmly established that the brains of RTT patients show no significant morphological abnormalities or tissue damage *(Panayotis et al., 2011*; *Zoghbi et al., 1989).* There is some reduction of brain size in Rett Syndrome which associated with reduction in dendritic branching of pyramidal neurons in the frontal, temporal and motor cortices *(Armstrong, 2005).* This may be related to decrease in number of dendritic spines in the frontal cortex *(Belichenko et al., 1994).* The size of neurons in the cortex, thalamus and hippocampus is reduced *(Kitt and Wilcox, 1995). Mecp2*$^{-/y}$ mice show also decreased cortical dendritic arborisation *(Kishi and Macklis, 2004)* and reduction in synapse number in the hippocampus *(Chao et al., 2007).*

Thus, mutation in *Mecp2* does not produce significant morphological changes in cellular and tissue organisation which can explain motor and emotional observed dysfunctions *(see Budden et al., 2005).*

Role of MeCP2 in Rett Syndrome. Mecp2 mutations have been identified not only in RTT children but also in males with schizophrenia, some forms of autism and other neurodevelopment disorders *(Shibayama et al., 2004).* Table 1.2 shows the symptoms in different syndromes as related to MeCP2 expression.

Mecp2 gene was first identified in mice in 1992 by the group of Adrian Bird (*Lewis et al., 1992).* The human gene was cloned in 1996 *(D'Esposito et al., 1996).* There are three different transcripts found in human *(Pelka et al., 2005)* with still unclear differences in their function or distribution.

Table 1.2 Phenotypes of MeCP2 dysfunction *(after Chahrour and Zoghbi, 2007*).

MeCP2 State	Sex-Associated Syndromes and Symptoms	
	Female	**Male**
Loss of Function	Classic RTT	Infantile encephalopathy
	Atypical RTT	Classic RTT (47,XXY or somatic mosaic)
	Angelman-like syndrome	Mental retardation with motor deficits
	Mental retardation with seizures	Bipolar disease, mental retardation, and tremors
	Mild mental retardation	Juvenile-onset schizophrenia, mental retardation, and tremors
	Learning disability	Mental retardation, psychosis, pyramidal signs, and macroorchidism
	Autism	
	Normal	
Overexpression	Preserved speech variant of RTT	Severe mental retardation and RTT features
		Nonspecific X-linked mental retardation

In spite of the fact that target genes of MeCP2 fully are not defined, such target genes are probably important for the normal function of the central nervous system *(Chahrour and Zoghbi, 2007).*

Moreover recent studies showed that MeCP2 may also function as a transcriptional activator, through recruiting the transcription factor CREB1 *(Chu et al., 2012)*. It is suggested that that MeCP2 is a key transcriptional regulator with potentially dual roles in gene expression *(Chu et al., 2012).*

1.2.1 Mouse model for Rett Syndrome.

There are several animal models for RTT examination *(Calfa et al., 2011).* Mouse models have been successfully used in study *in vivo* function of MeCP2 and RTT developing *(Guy et al., 2001; Viemari et al., 2005; Nelson et al., 2006; Wang et al., 2006).* Nevertheless every year appear the new more applicable mouse models for RTT examination *(Calfa et al., 2011; Guy et al., 2011; Ricceri et al., 2008).* Vast majority of the examinations using mouse models are concentrated on the loss of MeCP2 function in males, RTT female examinations served them as control *(Samaco and Neul, 2008).*

There are many similarities in Rett Syndrome of mice and humans *(Gaultier and Gallego, 2008).* Both in mice *(Larimore et al., 2009)* and humans *(Wenk, 1997)* with RTT, autopsy shows reduced brain size, a decrease in the size of individual neurons and a reduction of dendritic arborisation. It is well accepted that the mutant newborn mice with targeted deletions of *MeCP2* genes is an excellent research tool for understanding human Rett Syndrome that may provide new medicine and novel therapy in RTT (*Nelson et al., 2006*).

Similar to human RTT patients, homozygous female *Mecp2* murine mutants are not viable and heterozygous females are phenotypically heterogeneous due to variable patterns of X-chromosome inactivation (*Guy et al., 2001).* Therefore, most laboratories have studied the effects of MeCP2 loss of function in hemizygous males ($Mecp2^{-/y}$), which are completely devoid of MeCP2 and therefore tend to be more phenotypically homogenous than female heterozygotes *(see Bissonnette and Knopp, 2007).*

Several Rett Syndrome mouse models that at most often used in the laboratory examinations:

1) $Mecp2^{-/y}$ mice with extended exonic deletion of the *Mecp2* gene *(Chen et al., 2001; Guy et al., 2001; Pelka et al., 2006);*

2) *Mecp2*$^{308/y}$ mice with truncation of MeCP2 protein at amino acid 308, a human RS mutation *(Shahbazian et al., 2002);*

3) *Mecp*$^{2Flox/y}$ mice expressing a hypomorphic *Mecp2* allele *(Samaco et al., 2008);*

4) *Mecp*^{2Tg1} mice that overexpress MeCP2 protein *(Luikenhuis et al., 2004).*

In present study, we used *Mecp2-null* mice (strain B6.129P2(C)-Mecp2tm1-1Bird further denoted as Mecp2$^{-/y}$) *(Guy et al., 2001*) as a model to examine the possible pathophysiological mechanisms that manifest in RTT. Guy and co-workers made as follows: using cre/loxP system exons 3 and 4 were excised in the early embryonic period of mice. *Mecp*2 $^{-/y}$ mice (males and females) have a normal development until 3-5 weeks *(Guy et al., 2001; Kerr et al., 2010).* The anxiety both in males and heterozygous females *(Santos et al., 2007; Temudo et al., 2007)* or social abnormalities *(Kerr et al., 2008*) were not observed. It was detected some motility decreasing *(Panayotis et al., 2011)* and hyperventilation *(Guy et al., 2011).* Then (usually from 7-8 weeks) male Mecp2 $^{-/y}$ null mice develop erratic breathing, increased variability in the duration of the respiratory cycle, alternating periods of fast and slow breathing frequencies *(Viemari et al., 2005).* Initial breathing disturbances worsen between the first and second months and the mice eventually die from fatal respiratory *arrest (Viemari et al., 2005; Stettner et al., 2007; Zanella et al., 2008; Voituron et al., 2009).* Also they have: uneven teeth, misalignment of jaws, rapid weight loss *(Viemari et al., 2005).*

Morphologically the Mecp2 $^{-/y}$ mice show the reduction in the number of tyrosine hydroxylase-expressing neurons in *substantia nigra (Panayotis et al., 2011),* significant decreasing of NMDA receptors expression (from 2 to 7 weeks) in several brain regions *(Blue et al., 2011),* decrease in GABAergic transmission in brainstem *(Medrihan et al., 2008).*

1.3 Glutamatergic neurons and K-ATP channels.

Glutamate-mediated synaptic transmission plays a dominant role in the brain and the vast majority of CNS neurons are glutamatergic. Glutamate receptors (GluR) are the key elements in synaptic transmission of information between neurons. Unlike acetylcholine, it is not destroyed within synaptic cleft and slowly removed through diffusion and uptake by glial cells. The long-time presence of glutamate in extracellular space after periods of excitation can disturb operation of vitally important networks. More acute effect is excitotoxicity that is nowadays is linked to a number of diseases including depression and anxiety, drug addiction, tolerance, and dependency; schizophrenia, Rett syndrome, Alzheimer's disease and AIDS dementia complex; amyotrophic lateral sclerosis; mitochondrial abnormalities; multiple sclerosis; neuropathic pain syndromes; ischemia/stroke, seizures, Parkinson's and Huntington's disease, and aching. It is possible that every disease involving GluRs may have very similar if not identical pathways, with only slight regional variations between different areas in the brain.

A widespread occurrence of non-synaptic glutamate effects in the CNS raises an obvious issue whether there are intrinsic mechanisms that can compensate deleterious effects of glutamate. During ischemia, the brain has an unnaturally high concentration of extracellular glutamate; overstimulation of GluR may produce severe disturbances in ion distribution across plasma membrane. Restoration of ion balance requires ATP and leads to its depletion. This can activate ATP-sensitive K^+ (KATP) channels.

Brief outline. ATP-sensitive K^+ channels, or K-ATP channels, are inward-rectifier potassium channels *(Noma, 1983)*, whose their activity is inhibited by intracellular ATP concentration *(Noma et al., 1983).* In excitable cells such as neurons, they can shape action potentials and set the resting membrane potential, i.e. their role can be to dampen neuronal excitability through membrane hyperpolarisation *(Karschin et al., 1997; Zawar et al., 1999).*

Differences in whole-cell conductance, density and sensitivity to intracellular ATP may play important role in different tissues *(Kakei et al. 1985; Rorsman and Trube 1985; N. Fujimura et al. 1997).*

Structurally K-ATP channel in the brain is an octameric complex that consists of four inwardly rectifying potassium channel subunits Kir6.2 and four sulphonylurea receptors SUR1 (*Clement et al., 1997; Karschin et al., 1998).* In the CNS, Kir6.2-containing K-ATP channels are ubiquitous found in hippocampus *(Pelletier et al., 2000; Zawar et al., 1999),* hypothalamus (*Miki et al., 2001*) and in other brain parts. Hippocampal neurons express a particularly high density of K-ATP channels *(Karschin et al., 1997; Zawar et al., 1999*).

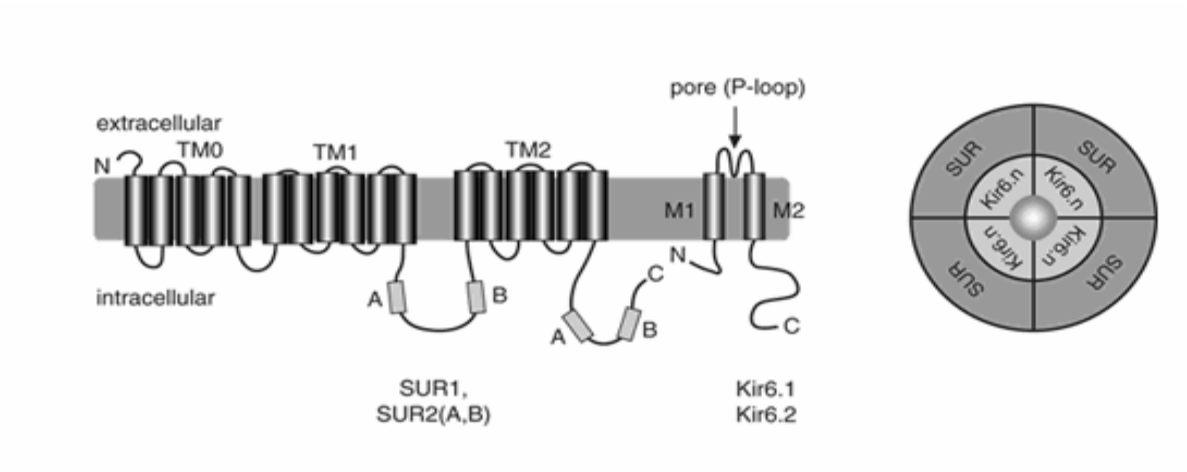


Fig 3.1 K-ATP channel structure (after Neuroamer Blog)
ATP-sensitive potassium channel is octameric proteins formed by 4 sulfonylurea receptor subunits (SUR1, SUR2A, or SUR2B) and 4 pore-forming inwardly rectifying potassium channel subunits (Kir6.1 or Kir6.2).

An increase in the intracellular ATP level in the membrane vicinity closes K-ATP channels *via* interaction with Kir6.2 subunits. An increase in ADP intracellular level activates K-ATP channels, by acting on SUR subunits. Opening of K-ATP channels shifts membrane potential toward the potassium equilibrium potential.

The physiological role of the K-ATP channels and the factors that regulate them in neurons under various physiological and pathological conditions are not yet fully understood. Up-to date the most studies have been performed by examining functional responses after pharmacological activation or blockade of K-ATP channels *(Lipton 1999; Ben-Ari & Cossart 2000; Avshalumov et al. 2001; Krnjević 2008)* or genetic modification *(Hernández-Sánchez et al. 2001; Yamada et al. 2001; Soundarapandian et al. 2007).*

The most energy in the brain is spend in restoration of ion homeostasis at glutamatergic synapses that limits the speed of information processing in the CNS *(Attwell & Laughlin, 2001)* and the estimates shows that ATP supplies can be readily exhausted within brief episodes of neuronal activity. The predictions are supported by the experimental data which show increases in open probability of K-ATP channels after action potentials *(AP, Tanner et al. 2011)* and their bursts *(Haller et al. 2001*). Activation of KATP channels negates, in part, the excitotoxic effects of glutamate *(Lipton 1999; Ben-Ari & Cossart 2000; Yamada et al., 2001; Krnjević 2008*). Genetically encoded enhancement of their activity is beneficial in the models of schizophrenia and epilepsy *(Hernández-Sánchez et al. 2001; Yamada et al, 2001)* and glutamate excitotoxicity *(Soundarapandian et al. 2007).* Thus, a current knowledge strongly supports the chain of events GluR activation → ATP depletion → K-ATP activity, but this pathway has been never examined on the level of single K-ATP channels.

Single K-ATP channels are potentiated by hypoxia *(Mironov et al. 1998)* in neurons of functionally intact preparation of the 'respiratory' network *(preBötzinger complex, preBötC, Smith et al. 1991).* In this study we found that AMPA/Kainate produced identical effects. Because the underlying mechanisms are different to study in slices due to various external factors produced by the presence of other cells in the tissue, we performed a detailed study in cultured hippocampal neurons, an established object in studies of glutamate excitotoxicity. Stimulation of GluRs induced activation of K-ATP channels that achieved maximum about 2 min after the stimulation and persisted over 20 min. Blockade of ATP consumption by ion

pumps, especially Na^+-K^+-ATPase, suppressed stimulation of K-ATP channels through GluRs. We measured ATP levels with genetically encoded ATP sensor *(Imamura et al. 2010)* and showed a close correspondence between single K-ATP channels and ATP in the cytoplasm. Robust and long-lasting potentiation K-ATP channels due to GluR stimulation observed in the hippocampal neurons suggest possible involvement of the effects in dampening uncontrolled excitation and excessive release of glutamate. This previously unexplored feedback between GluR and K-ATP channels may play important role in minimization of pathological consequences of glutamate insults in the CNS.

GluR and Rett Syndrome. The role of GluR and especially K-ATP channel referred to RTT is very poor understood.

Earlier using autoradiography method Blue and colleagues *(Blue et al., 1999; Johnston et al., 2001)* showed that the densities of NMDA and AMPA/Kainate receptors in frontal cortex of females with RTT are age–related. They showed that younger RTT females (≤8-10 years old) have higher NMDA receptor density those in older girls. Blue, Johnston and colleagues supposed that the Mecp2 mutations cause age-dependent disturbance of synaptic proliferation during first years of life.

Silverman and colleagues showed that the drug AMPAKAINE that acts as a positive modulator of AMPA receptors, can increase open state of the ion channels and subsequently augment excitatory glutamatergic neurotransmission. This enhancement can counteract some social RTT and autism abnormalities in mice and authors suggested a new pharmacological therapy approach for human patients *(Silverman et al., 2013)*.

A glutamate levels in cerebrospinal fluid from patients with RTT are elevated (*Hamberger et al., 1992; Lappalainen and Riikonen, 1996).* Using magnetic resonance spectroscopy (MRS) Maezawa and Jin (2010) showed increasing of glutamate level in RTT

brains, especially in young RTT patients and demonstrated the neurotoxic effect of glutamate in RTT brain.

Thus the patients with Rett Syndrome and especially young females' ≤8 years may have higher glutamate level in the brain and in CSF and higher glutamate receptor density. Enhanced sodium and calcium influx may cause increased glutamate excitotoxicity.

1.4. K-ATP channels and epilepsy.

In 1990 Amoroso and colleagues showed that opening state of K-ATP channels decreases GABA release in Substantia Nigra *(Amoroso et al., 1990; Schmid-Antomarchi et al., 1990).* They supposed that K-ATP channels can be involved in seizure propagation Using Kir6.2 knock-out mice Yamada and colleagues experimentally proved Amoroso hypothesis *(Yamada et al., 2001; Yamada, 2005).* They also showed that only facilitation of postsynaptic K-ATP channel activity is sufficient to reduce or abolish the activity of the GABA-containing neurones in response to hypoxia and subsequently decrease seizure propagation. Kir 6.2 knock-out mice are extremely vulnerable to epilepsy *(Seino and Miki, 2004*). Hernández-Sánchez and colleagues *(Hernandez-Sanchez* et al., 2001*)* generated mice with overexpressed K-ATP channels (namely SUR1 was overexpressed). The threshold for Kainate-induced seizures in overexpressed neurons was significantly higher and the resistance to excitotoxic damage was also increased.

