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Unraveling Molecular, Cellular and Cognitive Defects in the Mouse Model for Mental Retardation Caused by Rsk2 Gene Mutation

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Tahir Mehmood

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Abbreviations

Abbreviations:

A

ADAR	Adenosine Deaminase Acting on RNA
ADF	Actin depolymerizing Factor
AGC	Group of protein kinases including PKA, PKG and PKC
AHR	Aryl hydrocarbon receptor
AKT	Rac-alpha serine/threonine protein kinase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptor
AP2	Activating Protein 2/ Adaptor complex
APV	Amino Phosphono Valerate
Arc	Activity-regulated cyctoskeleton-associated protein
Arp2/3	Actin related protein 2/3
Asp	Aspartic Acid
ATF	cAMP-dependent Transcription Factor
ATF4	Activating Transcription Factor 4
ATP	Adenosine-5'-triphosphate
ATR-X	alpha-thalassemia X-linked mental retardation syndrome

B

Bad	Bcl-2-associated death protein
Bcl	B cell-lymphoma
BDNF	Brain Derived Neurotrophic Factor
bZIP	basic region leucine Zipper

C

C/EBPβ	CCAAT/Enhancer Binding Protein β
CA1-3	Cornu Ammonis
Ca^{2+}	Calcium
Cacnb4	Calcium channel voltage-dependent beta 4 subunit
Cacng8	Calcium channel voltage-dependent gamma subunit 8
CaMK	Ca^{2+} / Calmodulin-dependent protein kinase
CaN	Calcineurine
CBP	CREB-Binding protein

CDK	Cyclin-Dependent Kinase
CDKN1B	cyclin-dependent kinase inhibitor p27
c-Fos	Cellular FBJ murine osteosarcoma viral oncogene homolog
CLS	Coffin-Lowry syndrome
Cm	Centimeter
CNS	Central Nervous System
Cox	Cyclooxygenase
CRE	cAMP Response Element
CREB	cAMP Response Element Binding
CTD	Carboxyl-Terminal Domain
CTKD	Carboxyl-Terminal Kinase Domain

D

DA	Dopamine
Dapi	4', 6-diamidino-2-phenylindole
DAT	Dopamine Active Transporter
DIABLO	Direct inhibitor of Apoptosis Binding protein with low pI
DIV	Day in Vitro
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOPAC	3, 4-Dihydroxyphenylacetic acid
DrD2	Dopamine receptor D2
DUSP	Dual Specificity phosphatase

E

E2F	Eukaryotic transcription Factor 2
EEA1	Early Endosome Antigen 1
EF2K	Elongation Factor-2 Kinase
EGF	Epidermal Growth Factor
eIF	Eukaryotic translation Initiation Factor
ELK1	Ets-Like protein-1
E-LTP	Early long-term potentiation
EPSC	Excitatory Post-Synaptic Current
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinases

ESM	Extrasynaptic Membrane
Etv3	Ets variant gene 3
Exip	Exon-Skip

F

F-actine	Filamentous actin
FGF	Fibroblast Growth Factor
FGFR3	Fibroblast Growth factor Receptor-3
FMRP	Fragile X Mental Retardation Protein
Fxn	Frataxin
FXS	Fragile X Syndrome

G

G1/2	First/Second Gap
GABA	γ-Aminobutyric acid
GABA	gamma-Aminobutyric acid
G-actin	Globular actin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphatg
GEFs	Guanine nucleotide exchange factors
GFP	Green Fluorescent Protein
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
GluR	Glutamate receptor subunit
Gly (G)	Glycine
Grb2	Growth factor receptor-bound protein 2
Gria2	Glutamate receptor ionotropic AMPA2
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine Triphosphate Hydrolase

H

HAT	Histone Acetyl Transferase
HEK-293	Human Embryonic Kidney 293
Hela cell	Henrietta Lacks cell
HIAA	5-Hydroxyindole-3-acetic acid

HMGN1	High-mobility group N1
HPLC	High performance Liquid Chromatography
HSP90	Heat Shock Protein 90
HT29	Human colon adenocarcinoma grade II cell line
HVA	3-Methoxy-4-hydroxyphenyl acetic acid
I	
IAP	inhibitor of Apoptosis
ID	Intellectual disability
IEGs	Immediate early genes
IGF	Insulin-like growth Factor
iGluR	Ionotropic Glutamate Receptor
IL-1β	Interleukin 1 β
Ile or I	Isoleucine
IQ	Intelligence quotient
IκBα	inhibitor protein kappa B
J	
JNK	c-jun NH_2^- terminal Kinase
K	
KAR	Kainate Receptor
Kb	Kilobase
kDa	KiloDalton
KO	Knock Out
KSR	Kinase Suppressor of Ras
L	
L1CAM	L1 cell adhesion molecule
LBD	Ligand Binding Domain
Leu or L	Leucine
LIMK	Lin-11, Isl-1 and Mec-3
L-LTP	late- long-term potentiation
LTD	Long Term Depression
LTP	Long-term potentiation
LTP	Long Term Potentiation
Lys or K	Lysine