Thus, it is direct link between K-ATP channel activity and vulnerability to epilepsy.

1.5 Aim of the study.

To date the exact function of the MeCP2 protein is yet unknown. One of the long-standing questions in Rett Syndrome studies is the association of MeCP2 mutation and the incidence of epileptic seizures. Our knowledge on the molecular basis underlying the incidence of epilepsy, in general, is much limited. Over the years, many studies have shown a possible link between glutamate receptors and incidence of epileptic events. Although the physiological role of glutamate receptors in neurotransmission in the brain is well understood, their role and other factors that regulate them under pathological conditions of Rett Syndrome are ill-defined. Equally poorly understood is the role of K-ATP channels together with glutamate receptors either as effectors or regulators of the pathological state that manifests as Rett Syndrome.

In present work I aimed:

- To examine the role of K-ATP channels and their interdependence with glutamate receptors in the development of the pathological state that manifests as Rett Syndrome.
- To examine the cross-talk between K-ATP channels and intracellular ATP in relation to epileptic seizure.

To address these questions, I used *MecP2*-null mice as a model of Rett Syndrome.

2. Methods

2.1 Ethical approval

All animals were housed, cared for and euthanized in accordance with the recommendations of the European Commission (No. L358, ISSN 0378-6978), according to the protocols approved by the Committee for Animal Research, Göttingen University.

2.2 Mouse strains

Experiments were performed using the wild type (WT) and *Mecp2*-null mice (denoted hereafter as *Mecp2*$^{-/y}$ or KO) mice as a model for RTT. Both lines were maintained on a C57BL/6J background. The *Mecp2*-null mouse strain B6.129P2(C)-*Mecp2*$^{tm1\text{-}1Bird}$ (*Guy et al., 2001*) was obtained from the Jackson Laboratory (Bar Harbor, ME, USA). B6.129P2(C)-*Mecp2*$^{tm1\text{-}1Bird}$ mice with deleted *Mecp2* exons 3 and 4 are known to have normal development until 3-8 weeks. After approximately one month first signs of Rett Syndrome symptoms appear *(Guy et al., 2001*; *Viemari et al., 2005)*.

All mice were genotyped in accordance with the Jackson Laboratory genotyping protocol *(Stettner et al., 2007).* Studies were carried on hemizygous males because individual differences in X-inactivation pattern among heterozygous mice can result in variability in phenotype.

Hemizygous mutant *Mecp2*$^{-/y}$ males were generated by crossing C57BL/6J WT males with heterozygous *MeCP2* $^{+/-}$ females. The mice were examined blind and the data were then distributed into the two pools - the knock-outs (KO) and wild type (WT), as defined by their genotype.

2.3 Cell culture

Hippocampal neurons were obtained from P5 - P7 animals. They were decapitated, the brains were removed, and hippocampi isolated and cut in several parts. Then the hippocampi were washed 3 times in cold Hank's +20% Horse Serum (HS) (Invitrogen) solution and 3 times in cold Hank's-sol without serum. 0.5 % trypsin and 0.5mg/ml DNaseI in Solution I (in 100 ml ddH_2O: 0.8g NaCl (137 mM), 0.037g KCl (5 mM), 0.099 g Na_2HPO_4 (7 mM), 0.6g Hepes (25 mM), pH-7.2) at 4°C was added to the dissected tissue and incubated at 37 ^oC for 10 min. Then the solution with enzyme was removed and the tissue was washed again as described before. The tissue was then triturated gently with a fire-polished Pasteur-pipette in cold Solution II (100 ml Hanks + 0.166 g $MgS0_4$ (12 mM)) with 0.5 mg/ml DNase I and centrifuged for 10 min at 1200 rpm at 4°C. The suspension was then diluted in 1 ml of cold Medium I (90 ml MEM Earle´s without L-glutamate, 10 ml HS, 1 ml L-Glutamine (200 mM), 1 ml Penicillin/Streptomycin (10kU/20 kU), 0.5g D-Glucose, 10 mg Transferrin, 200⇐l Insulin (12.5 mg/ml) and 25000 - 35000 cells were plated on 12 mm glass coverslips covered with poly D-lysine and allowed to settle for up to 1 hour in the incubator at 37°C, where was supplied by 5 % CO_2. Then 700 µl pre-warmed Medium I was added. After 2 days the half of Medium I was replaced with Medium II (100 ml Neurobasal-A-Medium, 2 ml B-27 Supplement, 250⇐l L-Glutamine (200 mM), 1 ml Penicillin/Streptomycin (10kU/20 kU), 12.5⇐l b-FGF (12.5 ng/ml), 12.5⇐l AraC (added from the 4mM stock solution).

Experiments were usually performed from 3 to 10 days *in vitro* (DIV) at either room or at 32°C. The coverslips with hippocampal neurons were placed on the microscope stage in a chamber continuously superfused at 5-10 ml/min with bath solution contained (in mM): 136 NaCl, 5 KCl, 1.25 $CaCl_2$, 0.8 $MgCl_2$, 6 glucose, 10 HEPES, pH 7.4.

Data were collected only from functionally viable cells defined as showing intracellular calcium increases in Fluo-3 or Fura-2-stained cells after brief challenges to membrane

depolarisations elicited by application of 50 mM K^+ (high-K^+). High-K^+ solutions were prepared by exchanging for equivalent amount of Na^+ in ACSF.

2.4 Organotypic culture

Organotypic slice cultures were prepared at P5 –P7 in the form of 250 μm-thick hippocampus slices (Fig 2.1). Mice were prepared at P5–P7. The preparation itself and all further manipulations were conducted on ice (at 4°C) in ACSF solution saturated with 95% O_2, 5% CO_2.

Slices after preparation were placed on support membranes and Neurobasal-A medium (1 ml) was added such as the surface of the slice was continuously exposed to the incubator gas mixture that allowed consistent respiration and nutrition.

According to our experience, the organotypic slice cultures survived best in the culture medium which contained 50% MEM with Earle's salts, 25 mM HEPES, 6.5 mg/ml glucose, 25% horse serum, and 25% Hanks solution buffered with 5 mM Tris and 4 mM $NaHCO_3$, pH 7.3. The medium was changed every second day. Under these conditions organotypic slices could be maintained alive for more than a month without significant changes in viability or morphology of neurons. Organotypic slices were examined between 10 and 45 DIV, corresponding to postnatal days P14–P49.

Functional properties of neurons from WT and KO mice were examined in groups at the interval of 1 week starting from P14. Approximately equal numbers of neurons from the respective groups were measured in parallel. All data were acquired and analysed blinded to genotype. Each test in this study was repeated with at least five different preparations and the mean data were obtained by analysing responses of 6–12 neurons in the image field.

For the experiments the membrane around the slice was carefully removed. After that

slices were fixed on the 13 mm glass coverslip and immobilised with heavy platinum thin ring. Then slices were mounted in the experimental chamber on a microscope table.

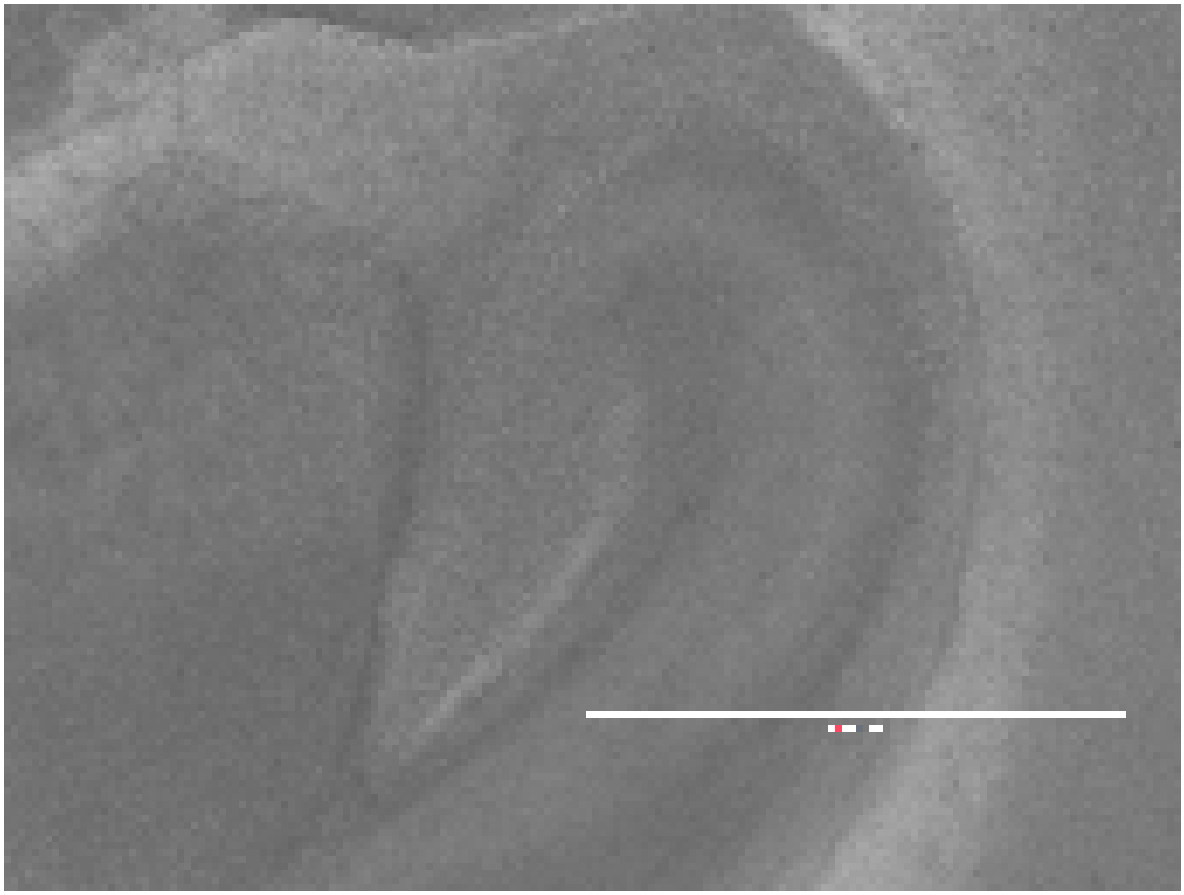


Fig 2.1 Transmission image of the organotypic hippocampus slices (at ×10 magnification).

2.5. Sensors

Calcium imaging was performed with commercially available chemical sensors (fura-2 AM, from Molecular Probes) and genetically encoded indicator D3cpv designed in the lab of Roger Tsien *(Palmer et al., 2006; Palmer and Tsien, 2006).* ATP imaging was performed with genetically encoded ATP indicator Ateam 1.03 *(Imamura et al., 2009) and* Reactive Oxygen

Species (ROS) levels were measured with mitochondrial redox sensor, ro-GFP1 *(Hanson et al., 2004*).

After loading the neurons with a sensor protein or expressing the protein indicator (see below), the fluorescence was excited at appropriate wavelength. The emission was collected using Zeiss Axioscop microscope and captured by a CCD Andor iXon camera (ANDOR, Offenbach).

2.5.1 Chemical indicators

For imaging of intracellular calcium, 1 µM fura-2/AM was added to ACSF as the aliquot of DMSO-based stock solution. The staining mixture was sonicated and slices were incubated with the dye from 20 to 30 min at room temperature.

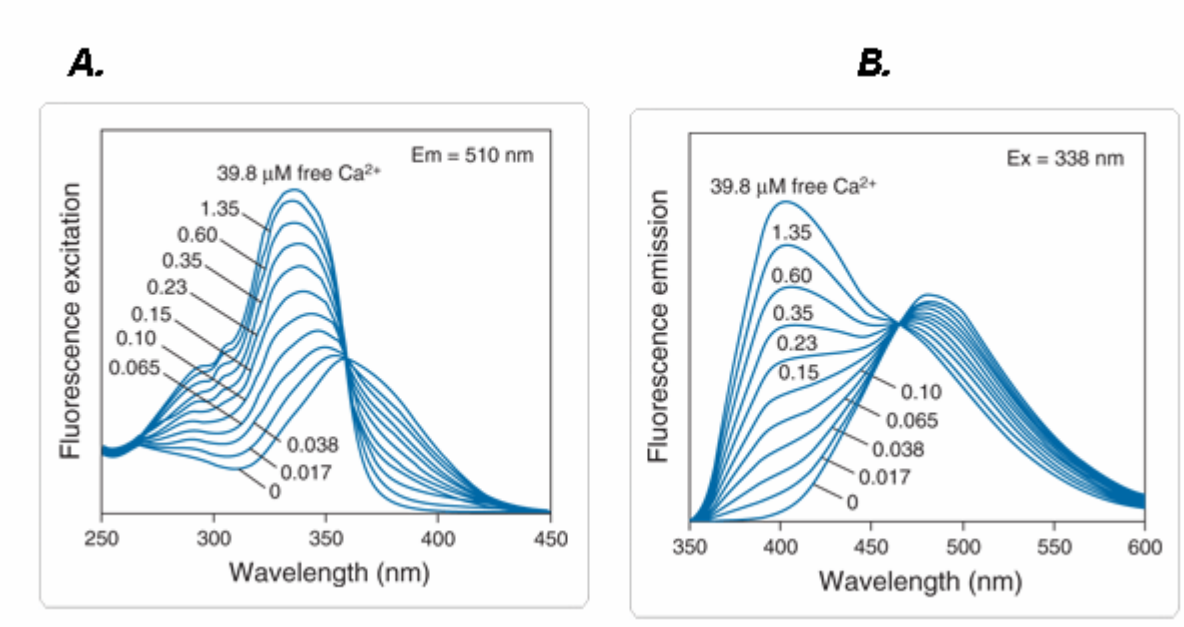


Fig 2.2 Fluorescence excitation (A) and emission (B) spectrums of fura-2 at 0–39.8 µM free Ca^{2+}. (From *Life Technology* home page).

Spectra of excitation and emission of Fura-2 for presented in Fig 2.2. Fura-2 was excited at 350 and 380 nm and the emission of the dye was collected at 535 nm. Exposure times ranged

from 300 to 500 ms and the frames were collected every 1 second. They were analysed off-line using MetaMorph software (Universal Imaging Corporation, West Chester, PA). $[Ca^{2+}]_i$ increases were estimated from fura-2 ratios as described previously *(Mironov and Hermann, 1996; Mironov and Langohr, 2005).*

2.5.2 Genetically encoded calcium indicators

Calcium sensors are fluorescent proteins derived from green fluorescent protein or its variants (YFP and CFP), fused with calmodulin (CaM) and the M13 domain of the myosin light chain kinase, which binds CaM. Genetically encoded indicators are produced within cells after their transfection.

We used calcium sensor D3cpv designed in the lab of Roger Tsien *(Palmer et al., 2006).* In order to obtain a neuron-specific transduction *(Kügler et al., 2003; Shevtsova et al., 2005)* the sensor was embedded into a recombinant adeno-associated virus (AAV) vector. For transduction, we applied 1 µl of AAV solution (≈1 x 10^9 viral genomes) directly at the surface of the slice. The expression of D3cpv was detectable from two to four days after transduction, reached a steady state after four to six days and then remained constant over six weeks. D3cpv was excited at 430 nm by the LED (20 mW, Roithner Lasertechnik).

Corresponding signals were separated with Optosplit (BFI Optilas, Puchheim) using dichroic mirror (495 nm) and 470 ± 12 and 535 ± 15 nm filters. Free calcium levels were obtained by rationing the emission of cyan fluorescent protein (CFP) at 470 nm to FRET signal between CFP and yellow fluorescent protein (YFP; emitted at 535 nm) (Fig.2.3). Acquisition time was 0.3 s. The signal was measured at 3 frames per second (fps).

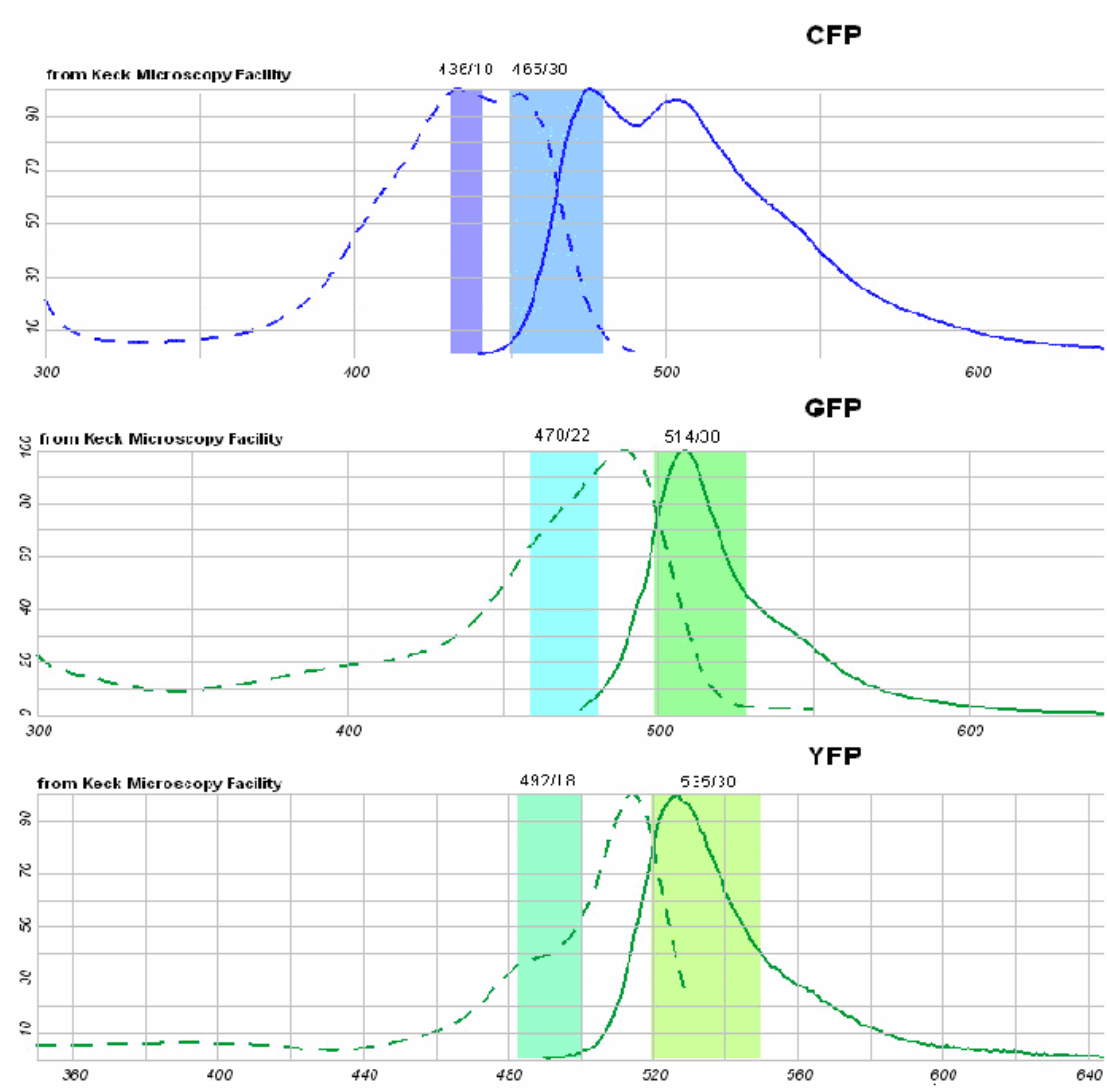


Fig 2.3 Spectra of excitation and emission of green fluorescent protein or its variants (YFP, CFP) (from Keck Microscopy Facility).

Images were captured by a cooled CCD iXon camera (ANDOR, Offenbach) and collected with ANDOR software (500 x 500 pixels at 12 bit resolution). Fluorescence signals were analysed offline with MetaMorph software (Universal Imaging Corporation, West Chester, PA). Cytoplasmic calcium levels were obtained from ratios of FRET and CFP signals (*R*) using the formula derived for fura-2 *(Grynkiewicz et al., 1985).*

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

The dissociation constant K_d = 0.6 μM was taken from *(Palmer et al., 2004)* and the values of R_{min} = 1 and R_{max} = 80 were determined from calibration experiments (*Palmer and Tsien, 2006*). D3cpv has brighter fluorescence than fura-2, D3cpv selectively expresses only in neurons, and it has a broader range on Ca^{2+} dynamic detection (0.6–6 μM) and greater resistance to photobleaching.

2.5.3 Genetically encoded ATP indicator

For ATP imaging experiments the cultures were transduced 2 days after plating with neuron-targeted ATP sensors (Ateam 1.03) using recombinant adeno-associated virus (AAV). Experimental protocols were similar to that used previously for transduction with calcium sensor D3cpv.

Initial overview image was captured at 5x magnification (Epiplan 5x/0.13 M27, Zeiss). Three hippocampus areas (DG, CA1 and CA3) were selected for imaging. Then we switched to higher magnification (W N-Achroplan 10x/0.3 M27, Zeiss) that allowed us to examine the network activity.

The slices were placed in a heated chamber (RC-26GLP, PH-1, Warner Instruments) of an upright microscope stage (Examiner, Zeiss).

Excitation light from LED source (455 nm, Colibri, Zeiss) was attenuated to 25%. Changes in fluorescence emission intensities at 535 nm (FRET acceptor) and at 470 nm (Enhanced Cyan Fluorescent Protein (ECFP); FRET donor) were measured after their separation

with an appropriate filter set (78ms/wl, Zeiss). Acquisition time was 0.3 s. For Ateam 1.03 the interval between acquisitions was 5 s.

Images were captured by two spatially aligned MRm cameras (Zeiss) and signal was analyzed offline with MetaMorph software (Universal Imaging Corp., Downingtown, PA, USA) and custom-made programs. Images for analysis were background subtracted and then the mean cytoplasmic ATP was obtained from ratioed signal in regions of interested centred on single neurons. The data was averaged from 8 to 12 neurons. The standard deviation from the mean was less than 10 %.

2.6 Transduction of neurons

Adeno-associated virus of the mosaic serotype 1/2 was constructed as described (*Shevtsova et al., 2005; Kügler et al., 2007).* The vector genome consisted of AAV-2 inverted terminal repeats flanking the neuron-specific human synapsin1 gene promoter (Kügler et al., 2001); EGFP, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) for enhanced transgenic expression and a bovine growth hormone polyadenylation site (Fig. 2.4). This virus provided greatly enhanced transduction of neurons in primary culture *(Shevtsova et al., 2005)* and was thus chosen for gene transfer into living slice cultures *(Hartelt et al., 2008).*

The slices were transduced at 2 to 4 DIV. At this time cells, which were damaged during dissection, recovered and the slice was yet not covered by the dense film of cells that might hamper the transduction. For transduction, we applied a drop of AAV solution (1×10^9 viral genomes) directly onto the slice surface. The protein expression reached steady state 5 to 7 days after transduction and it remained stable thereafter. The slices were taken into experiments at the times which correspond to about P12–P18 *in vivo*.

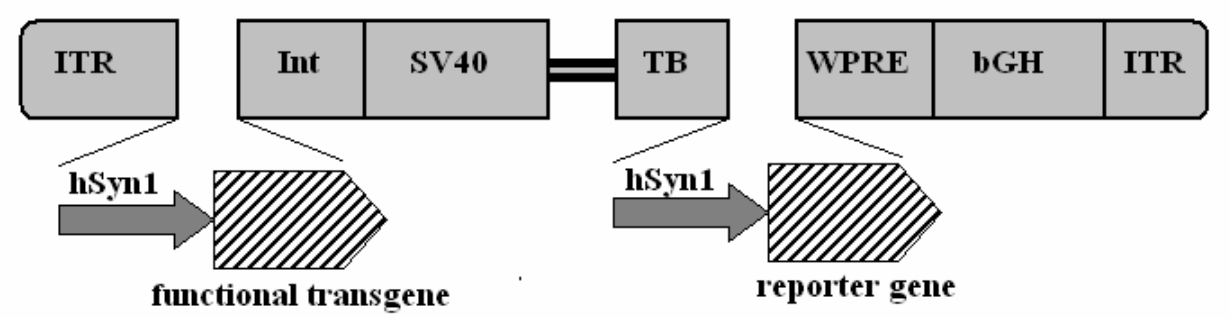


Fig 2.4 The structure of AAV vector *(after Shevtsova et al., 2005).*

ITR – Inverted terminal repeats of AAV-2; Int – 146 bp chimeric intron; SV40 – Simian virus 40 derived polyadenylation site; WPRE – Woodchuck hepatitis virus post-transcriptional 3′-control element; bGH – 3′-control element - bovine growth hormone derived polyadenylation site; hSyn1 – Short human synapsin-1 gene promoter; Reporter gene – Independent expression cassette for green fluorescent protein (EGFP).

All chemicals were from Sigma (Deisenhofen) and the fluorescent probes were from Molecular Probes (Leiden, Netherlands).

4.7 Patch-clamp.

Brief outline. Ion channels are pore-forming membrane proteins whose functions include establishing resting membrane potential, shaping action potentials as well as other electrical signals. They have different functional properties e.g. gating the flow of ions across the cell membrane, regulating the cell volume *etc.* Ion channels in excitable cells can be subdivided into ligand-or voltage-gated channels.Voltage-gated ion channels open or close depending on the voltage gradient across the plasma membrane, while ligand-gated ion channels open or close depending on binding of ligand to the channel. Nevertheless for any type of channel basic

pattern is the same: when the gate opens, current flows across the membrane and the current stops when the gate is closed. Openings and closings of the channel produce at least two different current levels that can be used to identify them by conductance. The probability of opening (which is a function of number of openings and their duration) of the channel depends on the control mechanism which depends on intrinsic properties of the channel protein, membrane potential itself and change that may underline specific cellular reaction to physiologically relevant events.

2.7.1. Cell-attached and whole-cell patch-clamp configurations.

In these studies I examined ATP sensitive potassium channels (K-ATP) that are abundant in neurons and other cells *(Noma, 1983*; *Standen et al., 1989; Ashcroft 1988; Bajgar et al. 2001).* Hippocampal cells express a high density of K-ATP channels *(Karschin et al., 1997; Zawar et al., 1999)*, such that single K-ATP channels can be recorded in 50%-70% of cell-attached patch recordings (*Pelletier et al., 2000).*

Pipettes were manufactured from a borosilicate glass capillary (Clark Instruments, Pangbourne, UK) using horizontal micropipette glass puller. Their tip openings had diameter of 1.5–2 μm and resistances of 1.5–2.5MΩ. The electrodes were filled with solution contained 140 mM D-gluconic acid, 0,5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 1 mM NaCl, 1 mM EGTA and 1 mM Na_2ATP, with pH adjusted to 7.4 with KOH.

For the formation of gigaseal electrode was placed above the chosen cell, slowly approached it and gentle sucking was applied to stimulate the formation of a gigaseal in cell-attached recordings. The whole-cell mode was obtained by rupturing the plasma membrane.

K-ATP channels were measured in the cell-attached mode at the range of holding potential, from +20 to -80 mV with or without action potentials. Intracellular signals were recorded with an EPC-7 patch-clamp amplifier (ESF, Friedland, Germany) as published

previously (*Mironov et al., 1998*). They were filtered at 3 kHz (-3 dB), digitized and transmitted with Pulse program (Acquisition interface LIH 1600 by HEKA Elektronik) and stored for further off-line analysis. Single-channel data measured in a cell-attached mode are in most cases presented as inverted currents i.e. their values were taken as inside the cell minus outside, according to conventional definitions of voltage and current directions.

2.7.2. Open-cell patch-clamp configuration.

Open-cell (OC) is a method where patch-clamp technique is combined with cell membrane permeabilisation. OC configuration patch was previously applied to study of egg cells *(Hagiwara, 1983),* myocytes *(Stanfield at al., 1981; Vandenberg,, 1986*) and pancreatic β-cells *(Edwards & Weston, 1995; Tarasov et al., 2006; Tarasov, 2008).* It has not yet been applied to neurons due to their small size and sensibility. I pioneered in applying of this patch-clamp configuration to neurons.

Briefly, after formation of cell-attached patch a second electrode filled with β-Escin (10-20 μM) was brought up to the downstream end of the cell and then permeation agent was locally applied to the cell patched (Fig 2.5A). B-Escin produces the pores with approximate diameter 1.5-2 nm (*Tarasov, 2008)*. The progress of permeabilisation was manifested as cell swelling (Fig 2.5B), and increase in the activity of K-ATP channels in ATP-free bath solution. In the test experiments we monitored a loss of fura-2 or fluo-3 from preloaded neurons and estimated that equilibrium between cytoplasm and bath solution was established within <1 min after addition of ß-Escin. Because Fura-2 molecules are bigger than ATP the wash-out of intracellular ATP likely occurs also within 1 min. K-ATP-channel activity in permeabilized neurons could be recorded from 5 min to 1 h.

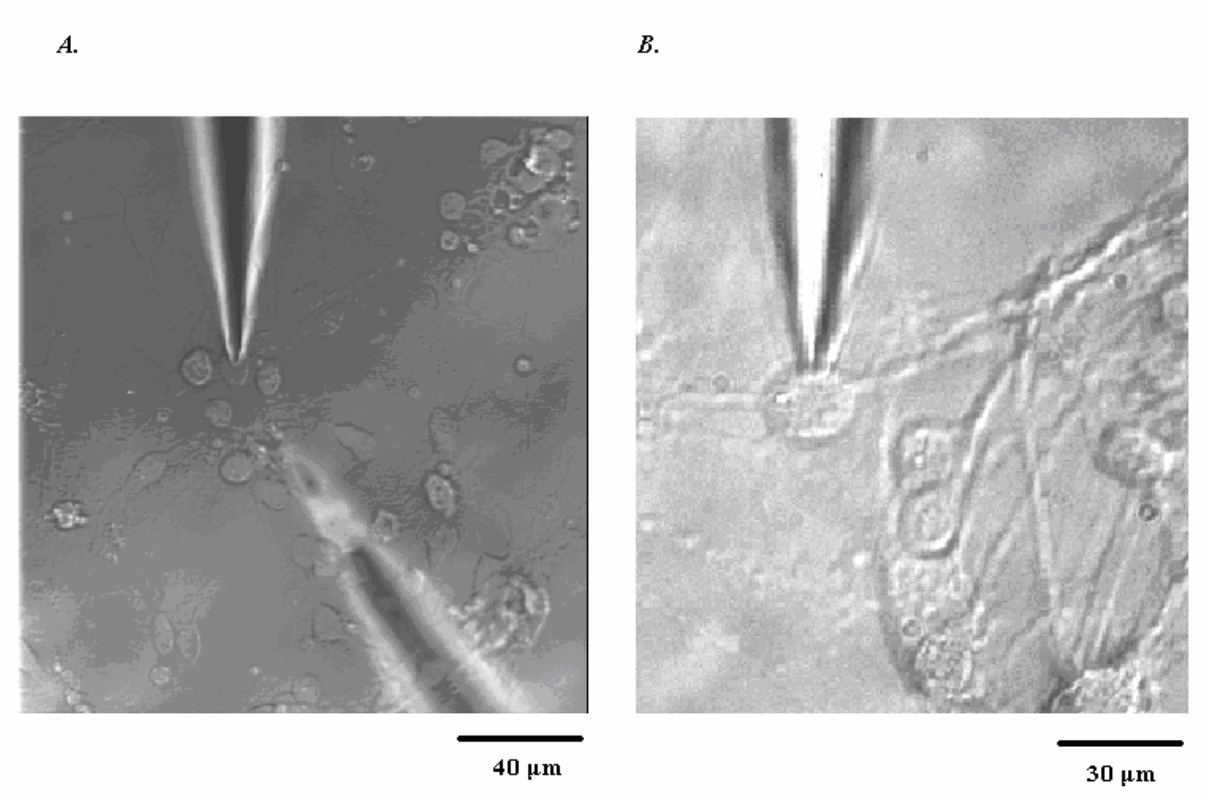


Fig 2.5 Open-cell patch configuration

A **-** Experimental design. The electrode in the upper part was gigaseal-attached to the plasma membrane; the electrode filled with β-Escin (10-20 µM) is in the lower part. Neurons were permeabilized in ATP, Ca^{2+}-, and Mg^{2+}-free bath solution. ***B*** **-** Neuron swallowing occurs in about 10 seconds after permeabilisation by ß-Escin.

2.8. Statistics

The data were obtained from primary cultures obtained from 144 mice and each treatment was performed on preparations obtained from at least 3 different mice. The data are given as mean ± standard deviation. In diagrams, significantly different changes are marked as * ($p < 0.05$) and ** ($p < 0.01$). Significant differences were determined using the non-parametric Mann–Whitney U test. Statistical calculations were done with the Excel software (Office 2003) and Statview (version 5.0.1, SAS Inc., NC, and USA).

3. Results

3.1. ATP-dependent inhibition of K-ATP channels

3.1.1 GluR induce long-lasting activation of K-ATP channels

In order to examine GluR effect on K-ATP channel activity we established a primary culture of hippocampal neurons. A rationale of using this preparation was several folds: (i) this is an established model to examining the effects of glutamate excitotoxicity in the CNS; (ii) the delivery of drugs to cultured neurons can be made local and well-controlled; (iii) detailed examination of GluR – K-ATP connection in the hippocampus might have important physiological implications such as epilepsy and stroke.