M

M	Mitotic phase
mAChRs	Muscarinic Acetylcholine Receptors
MAP1B	Microtubule-Associated Protein 1B
MAP2K	MAP kinase kinase
MAP3K	MAP2K Kinase
MAP4K	MAP3K Kinase
MAPK	Mitogen-activated protein kinase
Mcl-1	Induced Myeloid Leukemia Cell differentiation protein
MEK	MAPK ERK Kinase
mEPSCs	miniature Excitatory Post-Synaptic Currents
Mg^{2+}	Magnesium
mGluR	Metabotopic Glutamate receptor
MK	MAPK-activated Kinase
MKP	MAPK phosphatase
MLCK	Myosin Light Chain Kinase
MM	Multiple myeloma
MNK1	MAPK signal-integrating Kinase 1
MOR	μ Opioid Receptor
MPEP	2-Methyl-6-(phenylethynyl) pyridine
MR	Mental Retardation
MRCK	Myotonic dystrophy kinase-related Cdc42-binding kinase
mRNA	messenger-RNA
MSK	Mitogen and stress activated protein kinase
mTOR	Mammalian Target of Rapamycine

N

Na^{+}	Sodium
Neo	Neomycin
NES	Nuclear Export Signal
NF1	Neurofibromin 1
NF-1	Neurofibromatosis type-1
NF_kB	Nuclear Factor Kappa B
NHE1	Sodium/hydrogen exchanger Isoform-1

NLS	Nuclear Localization Signal
NMDA	N-Methyl-D-aspartic acid
NMDARs	N-Methyl-D-aspartic acid Receptors
nNOS	Neuronal NO synthase
NO	Nitric Oxide
NR	NMDA receptor subunit
NRF-1	Nuclear Respiratory Factor-1
NRSE	Neuronal Restrictive Silencing Element
NS-XLMR	Non-Syndromic X-Linked Mental Retardation
NTD	N-terminal domain
NTKD	N-terminal Kinase domain

O

OPHN1	Oligophrenin-1

P

$P27^{kip1}$	Cyclin-dependent kinase inhibitor 1B
$p70^{S6K}$	70kDa ribosomal protein S6 Kinase
PA	Phosphatidic acid
PAK	p21- activated Kinase
PARP	Poly (ADP-ribose) polymerase
PARs	Proteniase-Activated Receptors
PC12	Pheochromocytoma Cells
PDGF	Plateled- Derived Growth Factor
PDK1	3-phosphoinositide dependent protein kinase-1
PDZ	Postsynaptic density fraction Discs large ZO-1
pH	Potential Hydrogen
PI3K	PhosphoInositide-3-Kinase
PKA	Protein kinase A
PKA/C	Protein Kinase A/C
PKD1	Protein Kinase D1
PLD1	Lipid modifying enzyme phospholipase D isoform 1
PMA	Phorbol 12-Myristate 13- Acetate
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A

PP2Ac	Protein Phosphatase 2A catalytic subunit
PS	Postsynaptic
PSD	Post-Synaptic Density
PSD	post-synaptic density
PSD95	Post- Synaptic Density Protein 95
Ptgs2	Prostaglandin-endoperoxidase synthase 2

Q

QRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction

R

Rac	Ras-related C3 botulinum toxin substrate
RanBP3	Ran-Binding protein-3
Ras	Rat Sarcoma
RE1	Restrictive Element 1
RNA	RiboNucleic Acid
ROCK	Rho-associated Coiled-coil forming protein kinase
RpS6	Ribosomal Protein S6
Rps6ka3	Ribosomal protein S6 kinase 90kDa polypeptide 3
RSK	Ribosomal S6 Kinase
Runx2	Runt-related transcription factor 2

S

SAPK	Stress-activated protein kinase
Ser	Serine
SH2	Src Homology 2
SH3	Sarcoma Homology 3
Shnak	SH3 and multiple ankyrin repeat domains protein
shRNA	short hairpin RNA
SIDAs	Stimulus Induced Drop Attacks
siRNA	small interfering RNA
SMAC	Second Mitochondria Derived Activator of caspases
SNAP25	Synaptosomal Associated protein 25
SNARE	Soluble NSF attachment protein receptor
Sod2	Superoxidase dismutase 2

SOS	Son of Senvenless
Sp1	Specificity Protein 1
SRF	Serum Response Factor
SSH	Slingshot Phosphatase
STEP	Striatal Enriched tyrosine phosphatase
Stk3	Serine/threonine kinase 3
S-XLMR	Syndromic X-Linked mental retardation
SynGAP	Synaptic Ras GTPase- Activating Protein