Single K-ATP channels in the hippocampal neurons were potentiated after GluR stimulation. The response had slow onset, after which the increase in activity developed within 2 min and was maintained for >20 min after washing out the agonist (Fig.3.1A).

Both spontaneously active and quiescent neurons showed GluR-mediated potentiation of K-ATP channels (Fig.3.1 B and C). In neurons generating action potentials (AP), we observed more frequent openings of K-ATP channels after AP generation that resembles the causal relationship in the inspiratory neurons *(Haller et al., 2001)* and in hippocampal slices *(Tanner et al., 2011).*

Application of Kainate/ AMPA (aminomethyl phosphonic acid) resembled the effects of hypoxia *(see Mironov et al. 1998).* After 3 min after application of GluR agonists (Kainate, AMPA and N-Methyl-D-aspartic acid (NMDA) open probability (P_{open}) of K-ATP channels increased from 0.08 ± 0.02 to 0.31 ± 0.03 (3 μM Kainate, n = 6) and from 0.09 ± 0.02 to 0.29 ± 0.04 (3 μM AMPA, n = 6). The effects were abrogated by AMPA/Kainate blockers, 6-Cyano-7-nitroquinoxaline-2,3-dione **(**CNQX) and 2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo[f]quinoxaline

(NBQX) (n = 4). The effect of hypoxia induced by replacement O_2 by N_2 in the bubbling gas mixture and AMPA/Kainate actions were mutually exclusive

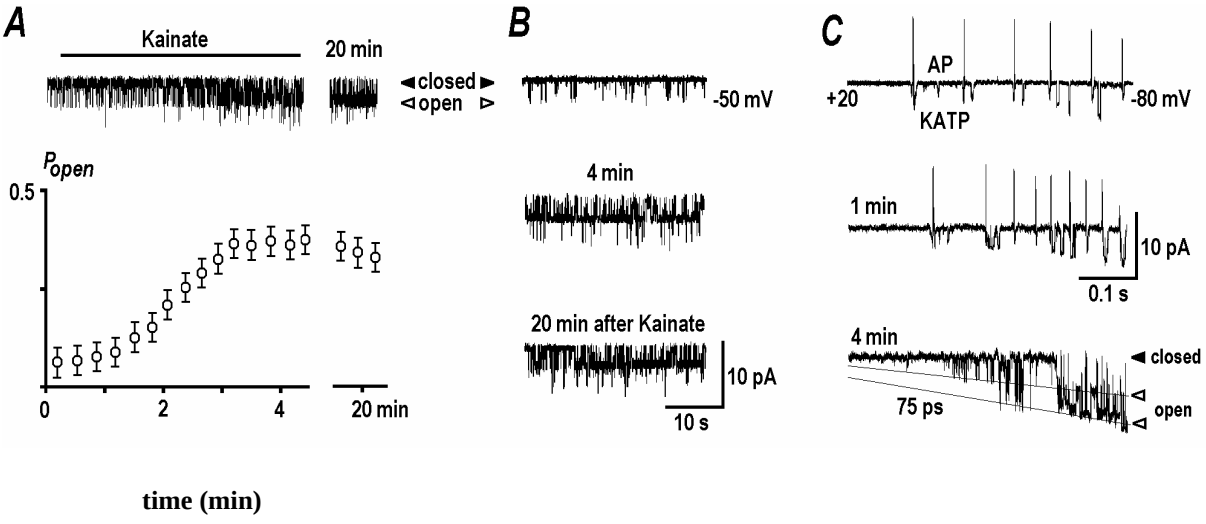


Fig.3.1 Long-lasting activation on K-ATP channels by Kainate in the isolated hippocampal neurons.

A – 10 μM Kainate slowly potentiated K-ATP channels that were maintained long after washout of the agonist. Top inset shows continuous membrane current recording at holding potential -50 mV and the graph below shows changes in the open probability of K-ATP channels.

B – Three traces show K-ATP channel activities in the control, at the maximum of Kainate effect and 20 min after its removal from medium. The recordings were made at holding potential -50 mV in neuron without spontaneous activity.

C – Kainate actions in the spontaneously active neuron (the upwardly directed inflections indicate action potential (AP). The recordings were made during a voltage ramp from +20 to -80 mV. The frequency of AP transiently increased 1 min after application of 10 μM Kainate and then disappeared when the activity of K-ATP channels attained maximum. Observed single K-ATP channel conductance was 75 pS. (n = 35).

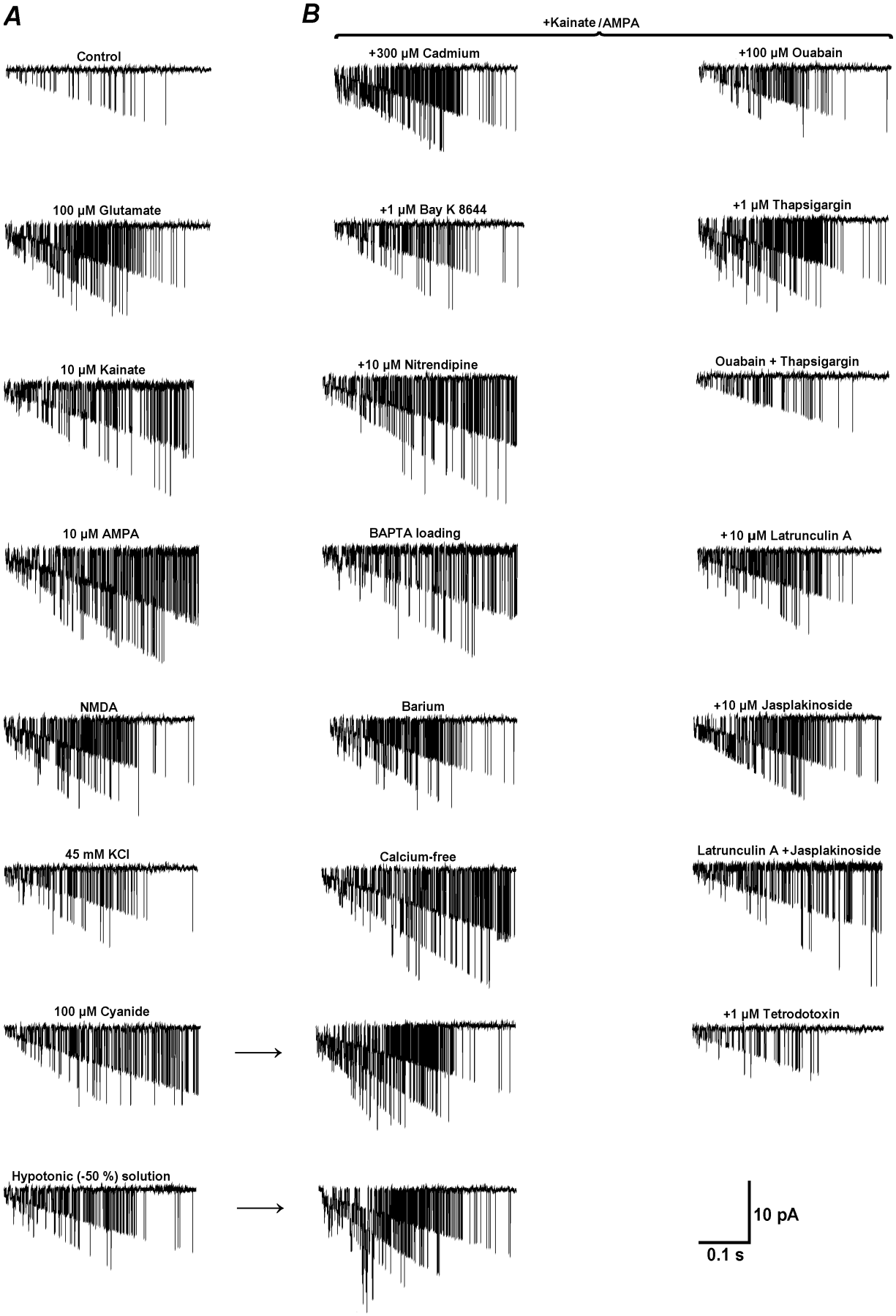

A
B
+Kainate /AMPA
Control
+300 µM Cadmium
+100 µM Ouabain
100 µM Glutamate
+1 µM Bay K 8644
+1 µM Thapsigargin
10 µM Kainate
+10 µM Nitrendipine
Ouabain + Thapsigargin
10 µM AMPA
BAPTA loading
+10 µM Latrunculin A
NMDA
Barium
+10 µM Jasplakinoside
45 mM KCl
Calcium-free
Latrunculin A +Jasplakinoside
100 µM Cyanide
+1 µM Tetrodotoxin
Hypotonic (-50 %) solution
10 pA
0.1 s

Fig.3.2 GluR-mediated potentiation of K-ATP channels.

A - Representative traces showing the activity of K-ATP channels in the control and 2 min after the treatments with 100 μM glutamate, 10 μM Kainate or AMPA, 45 mM KCl (membrane depolarisation), 100 μM cyanide (to examine the effects of GluR after metabolic poisoning); and hypotonic solution (50 % dilution of the control solution).

B - Results of experiments when 10 μM AMPA or Kainate were applied in the presence of agents indicated: 1 μM Bay K 8644 and 10 μM Nitrendipine to modulate L-type calcium channels, 300 μM cadmium (blockade of all pathways of calcium entry into the cell); barium instead of calcium in the bath solution, calcium-free solutions (all treatments lasted 10 min and the agent was left in the bath), after loading with calcium chelator BAPTA (10 μM for 30 min) and after 2 min with 100 μM cyanide and hypotonic solution. The rightmost panel shows the traces obtained after blockade of ion pumps with 100 μM ouabain and 1 μM thapsigargin (also in combination); modification of actin turnover with 10 μM Latrunculin A and 10 μM Jasplakinolide (also in combination); blockade of sodium influx with 1 μM tetrodotoxin (TTX). In 'open-cell' configuration we measured the effects of GluR in solutions contained 0 and 1 mM ATP.

n = 4 - 20.

The effects evoked by GluR agonists on K-ATP channels were robust and observed in all examined cells. They are partly summarized in Fig. 3.2 and show representative traces recorded for different treatments (see details in figure legends and in text). Figure 3.2 is representative for experiments performed in more than 700 completed patches.

Because GluR effects have not been previously described, I decided to examine the underlying pathways.

3.1.2 Possible candidates for GluR effect.

A slow onset and endurance of GluR on K-ATP channels indicate the involvement of specific mediator of the effects. To examine the GluR effect in detail and based on earlier published works, in this study I examined the most likely candidates – calcium, osmotic stress, free radicals (ROS) and ATP depletion. I used both single-channel patch-clamp recordings and optical imaging. The effects of AMPA and Kainate in the control were identical; in further experiments I interchangeably used the agonists at concentration 10 μM.

3.1.2.1 Intracellular calcium

An immediate effect of GluR stimulation is the membrane depolarization leading to activation of voltage-sensitive calcium channels and elevation of cytoplasmic calcium. The main route of calcium into the neuronal soma is the slowly inactivating dihydropyridine-sensitive CaV channels *(Nowycky et al., 1985).* The effects of AMPA/Kainate on K-ATP channels were not modified in the presence of blocker, Nitrendipine (10 μM), and activator, BAY K 8064 (1 μM, both applied for 30 min before the agonist challenge that was left in the bath). AMPA and Kainate were equally effective after replacement of external calcium with barium and in the neurons, which were loaded with the calcium-chelator BAPTA (Fig. 3.3 B).

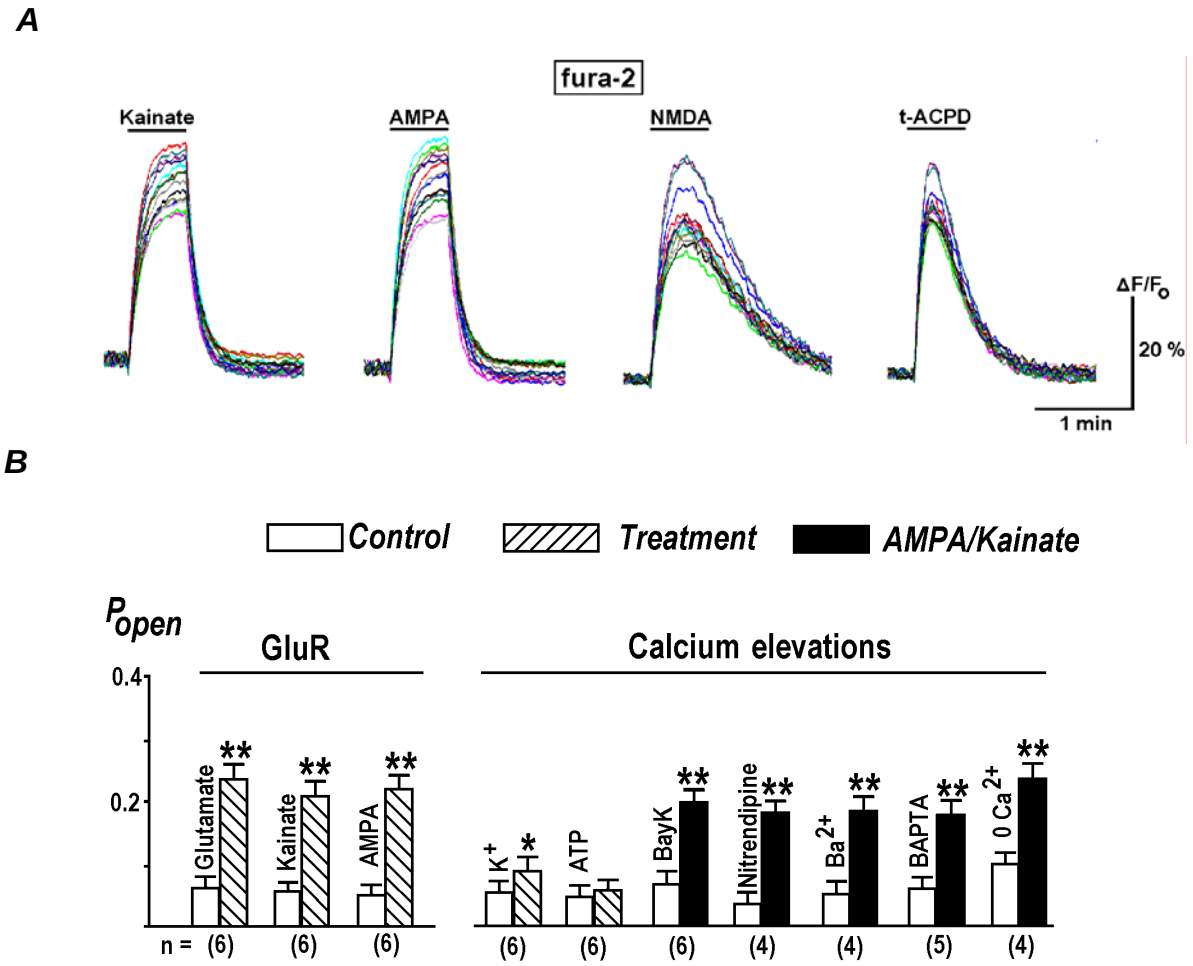


Fig. 3.3 GluR-mediated changes in intracellular calcium.

A - The imaging experiments were performed as described in Methods. $[Ca^{2+}]_i$ presented as relative changes in fluorescence (the basal level taken for 100 %). Different traces code individual responses of 8 – 12 neurons present in the image field. Intracellular calcium changes evoked by brief applications of GluR agonists (fura-2 loaded neurons; excitation, 380 nm).

B - Shown are the means of maximal P_{open} values for each experimental protocol (for AMPA/Kainate they were measured 3 min after addition of the agonist). Differences are significant at $p < 0.01$ (**) and $p < 0.05$ (*) as indicated and the number of experiments is indicated below each couple of bars.

The empty bars show the data in the control, and hatched bars show maximal P_{open} values after the application of 100 μM glutamate, 10 μM Kainate or AMPA; 100 μM NMDA; 45 mM KCl and 1 mM ATP to elevation cytoplasmic calcium; 100 μM cyanide to examine the effects of GluR after metabolic poisoning; and hypotonic solution (50 % dilution of the control solution). In other experiments the activity of K-ATP channels was measured after pretreatment with the agents indicated (10 to 30 min incubation time). In all cases P_{open} did not change significantly and served as control (empty bars). Filled bars show mean P_{open} 3 min after application of 10 μM AMPA or Kainate. We did not distinguish between the agonists, because their effects were identical (the leftmost diagram). Possible effects of intracellular calcium were examined by applying 1 μM Bay K 8644 and 10 μM Nitrendipine to modulate

L-type calcium channels, replacement of calcium with barium, calcium-free solutions and loading the neurons with calcium chelator BAPTA.

Application of cadmium (300 μM) that can block all pathways of calcium entry into the cell led to a slight suppression but not the abolishment AMPA/ Kainate actions. Imaging of calcium (Fig. 3.3 A) showed typical responses for GluR agonists *(Zorumski et al., 1989; Berg et al., 1995; Alici et al., 1997*). Kainate and AMPA rapidly increased calcium, which decayed fast after removal of the agonist. The applications were brief to avoid excitotoxicity that is manifested as a severe prolongation of calcium recovery, especially when higher doses and longer times are used (*Delorenzo et al., 2005).*

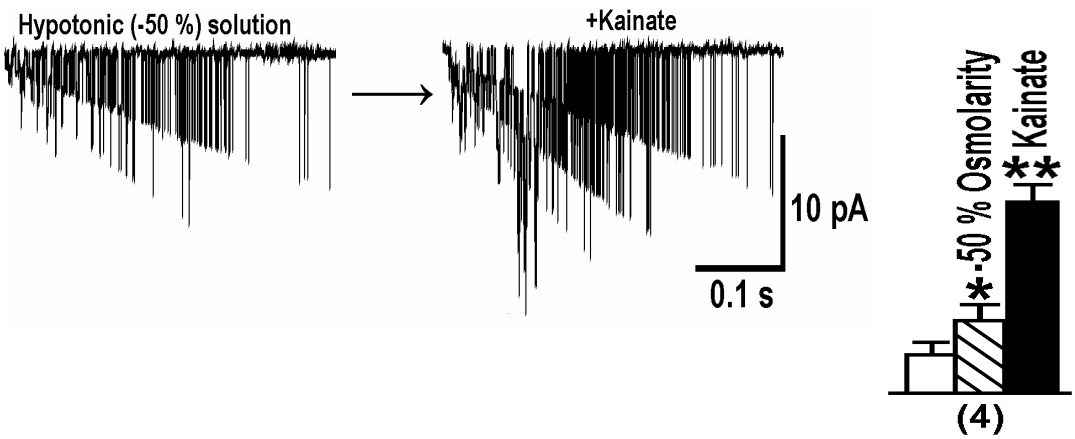
A fast development and decay of calcium elevations due to Kainate and AMPA are consistent with depolarization-induced calcium influx and physiological recovery to basal levels after its termination. Calcium responses to NMDA had different kinetics that might reflect slow calcium permeation through the channels. In no case I observed high-amplitude calcium transients and minutes-long calcium recovery, a hallmark of excitotoxicity.

3.1.2.2 Osmotic stress and reactive oxygen species.

Stimulation of GluRs can disturb ion homeostasis that may cause the swelling of neurons. Single K-ATP channels in the neurons increase their activity in response to osmo-mechanical stress *(Mironov & Richter, 2001)*.

Application of 5 μM glutamate for 10 min to hippocampal neurons increase their volume by about 30 % that remains elevated hours after the application *(Delorenzo et al., 2005).* Exposure to 10 μM Kainate for 2 min did not produce significant volume effects (>12 neurons for >3 different preparations analyzed (Fig 3.4 B).

A



B

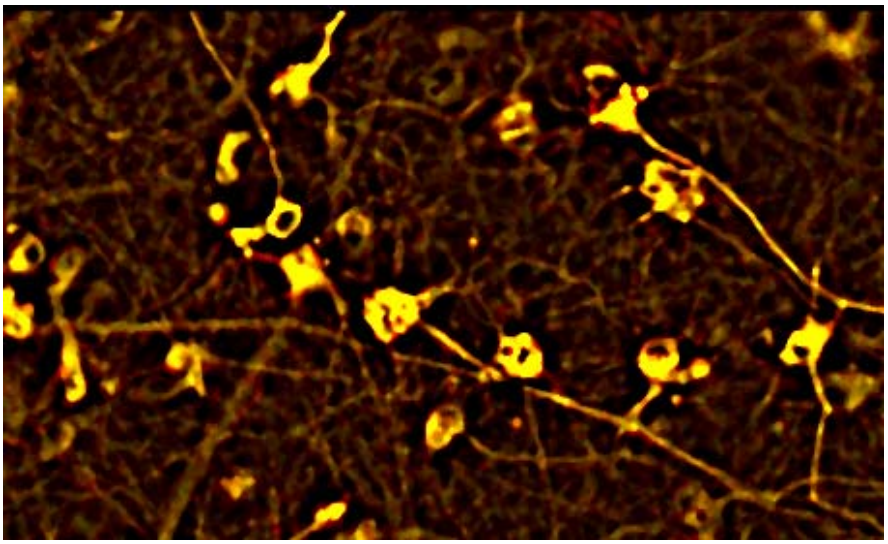


Fig.3.4 Osmo-mechanical stress and Kainate have additive effects.

A - Hypotonic solution increased P_{open} about 2-fold within 2 min and subsequent application of AMPA/Kainate produced further potentiation, in the same proportion as in the control.

B - The plot presents an overlay of images obtained before (red) and 3 min after addition of 10 μM Kainate (green). Considerable overlap between the images (yellow) indicates no substantial changes in the volume of neurons after Kainate.

Hypotonic solution increased P_{open} about 2-fold within 2 min and subsequent application of AMPA/Kainate produced further potentiation, in the same proportion as in the control (Fig.

3.4 A).). Thus I conclude that changes in the activity of K-ATP channels due to osmo-mechanical stress and GluR are additive.

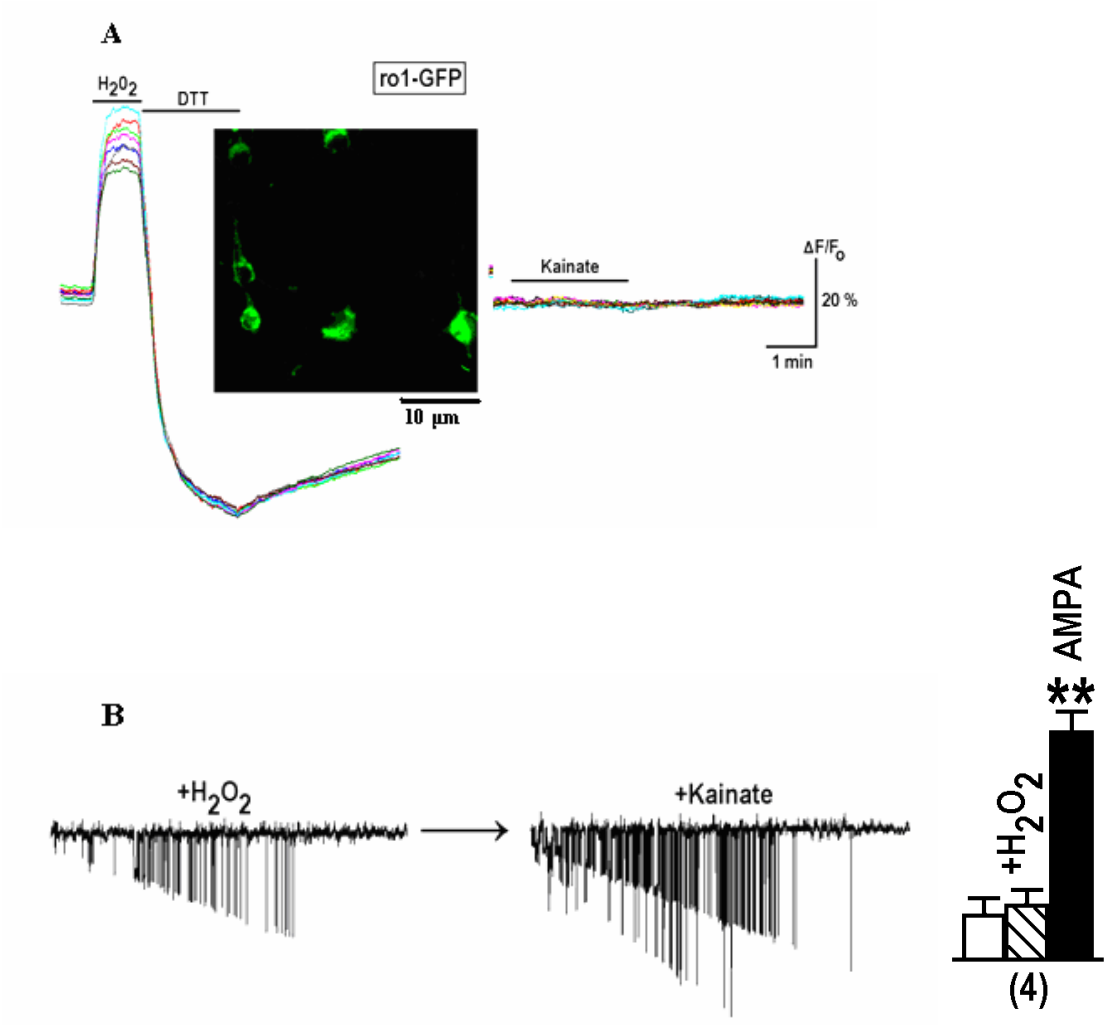


Fig.3.5 Hydrogen peroxide and GluR-mediated changes in intracellular ROS and K-ATP channel activity.

The imaging experiments were performed as described in Methods and the data presented as relative changes in fluorescence (the basal level taken for 100 %). ***A*** – The traces on the left show responses to oxidizing agent H_2O_2 (0.1 mM) and reducing agent dithiotreitol (DDT) (1 mM) that should cause opposite changes in redox status of the cell. The traces on the right one show the absence of Kainate effects on ROS levels. The inset shows neurons virally transduced with neuron-targeted ro-GFP1.

B **–** AMPA/Kainate application after peroxide produced further potentiation, in the same proportion as in the control.

ROS modulate the activity of K-ATP channels in preBötC neurons *(Mironov & Langohr, 2005)* and in other cell types *(Liu & Gutterman, 2002)*. I examined ROS production in neurons, which were virally transduced with mitochondrial redox sensor ro-GFP1 *(Hanson et al., 2004*).

Oxidizing agent H_2O_2 increased fluorescence of the sensor that was reversed below basal levels with reducing agent dithiotreitol (DTT, Fig.3.5A), but ROS was unchanged during application of 30 μM Kainate ($n = 6$).

At the same time in the patch-clamp examinations oxidizing agent H_2O_2 increased P_{open} about 2-fold within 1 min. The subsequent application of AMPA/Kainate produced further potentiation, in the same proportion as in the control with or without ROS scavengers (Fig. 3.5 B).

3.1.2.3 Patch-clamp and optical examination of intracellular ATP.

The negative results obtained with potential candidates of GluR effects – calcium, ROS and osmo-mechanical stress – that might exert long-lasting effects on K-ATP channels, pursued me to examine a possibility of modulation of K-ATP channel activity directly through changes in cytoplasmic ATP. A massive ion flux arising after GluR stimulation might considerably distort ion homeostasis which in turn needs ATP. ATP depletion in submembrane space then should disinhibit K-ATP channels. Although the scenario is self-evident, it has not been experimentally tested on the single channel level so far.

According to Rolfe and Brown *(1996),* the most part of ATP in cells is consumed by the three mechanisms – Na^+/K^+-ATPase in the plasma membrane, the calcium pump in the endoplasmatic reticulum (ER) and actin turnover. In the presence of 1 μM thapsigargin, an inhibitor of calcium ATPase (SERCA) the effects of AMPA/Kainate on K-ATP channel activity became smaller (Fig. 3.6B).

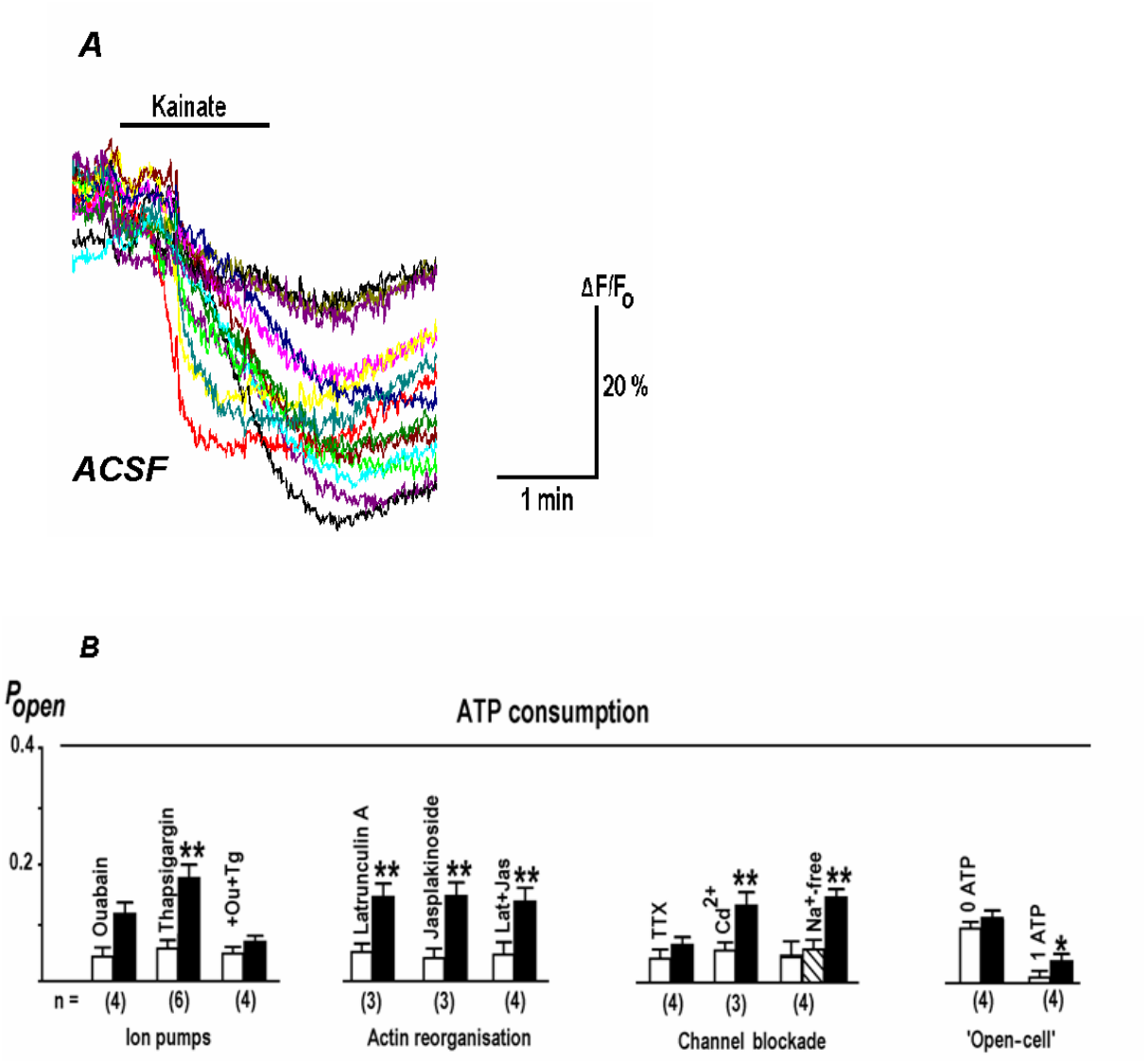


Fig.3.6 GluR-mediated changes in ATP and K-ATP channel activity.

A - The traces present ATP changes obtained during application of Kainate. ***B*** - Shown are the means of maximal P_{open} values for each experimental protocol (for AMPA/Kainate they were measured 3 min after addition of the agonist). Differences are significant at $p < 0.01$ (**) and $p < 0.05$ (*) as indicated and the number of experiments is indicated below each couple of bars.

For examination of ATP consumption I blocked the ion pumps with 100 µM ouabain (Na^+/K^+-ATPase) and 1 µM thapsigargin (SERCA); modified actin turnover with 10 µM Latrunculin A and 10 µM Jasplakinolide; abolished sodium and calcium influx with 1 µM tetrodotoxin (TTX) and 300 µM cadmium, respectively. In 'open-cell' configuration I measured the effects of AMPA/Kainate in solutions contained 0 and 1 mM ATP.

In neurons treated with 10 μM ouabain, a blocker of Na^+/K^+-ATPase, the effects were significantly stronger and combined application of thapsigargin and ouabain completely abolished the potentiation of K-ATP channels (Fig. 3.6 B).

Actin turnover is ATP dependent. It was recently proposed to represent a major energy drain for neurons (*Bernstein & Bamburg, 2003)*. I reproduced the treatments used in this study i. e. jasplakinolide to slow actin filament turnover by inhibiting actin disassembly and latrunculin A to prevent actin assembly by sequestering actin monomers. Both drugs (apart or in combination) slightly suppressed the activation of K-ATP channels by AMPA/Kainate (Fig. 3.6B).

Then I targeted the genetically encoded ATP indicator, Ateam 1.03, *(Imamura et al., 2009)* to neurons and imaged cytoplasmic ATP levels (Fig. 3.6 A). The time-course of ATP decreases due to Kainate closely corresponded to potentiation of K-ATP channels.

3.1.3. 'Open-cell' patch clamp

To gain more insight into GluR – K-ATP crosstalk in neurons I recorded the activity of the K-ATP channels in 'open-cell' (OC) patch-clamp configuration. OC served also as an additional indicator for K-ATP channel identification. The technique has been applied for cardiomyocytes and ß-cells *(Nichols & Lederer, 1989; Vandenberg, 1987; Carrasco et al., 2003; Tarasov et al., 2006; Tarasov, 2008)*. Actually open-cell is a variety of inside-out patch-clamp configuration but without destroying cell composition. It is a method *per se* where patch-clamp technique is combined with cell membrane permeabilisation.

The method allows examining K-ATP channels in cell-attached patches after permeabilisation of the rest of the membrane (Fig.3.7A). It has not yet been applied to neurons, perhaps because they are smaller and more fragile.

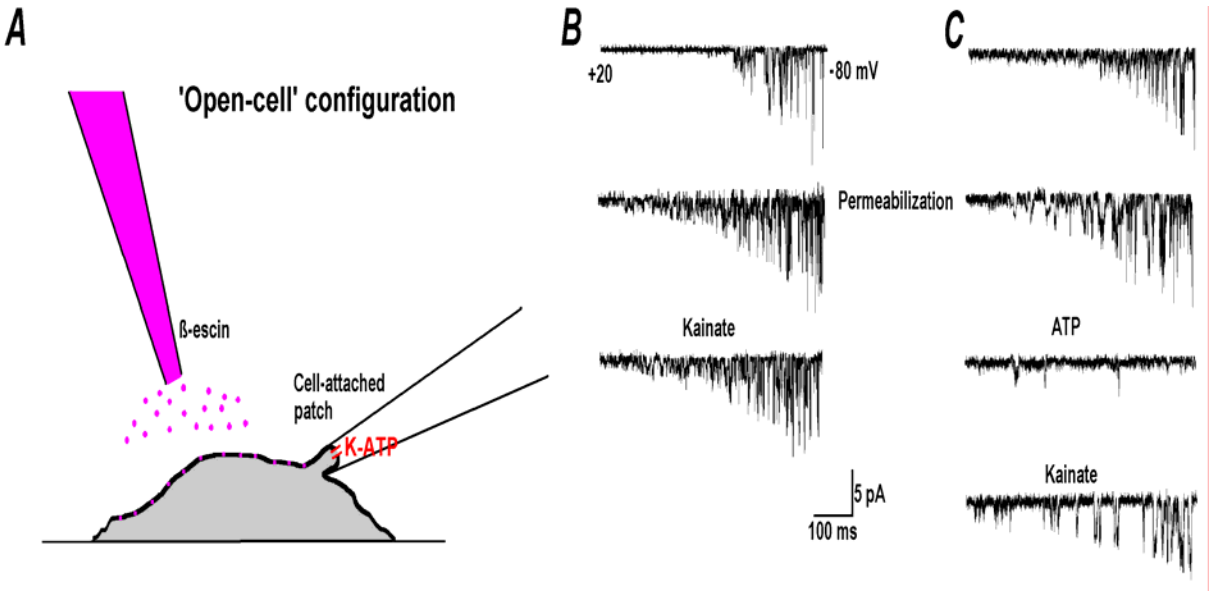


Fig.3.7 ATP and Kainate effects on K-ATP channels in the 'open-cell' configuration.

A - Experimental design. Neurons were permeabilized with ß-escin in ATP, Ca-, and Mg-free bath solution. ***B*** – Permeabilisation led to fast (< 1 min) depletion of cytoplasmic ATP and increase in the activity of K-ATP channels. Subsequent addition of 10 μM Kainate produced only slight potentiation of activity, much smaller than in intact cells. ***C*** - Application of 1 mM ATP to the neuron permeabilized in ATP-free solution inhibited K-ATP channels and subsequent application of 10 μM Kainate substantially increased their activity. The traces in ***B*** and ***C*** were obtained by applying voltage ramp from +20 to -80 mV as indicated. N= 6-18.

I established the protocol to record K-ATP channels in this configuration (see Experimental Methods). To maintain the viability of neurons and exclude possible side effects that might have influenced the activity of K-ATP channels, I used calcium- and ATP-free bath solution. Because β-escin produces big pores (approximate diameter 1.5-2 nm) I could wash out intracellular ATP completely. In the test experiments I monitored a loss of fura-2 or fluo-3 from preloaded neurons and estimated that equilibrium between cytoplasm and bath solution was established within <1 min after addition of ß-escin. Cell-attached patches survived from 5 min to 1 h after permeabilisation. Permeabilisation in ATP-free solutions increased the activity

of K-ATP channels and subsequent application of 10 μM Kainate did not produce significant changes (Fig. 3.7B). When ATP after permeabilisation was raised to 1 mM, the activity was suppressed and application of Kainate increased it (Fig. 3.7C).

3.1.4 Kainate increases ATP consumption in hippocampus neurons.

Next I (in cooperation with Johan Tolö) showed that Kainate application increased ATP consumption in hippocampus neurons. For this purpose I used FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) which uncouples oxidative phosphorylation in mitochondria *(Mattson and Partin, 1999; Shindo et al., 2010).*

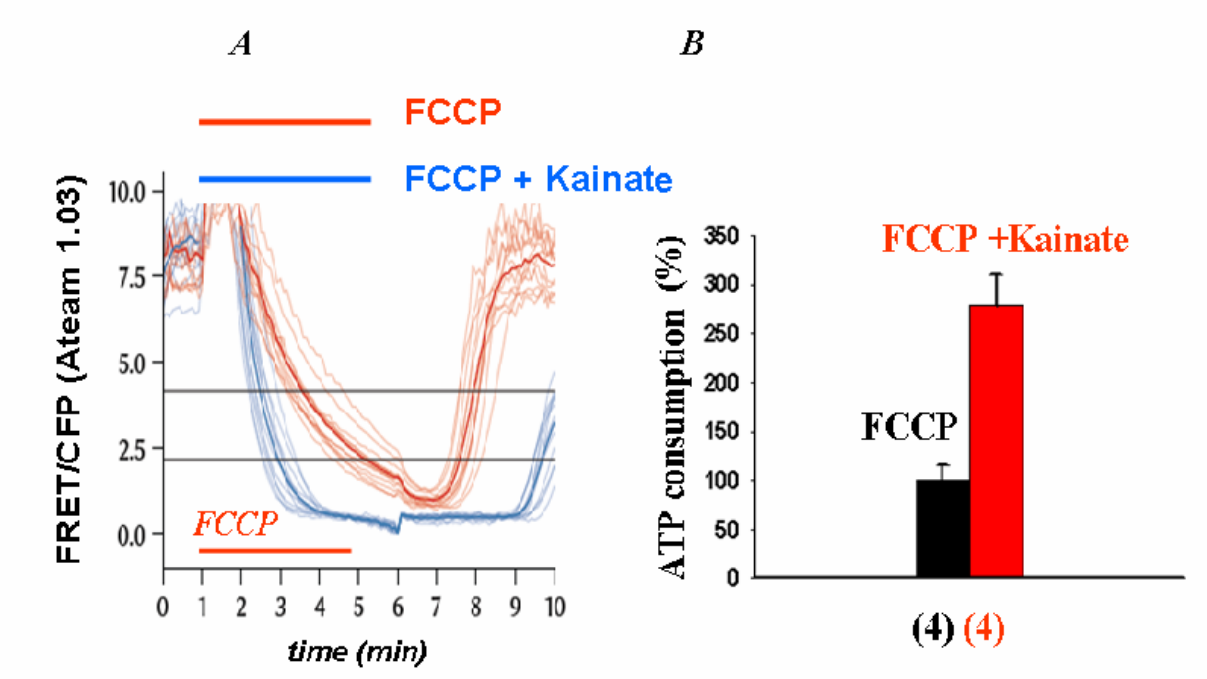


Fig.3.8 ATP production by mitochondria was uncoupled by protonophore FCCP. In the presence of Kainate the rate of consumption increased ~3-fold.

The imaging experiments were performed as described in Methods and presented as relative changes in fluorescence (the basal level taken for 100 %). Hippocampus cell cultures were transduced 2 days after plating with neuron-targeted ATP sensor (Ateam 1.03) using recombinant adeno-associated virus (AAV). ***A*** **-** ATP production by mitochondria was uncoupled by protonophore FCCP (2 μM) and combined FCCP + Kainate (10 μM) application increased ATP consumption. ***B*** **-** In presence of Kainate (10 μM) the rate of consumption showed ~3-fold increase. ATP consumption with FCCP application was accepted as 100%. n=4.

According Rolf and Brown model (1997*)* the main source of ATP production in neurons is mitochondria (~ 95%). FCCP that enters the cytoplasm accumulates in the mitochondrial membrane and removes the proton gradient thus resulting in the inhibition of ATP production in mitochondria. The minimum level of ATP was reached within several minutes and when FCCP was washed out; ATP levels were restored and arrived at initial level. The combined treatment with Kainate and FCCP increased ATP consumption at least 3 times in comparison to only FCCP application (Fig.3.8). I can conclude that K-ATP channel activation occurs just due to ATP depletion but not due to other reasons.

3.1.5 ATP-dependent inhibition of K-ATP channels in Mecp2 null mice

Earlier it was shown that the activity of K-ATP channels in WT preBötC neurons is higher than in *Mecp2* $^{-/y}$ *(Mironov et al., 2011)*. In the patch-clamp studies of *Mecp2* null mice, the initial activity of K-ATP channels in hippocampal neurons (hereafter denote also as resting hippocampal neurons) was slightly reduced as manifested by distinctly smaller probability of opening (Fig.3.9 A). Also I showed a difference in the K-ATP channel activation after AMPA/Kainate applications (Fig.3.10 B). In MeCP2 $^{-/y}$ mice the onset and development of K-ATP channel activation after AMPA/Kainate application began later by an average of 10-15% (82±8 sec for WT, n=89 and 115±14 sec for MeCP2 $^{-/y,}$ n=27).

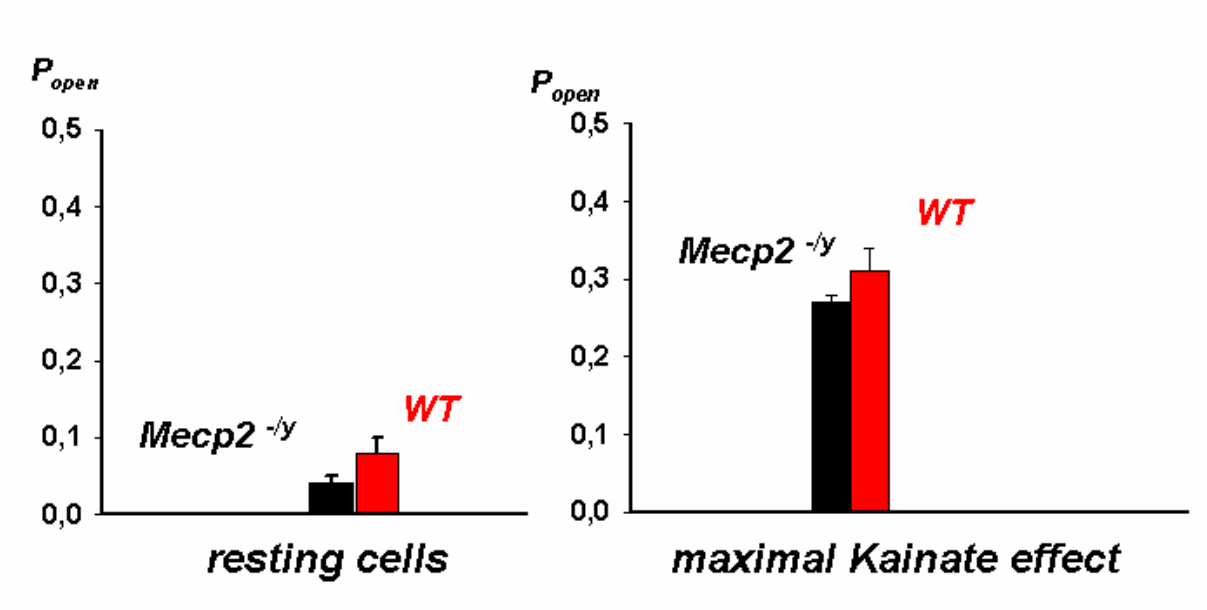


Fig.3.9 Weaker K-ATP channel activity in resting and potentiation after GluR stimulation in MeCP2 $^{-/y}$ hippocampal neurons.

The comparison of changes in the open state probability of K-ATP channels in WT and MeCP2 $^{-/y}$. Left graph show the difference in resting cells, right graph denote the difference at the maximal Kainate (10 μM of Kainate, 4 min after application) effect The calculations were made for recordings at holding potential -50 mV in neurons without spontaneous activity (the special-purpose program for this calculations was written and kindly rendered by Dr. Polyakov). n = 16.

3.1.6 ATP imaging in the resting WT and Mecp2 $^{-/}$y hippocampal neurons.

The open state probability of K-ATP channels depends on ATP intracellular level (in the strict sense ATP/ADP ratio) in the cells *(Liss and Roeper, 2001).* In previous chapter I showed that the initial activity of K-ATP channels and activity after GluR stimulation in RTT hippocampal neurons were significantly less with comparison with WT ones. It suggested us to measure ATP intracellular level in WT and Mecp2 $^{-/y}$ hippocampal neurons.

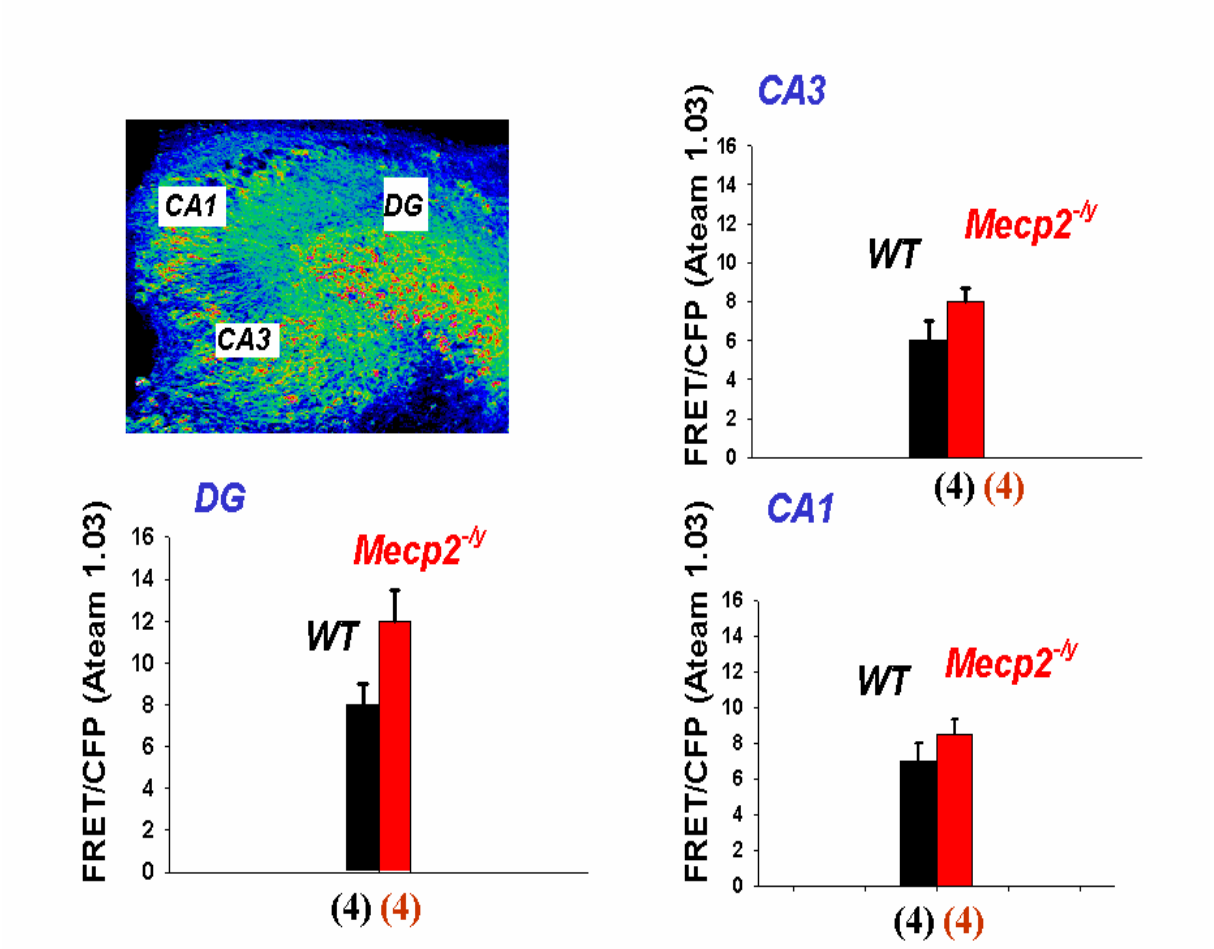


Fig13.10 Intracellular ATP levels differ in the resting WT and the Mecp2 -/y hippocampal neurons.

The graph shows ATP level in the WT and RTT hippocampal neurons. Hippocampal slices were transduced with neuron-targeted ATP sensor (Ateam 1.03) using recombinant adeno-associated virus (AAV). The ATP level in MeCP2$^{-/y}$ hippocampus neurons was higher: 30 % in CA3 region, 15% in CA1 region and 44 % in DG region as calculated from FRET/CFP signal ratio.

Briefly, WT and Mecp2 $^{-/y}$ slices were prepared in one day under the identical conditions. Next day they were transduced by ATP indicator, Ateam 1.03, under the same standard conditions. On the 7th day the samples were examined. It was made in as on a chess-board order, *viz*. at first was examined WT slice then RTT and so forth. Next (8th day) the examination was made in inverse order, RTT –WT-RTT-WT. In all cases the standard deviation of the mean was ≤ 8 %. The experimental data are shown in Fig. 13.10 as a summary on mean values.

It was observed a significant difference in the intracellular ATP levels in resting WT and MeCP2$^{-/y}$ mouse hippocampus neurons. The ATP level in MeCP2$^{-/y}$ hippocampal neurons was higher: 30% in CA3 region, 15% in CA1 region and 44 % in DG region as calculated based on the comparison of FRET/CFP signal ratio.

From obtained data I propose that:

(i) There exists a bilateral cross-talk between GluR and K-ATP channels in hippocampal neurons;

(ii) GluR actions were abolished in Na^+-free solutions and after blockade of Na^+-K^+-ATPase.

(iii) GluR stimulation enhanced ATP consumption that decreased submembrane ATP levels, whereas metabolic poisoning diminished bulk ATP

(iv) bilateral cross-talk between GluR and K-ATP channels for Mecp2 $^{-/y}$ basically is the same as for WT;

(v) ATP level in Mecp2 $^{-/y}$ is higher than in WT hippocampal neurons;

(vi) weaker activation of K-ATP channels in resting RTT hippocampal neurons and after AMPA/Kainate application occurs due to higher ATP intracellular levels in comparison with WT;

3.2. Ca and ATP imaging in the hippocampal neurons during the epileptogenic treatment.

3.2.1 Calcium imaging in the hippocampal neurons during the epileptogenic treatment.

The next part of my experiments is closely related to ATP intracellular changes during epileptogenic treatment (induced by disinhibition of NMDA receptors) and *pro tanto* probable role of K-ATP channels in metabolic control of seizure origin and seizure spreading in hippocampal neurons that may play an important role in RTT.

In introduction I mentioned about the experiments of Amoroso and Yamada where they showed interdependence between K-ATP channel activity and seizures *(Amoroso et al., 1990; Yamada et al., 2001).* Both examinations were related to reduction of GABA release in *Substantia Nigra.* The population of GABAergic neurons in hippocampus is much smaller than glutamatergic NMDA and AMPA/Kainate receptors *(Benson et al., 1994).* In hippocampus neurotransmission is mainly mediated by glutamate receptors *(Dingledine et al., 1990).* Earlier it was shown that removal of Mg^{2+} from bath solution induced spontaneous epileptiform activity in hippocampal acute slices *(Walther et. al., 1986*).

I first applied calcium imaging to examine responses of organotypic hippocampus slices transduced with genetically encoded calcium sensor, D3cpv. When Mg^{2+} ions were removed from the bath solution, a seizure-like activity appeared (Fig.3.11 B).

I observed consistent effect in all parts of hippocampus. Seizure-like activity developed within 1 min that may be caused by reorganisation of hippocampal network necessary for onset of seizures *(Kramer and Cash, 2012).* After reaching this state calcium transients became synchronous in all hippocampal neurons. After Mg^{2+} ions admission the seizure-like activity vanished.

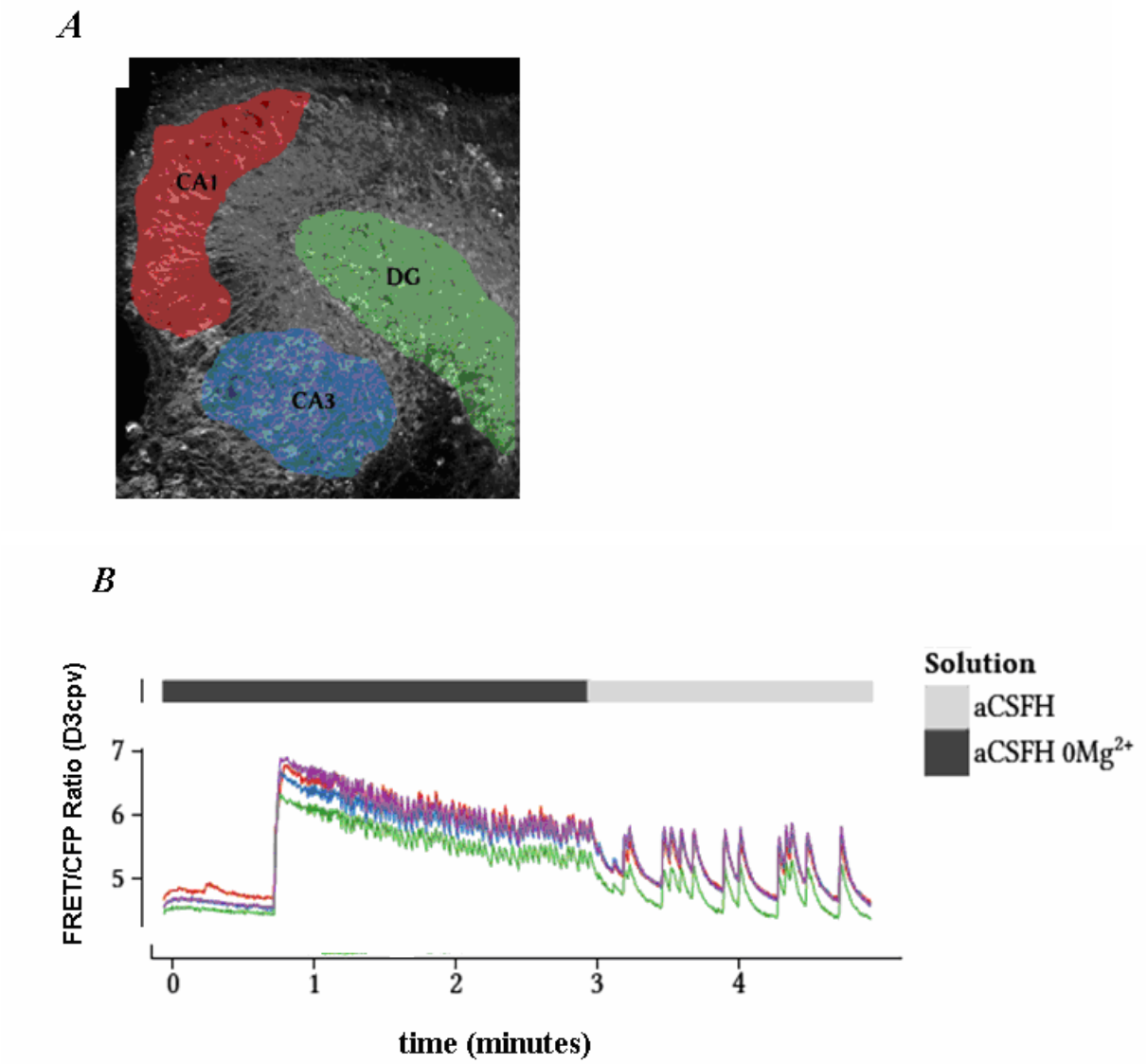


Fig 3.11 Exposure of neurons to Mg-free solution induces seizure-like activity.

A **-** Anatomically the CA1, CA3 and DG areas of hippocampus can be distinguished. ***B*** **-** The representative experiment which shows seizure like activity in slices transduced with D3cpv after removal of magnesium from the bath. Green, blue and red/violet traces show calcium transient in the soma of representative neurons in CA1, CA3 and DG respectively.

Then I examined calcium responses in MeCP2$^{-/y}$ organotypic hippocampal slices transduced with calcium indicator, D3cpv (not shown). No differences were observed in character and magnitude of calcium responses between WT and RTT neurons. In MeCP2$^{-/y}$

neurons, the response also developed within 1-2 minutes and after reaching this state calcium transients became synchronous in all neurons of hippocampus.

3.2.2 ATP imaging in the WT and Mecp2 $^{-/y}$ hippocampal neurons during the epileptogenic treatment.

Then, using this model I (in cooperation with Johan Tolö and Dr. Kügler) sought to measure ATP changes that might accompany seizure-like activity. I observed that in Mg-free solution, ATP levels decreased significantly. The decrease started with a delay that matched the slow development of seizures. I also observed that the decrease in ATP levels during seizures was reversible and completely recovered after the reapplication of Mg^{2+} ions. Unexpectedly in WT samples the effects were much weaker (or even absent) in DG than in CA areas Fig 13.13. Because the D3cpv transduced neuron responses showed a similar effect, I cannot give an intelligible explanation.

Then ATP changes that accompanied seizure-like activity were measured in $MeCP2^{-/y}$ hippocampus neurons transduced with Ateam 1.03. In spite of that in both mouse lines ATP decreased during the seizures, nevertheless, I showed a qualitative and quantitative difference in the decrease of ATP levels in WT and $Mecp2^{-/y}$ hippocampus neurons during seizure-like activity (Fig 3.12). Today I cannot explain these differences.

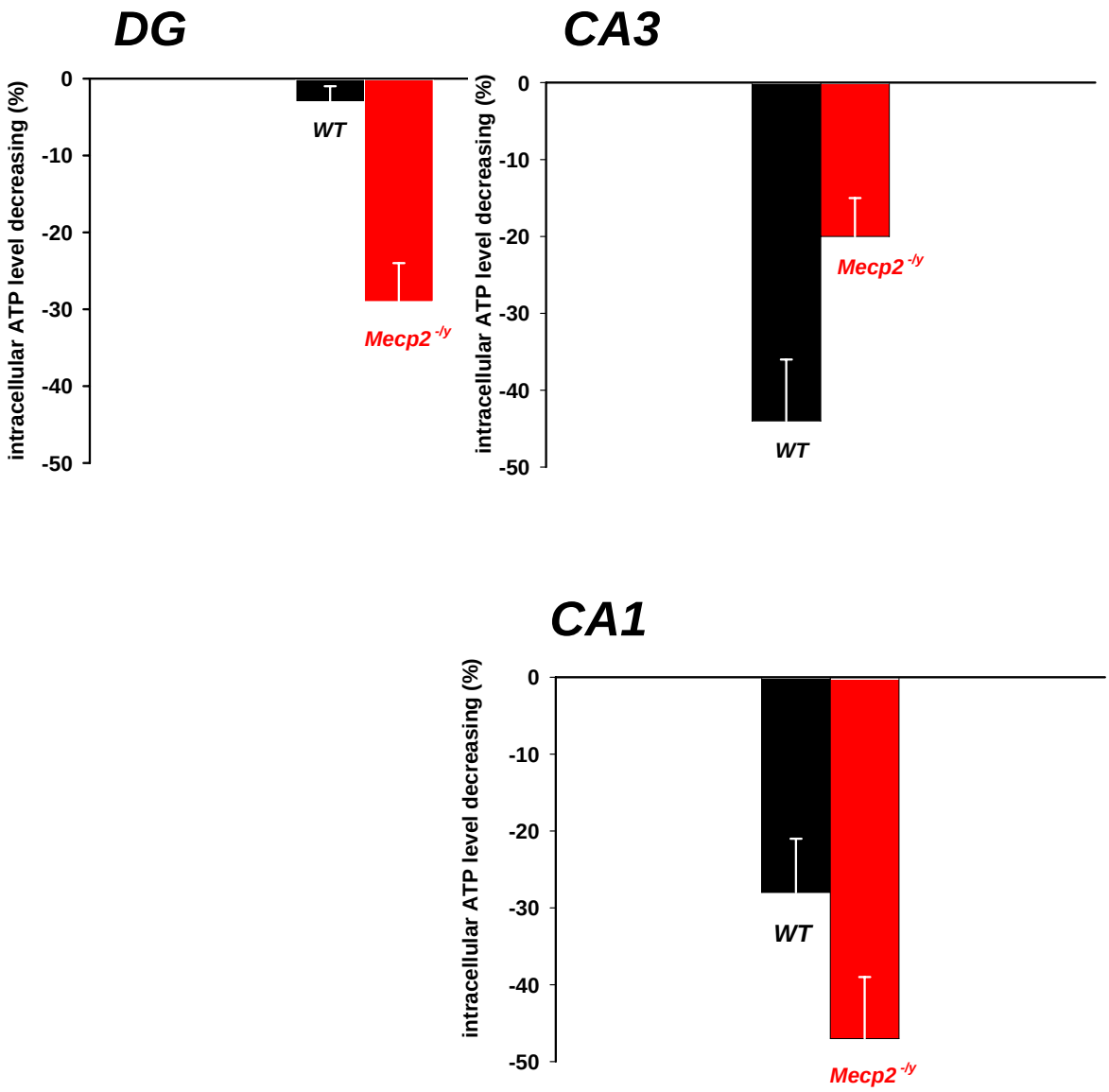


Fig 3.12. The comparison of intracellular ATP level changes in the WT and the Mecp2 -/y hippocampal neurons during epileptic-like activity.

The graph shows ATP level changes in different regions of WT and RTT hippocampus. Hippocampal slices were transduced with neuron-targeted ATP sensors (Ateam 1.03) using recombinant adeno-associated virus (AAV).

Resting ATP level was taken as 0%. The ATP changes in MeCP2$^{-/y}$ hippocampal neurons were bigger in CA1 region and in DG region and smaller in CA3 region respectively. The mean values and deviations were calculated using FRET/CFP signal ratio.

From the obtained data I concluded:

(i) seizure-like activity induced by removal of Mg^{2+} from the bath is accompanied by decline of intracellular ATP levels;

(ii) ATP levels vary significantly in different regions of the same hippocampus;

(iii) ATP change during epileptic seizure in each region of hippocampus has its particular characteristics.

(iv) the rates of ATP consumption/production in $MeCP2^{-/y}$ mouse hippocampus neurons were bigger than in WT neurons. It was bigger for resting cells as well as for neurons during seizures.

4. Discussion

Recent findings reveal multiple physiological consequences of Rett Syndrome, such as modification of synapses during the first two weeks of development *(Nelson et al., 2006; Chao et al., 2007),* impaired hippocampal plasticity *(Asaka et al., 2006),* increased neuronal vulnerability during excitotoxicity in the cerebellum *(Russell et al., 2007),* enhanced susceptibility to hypoxia in the hippocampus *(Fisher et al., 2008),* the age-related densities of NMDA and AMPA/Kainate receptors in frontal cortex of females with RTT *(Blue et al., 1999; Johnston et al., 2001*), the dependence of seizure frequency from age *(Jian et al., 2006; Glaze et al., 2005; Glaze et al., 2010; Nissenkorn et al., 210; Kim et al., 2012*) and others. Nevertheless the molecular mechanisms of Rett Syndrome development and long term prognosis are still a matter of investigation *(Hite et al., 2009; Chahrour et al., 2008, Gonzales and Salle, 2010; Cardoza et al., 2011).*

As was mentioned above the most of people with Rett Syndrome develop seizures *(Jian et al., 2006).* Epileptic seizures manifest in a variety of forms, encompassing a wide spectrum, which ranges from the easily controlled to the very severe *(Jian et al., 2006).* It is also accepted that an excessive stimulation of glutamate receptors often leads to the pathological effects, such as epilepsy, especially when energy supply is insufficient *(Greene and Greenamyre, 1996; Choi, 1990).* Implications of the interconnection between intracellular ATP levels and K-ATP channel functioning have not been, per se, previously examined.

In the present work, I examined the relationship between electrophysiological characteristics of single K-ATP channels with intracellular ATP levels and the mechanism of epilepsy in WT and *Mecp2*-null mouse hippocampal neurons.

4.1. ATP-dependent inhibition of K-ATP channels

Glutamatergic neurons embody the most abundant neuronal population in the brain and they are critical for processing information within the CNS. GluR stimulation elicits a "double-edged sword" effect, as, under certain conditions, GluR activity may become dysregulated and lead to pathological release of glutamate and to spillover in the neuronal tissue. When uncontrolled glutamate release occurs, it can trigger signalling cascades that disturb neuronal functions. Depending on damage progression, the disturbances may range from the malfunctioning of networks (epilepsy) to irreversible neuronal damage and death of neurons (stroke). Such pathological effects of glutamate have referred to as excitotoxicity *(Olney and de Gubareff, 1978).* Because of its versatile occurrence in the CNS, a question arises: do specific mechanism(s) exist, which are able to counteract the deleterious effects of glutamate, the most prevalent transmitter in the CNS? Around the time that excitotoxicity studies were gaining momentum, a novel type of channel was discovered in the heart *(Noma, 1983),* who's properties might fit those required for a desired protective agent.

These ATP-sensitive potassium (K-ATP) channels, found later in many neuronal types, show specific functions that are dependent on cell metabolism. These channels act as sensors for changes in nucleotide (ATP and ADP) levels and their metabolites (adenosine). K-ATP channels can dampen neuronal vulnerability, through membrane hyperpolarisation and changes in the input resistance. The role of K-ATP channels in neurons has been most frequently examined under hypoxic and ischemic conditions, when ATP production ceases (*Mironov et al., 1998; Mironov et al., 2000).* They, however, are functional under much milder conditions and increase activity after 'respiratory' bursts *(Haller et al., 2001)* and even after single action potentials *(Tanner et al., 2011).* Such responses are congruent with the theoretical estimates *(Attwell & Laughlin, 2001*), predicting that most of the energy in the brain is spent in the

restoration of ion homeostasis after action potentials and activation of glutamatergic synapses. Despite possible importance of interaction(s) that can exist between neuronal K-ATP channels and glutamate receptors, they have never been examined on the single channel level. Here, in this study, we analyze GluR – K-ATP cross-talk, using much milder conditions than those applied to examine excitotoxicity. The impetus for this study is driven by the discovery of a striking similarity among the effects of hypoxia and Kainate on K-ATP channels, within preBötC inspiratory neurons *(Mironov et al., 2011)* and hippocampal neurons. Because pharmacological and gating properties of single K-ATP channels in both preparations are similar *(Mironov et al., 1998; Pelletier et al., 2000),* we focused on the analysis of GluR – K-ATP interaction in clearly defined preparations of hippocampal neurons.

The role of K-ATP channels in linking metabolism to electrical activity is best understood in pancreatic ß-cells, where they maintain a hyperpolarized state and after closure, allow action potentials to fire and produce calcium influx and insulin release *(Edwards & Weston, 1993).* Yet K-ATP channels are also present in a large number of central neurons that do not act as glucose sensors. Instead, the channels are thought to remain closed but K-ATP channels show weak basal activity and are transiently potentiated after action potentials *(Tanner et al., 2011)* and their bursts *(Haller et al., 2001).* Both brainstem and hippocampal neurons express a particularly high density of K-ATP channels (*Karschin et al., 1997).* In performed experiments, they were observed in almost every patch. The activity was however low (P_{open} < 0.1) and the openings at rest were so rare that usually only one opening level was seen. After stimulation of GluR, P_{open} increased and several conductance levels (3-6 simultaneously open channels) were observed. The channels were present in the patch but they might be silent channels and become recruited. Nonetheless, four channels in the patch with 5 μm^2 square corresponded to approximately about 10^3 channels in the soma of neurons with a radius of 10 μm. Soma conductance is, thus, 75 nS and for $P_{open} \approx 0.3$ gives $I_{KATP} \approx 3$ nA for the membrane

potential -40 mV (E_K = 90 mV). This additional current is big enough to counteract most depolarizing actions.

I found that: approximately a 3-fold potentiation of K-ATP channels occurred, after application (or introduction) of GluR stimulation agonists; it developed slowly, attained maximum 2 min after stimulation and persisted for >20 min. The relative potency of GluR agonists was the following: Kainate ≈ AMPA > NMDA. GluR-mediated effects were not related to cytoplasmic calcium level, reactive-oxygen species or osmotic effects. GluR stimulation induced submembrane ATP depletion and subsequent disinhibition of K-ATP channels; the main ATP consumers were the Na^+/K^+-ATPase > SERCA > actin turnover.

GluRs induced slow decreases in cytoplasmic ATP during which, the time-course and magnitude correlated with changes in the activity of K-ATP channels. The pathways examined and their significance is schematically presented in Fig. 3.6 A.

Cytoplasmic calcium is an established factor which plays a central role in glutamate receptors mediated excitotoxicity *(Choi & Rothman, 1990)*. Thus I examined first the involvement of Ca^{2+} fluxes mediated by GluR agonist. GluR agonists elevated calcium levels (Fig. 3.2 and 3.3) but inhibition of various pathways of calcium elevation did not modify the GluR effects on to K-ATP channel activity (Fig. 3.3 B). The finding does not contradict previous studies on excitotoxicity, because in these studies, longer and stronger stimuli have been applied that resulted in massive overload of neurons with calcium, activation of intrinsic apoptotic pathways and the death of neurons several hours after stimulation *(Choi & Rothman, 1990; Mattson et al., 1997; Abramov et al., 2007).* It is generally accepted that calcium does not influence the activity of K-ATP channels directly *(Edwards & Weston, 1993).*

In my experiments, K-ATP channels attained maximal activation within 2 min and notably, the effects developed much faster than calcium-mediated excitotoxicity. Calcium imaging also showed normal recovery to resting Ca^{2+} levels whereas, during excitotoxicity calcium recovery becomes severely prolonged *(Delorenzo et al., 2005).* Nonetheless, to confirm

calcium independence in the effects observed, I blocked calcium influx with cadmium, replaced calcium with barium and loaded neurons with the calcium-chelator BAPTA.

None of these treatments modified the responses of K-ATP channels to GluR stimulation (Fig. 3.3). The calcium-hypothesis of glutamate excitotoxicity frequently invokes the effects of free radicals (ROS), also important in neurodegeneration. I used the genetically encoded mitochondrial neuron targeted, redox sensor ro-GFP1 *(Hanson et al., 2004),* to monitor ROS levels in mitochondria. Robust responses to oxidizing (H_2O_2) and reducing agents dithiotreitol (DTT) were observed but GluR stimulation did not elicit any changes in ROS levels (Fig.3.5).

Another possibility is the swelling of neurons that is often considered in excitotoxicity. Osmo-mechanical stress increases the activity of K-ATP channels in brainstem neurons *(Mironov and Richter, 2000)* and hippocampal neurons showed a similar response. This however did not preclude further activation of K-ATP channels by AMPA/Kainate. I also did not find significant changes in cell volume that can be related to stimulation of GluRs (Fig 3.4).

Experimentally rejecting calcium, ROS and swelling hypotheses as possible causes, I next examined the role of ATP, an intrinsic inhibitor of K-ATP channels. Metabolic stress on the cell may be sufficient to open K-ATP channels, via ATP depletion, above their low steady-state resting levels. Rolfe and Brown (1996) predict, for a 'general' cell that at about 25% of total ATP synthesis is used by the Na^+-K^+-ATPase, 5 % by the Ca^{2+}-ATPase, 5 % by the actomyosin ATPase, and that the rest is utilized by gluconeogenesis, substrate cycling, protein and mRNA synthesis. While blockade of SERCA and ATP-dependent actin turnover slightly suppressed GluR actions and inhibition of Na^+-K^+-ATPase, ouabain abrogated the effects completely (Fig.3.6). The data are, thus, are congruent with predictions of Rolfe and Brown (1996). Using neuron-targeted sensor, Ateam 1.03, I measured ATP levels and obtained a close correspondence between ATP depletion and changes in single-channel activity. Earlier Dr. Mironov developed a simple model of ATP homeostasis that predicted substantial ATP depletion in submembrane regions. He found that estimates did not match measured P_{open} values. He explained this as

follows: this discrepancy is neither novel nor disappointing, as it represented an expected stumbling block in studies of K-ATP channels, from the very beginning. Measured values of IC_{50} (or K_d) for K-ATP channels (overall mean, 30 μM) in different cells are far below the levels of ATP in the cytoplasm (overall lower limit, 0.3 mM). For bigger K_d values of ATP inhibition, most effects related to K-ATP channels in living cells are readily (and quantitatively) explained. There was an intense search of factors that can raise K_d values. Several candidates are noteworthy but the most promising are PIP2 *(Loussouarn et al., 2001*) and ADP *(Proks et al., 2010*), as discussed above. The exact mechanism of K_d increase in neurons remains to be established.

Evidence, gained in studies of glutamate excitotoxicity, rendered improbable the hypothesis, which postulates that activation of K-ATP channels is sufficient to completely abolish the effects of massive and long-lasting glutamate release and concomitant death of neurons. This study extends recent results *(Soundarapandian et al., 2007*), showing that neurons with deficiencies in expression of functional K-ATP channels have enhanced glutamate release, following activation of Kainate receptors. A bilateral cross talk between GluR and K-ATP channels has to be considered, however *(Mollajew et al., 2013)*. Such connection may also represent a general mechanism for tempering excitability during high-activity, epileptic-like states. There is an evidence *(Hernández-Sánchez et al., 2001; Yamada et al., 2001)* showing that an inhibiting effect of K-ATP channels on neuronal activity is pivotal in the control of seizures.

In summary, glutamate release and spillover that occurs under both physiological and pathological conditions can be regulated through modulation of the activity of K-ATP channels. As was mentioned above patients with Rett Syndrome and especially younger patients seemingly have higher glutamate level in brain and in CSF and higher glutamate receptor density. This may make them more prone to glutamate overexcitation and excitotoxicity.

The present data may contribute to the understanding of the mechanisms involved in processes ranging from modulation of activity of neuronal networks to brain injury and, potentially, to designing specific pharmacological interventions.

4.2. Calcium and ATP imaging in intact WT and Mecp2 $^{-/y}$ mouse hippocampal neurons and during the epileptogenic treatment. A link to K-ATP channels.

ATP is the main energy transfer molecule in the cell (*Lipmannin 1941),* acting as an intermediary molecule between energy-yielding exergonic and endergonic reactions. The adult human brain constitutes about 2% of body weight but receives 20% of blood supply *(Erecinska M. et al., 2004).* This requirement for metabolic energy has important implications for the brain's evolution and function. ATP is considered to be associated with accumulation of sodium and calcium ions within nerve cells *(Lux et al., 1986, Staley, 1994; Callewaert et al., 1996)* and activity-dependent potassium efflux *(Heinemann and Lux 1975; Lothman and Somjen, 1975).* In order to maintain neuronal excitability, ion transport processes at plasma membranes (Na^+/K^+-ATPase. Ca^{2+}-ATPase and other) must be activated. These processes require large amounts of ATP *(Erecinska et al 1996).* It is well known that most of the energy in the brain is spent on restoration of ion homeostasis at glutamatergic synapses *(Attwell & Laughlin, 2001)* and that glutamate can be very neurotoxic and can cause malfunctioning of the networks, especially when energy supply is insufficient *(Greene and Greenamyre, 1996; Choi, 1998).*

In previous studies, the changes in ATP levels were monitored using biochemical methods *(Koop and Cobbold, 1993; Kennedy et al. 1999; Mironov, 2007).* However, recent application of genetically encoded ATP indicators allowed precise measurement of ATP levels in the cytoplasm and within various intracellular compartments *(Imamura et al., 2009).* Based on our previous expertise with ATP level measurements using biochemical methods, in this study, I

(with Johan Tolö) designed a method using Ateam 1.03 based optical probe for ATP measurements in neurons of tissue slices. Using this method we were able to demonstrate a clearly ATP elevated in Mecp2$^{-/y}$ hippocampal neurons compared to that of the WT. The ATP level in intact Mecp2$^{-/y}$ hippocampal neurons was bigger: by 15% in CA3 region, 22% in CA1 region and 33% in DG region as calculated based on the comparison of FRET/CFP signal ratio (Fig 3.10). The higher ATP level can explain the smaller activity of K-ATP channels in the intact Mecp2$^{-/y}$ hippocampal neurons and after AMPA/Kainate stimulation (Fig.3.9).

Application of magnesium-free solution induced an epileptogenic stimulus, led to a decrease in ATP levels with regional variations (Fig 3.12). Reducing magnesium to below physiological level induces enhances excitability in hippocampal slices *(Walther et al., 1986)*. It has been suggested that N-methyl-d-aspartate receptors participate in the generation of this activity. When magnesium is removed, NMDA receptors became disinhibited that disturbs the balance between excitation and inhibition. The neurons enhanced their bursting activity, which was synchronized over time and translated into epileptic-form activity in the neuronal network.

Using D3-cpv transduced organotypic hippocampal slices I demonstrated a seizure-like activity (in all hippocampal regions CA1, CA3 and DG), which developed within 1-2 minutes. After reaching this state, calcium transients became synchronous in all hippocampal neurons with a similar response in amplitude and frequency.

Excitation of neurons is accompanied by sodium influx through NMDA and voltage-sensitive channels, resulting in sodium accumulation and that activates the sodium pump, which leads to ATP depletion. I did, indeed, observe a decrease in ATP levels, during seizure-like activity (Fig 3.12). ATP depletion is the highest in the membrane vicinity, where ATP consumption is higher due to intense sodium turnover. This ATP depletion can disinhibit K-ATP channels and diminishes neuronal excitability, through a decrease in membrane resistance and by shifting membrane potentials to more negative values.

Finally, I applied ATP imaging to analyze ATP homeostasis in hippocampal Mecp2 $^{-/y}$ neurons. The rate of ATP consumption/production was bigger in hippocampal Mecp2$^{-/y}$ neurons. This can explain higher resting ATP levels and larger ATP changes during seizures.

Greater ATP consumption in MeCP2$^{-/y}$ hippocampal neurons is seemingly compensated by higher production. Since the ATP level in Mecp2$^{-/y}$ hippocampal neurons is bigger, one can expect that K-ATP channels may be more inhibited, even under normal physiological conditions. We previously reported a weaker activity of K-ATP channels in Mecp2$^{-/y}$ neurons in the brainstem (*Mironov et al., 2011)* and in the hippocampus (this study; see Results). In the hippocampus, we show only slight differences in K-ATP behavior in WT and Mecp2$^{-/y}$ neurons after AMPA/Kainate application; the channel activation began only after a delay, which can be explained by a delay in submembrane ATP depletion.

4.3 Conclusions

The data presented in this study suggests that the observed increase in ATP handling in RTT may involve a compensatory mechanism that can maintain proper functions in Mecp2$^{-/y}$ mouse hippocampal neurons. This compensatory mechanism may inhibit and/or delay of K-ATP channel activation, resulting in a delayed control of neuronal excitability, through membrane hyperpolarisation *(Mironov et al., 2011; Russell et al., 2007; Soundarapandian, 2007*).

We showed for the first time the interdependence between GluR activation, intracellular ATP depletion and K-ATP channel activity and seizure-like activity. Obtained data suggest the following chain of events: GluR activation → ATP depletion → K-ATP channel activity. I suppose that high glutamate level in the brain of the patients with RTT, the glutamate neurotoxicity may induce damage the brain cells through neurotoxic sodium and calcium influx. ATP level in RTT neurons is higher consequently K-ATP channels are less active and may experience smaller activation by appropriate stimuli.

This chain of events, the difference in the initial activity of K-ATP channels in WT and Mecp2$^{-/y}$ hippocampal neurons and the difference in ATP intracellular levels in these mouse lines can explain the RTT neuron vulnerability towards epileptic seizures due to insufficient and/or late K-ATP channel functioning.

5. References

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Curriculum Vitae

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List of Publication

0. [Johan Tolö, **Rustam Mollajew**, Sebastian Kügler and Sergej Mironov]
[ATP imaging in the hippocampal neurons of mice Rett model reveals differences in ATP homeostasis during epileptogenic treatments] **Article is under construction**.

1. Rustam Mollajew, Johan Tolö and Sergej Mironov. Single KATP channel opening in response to stimulation of AMPA/Kainate receptors is mediated by Na+ accumulation and submembrane ATP and ADP changes. J. Physiol. Accepted March, 2013, [Epub ahead of print]

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