T

TARP	Transmembrane AMPAR Regulatory Proteins
TCF	Ternary Complex Factor
TDG	Thymidine DNA glycosylase
TESK	Testicular protein kinase
Thr	Threonine
TMD	Transmemebrane Domain
TMN	Tubero Mamillary Nucleus
TNFα	Tumor Necrosis Factor α
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Trp or W	Tryptophan
TSC2	Tumor-suppressor protein Tuberous Sclerosis complex-2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr or Y	Tyrosine

U

UO126	1, 4-diamino-2, 3-dicyano-1, 4-bis [2- aminophenylthio] butadiene
UTR	Un-translated Region
UV	Ultra Violet

W

WAIS	Wechsler intelligence Scale for Adults
WHO	World Health organization
WISC	Wechsler intelligence Scale for Children
WISC	Wechsler intelligence scale for children

X

XIAP	X-linked inhibitor of apoptosis protein
XLMR	X-Linked mental retardation

Z

Zif268	Zinc finger protein 268
Zn^{2+}	Zinc
3'5-DHPG	3'5-DihydroxyPhenylGlycine
4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
5-HT	5-hydroxytryptamine (Serotonin)

Introduction

1. Intellectual disability (ID) or Mental Retardation (MR):

1.1. Definition and classification:

The spectrum of the brain disorders covers hundreds of disorders that are listed either as mental or neurological disorder. Human brain is one of the most important organ that plays essential and effective roles in functioning, but also one of most fragile one. Disability is defined by the World Health Organization (WHO) (Oliver and Sapey, 1999:38) as a restriction of the ability to perform an activity in the manner or within the range considered normal for a human being. Mental retardation (MR) now called Intellectual disability, is a form of developmental disability characterized by significant limitation both in intellectual functioning and adaptive behavior, as expressed in conceptual, practical, daily living skills and social adaptive skills that onset in early age. (Inlow and Restifo, 2004; Tarpey et al., 2009).

1.2. Prevalence of ID:

ID is an important socio- economic problem of health care but unfortunately, it has received less attention than other disorders like autism etc. Many factors contribute the ID; cultural deprivation, malnutrition, fetal alcohol exposure, poor health care, and parental consanguinity. ID affects 1-3% of the total population of the world, out of which 0.3-0.5% are moderately affected (Chelly et al., 2006). The prevalence of ID is inversely correlated with socio-economic standards, both within and between countries. The poor or underdeveloped countries have more ID and its frequency is two- to threefold higher than in high-income countries (Gustavson, 2005). In industrialized countries, measures against the specific causes of ID have proved to be very effective (Silverman, 2009).

1.3. Diagnosis:

In order to diagnose ID and assess severity, an overall assessment of intellectual functioning is achieved through standardized psychometric tests (the best known are the Wechsler tests, adapted for children (WISC) or adults (WAIS)) (Ropers, 2010). These tests are used to estimate verbal and motor performance of individuals. The result of these tests is expressed by a score called the intelligence quotient (IQ). In the general population, the

average IQ is 100. An IQ value below 70 is synonymous with ID (Chelly and Mandel, 2001). WHO distinguishes four degrees of ID: light for IQ between 50 and 70, moderate between 35 to 49, severe between 20 and 34 and profound below 20 (they are totally dependent) (Source: WHO website). (Ropers, 2010). The disorder severities are given in (Table. 1) (Chelly and Mandel, 2001).

Table 1. ID Classification in terms of Degree of Severity

Terminology	IQ	Level of Functioning
Profound	<20	Fully reliant on caregivers Often diagnosed with a neurological disorder and epilepsy. Speech and self care skills impaired
Severe	20-34	They are seriously impaired in their motor and speech development, and only learn basic language, are dependent on others.
Moderate	35-49	They are trainable Able to perform academic activities
Mild intellectual disability	50-70	Individuals educable Can be minimally self-supporting Can acquire basic social and vocational Skills.

1.4. Causes of Intellectual disability:

The causes of ID are highly heterogeneous ranging from non-genetics to genetics or combination of both (McLaren and Bryson, 1987). Several non-genetics factors contribute to ID, including infection during pregnancy such as cytomegalovirus and toxoplasmosis. In western countries, excessive alcohol consumption (Fetal alcohol syndrome) also contributes to ID. Genetic causes include chromosomal abnormalities (such as aneuploidy, microdeletions, subtelomeric rearrangement) and single-gene defects (involvement of a single gene in the pathology) (Chelly et al. 2006; Ropers, 2010). While environmental factors are believed to be involved mainly in light to moderate ID, genetic abnormalities seem to be the cause of 25 to 50% of severe ID (Chelly et al. 2006; Ropers, 2010).

However, in about 50% of ID cases the precise cause of the deficit remains unknown (Chelly et al., 2006).

Monogenic causes of ID are caused by mutations in both autosomal and X-liked genes (X-linked Intellectual disability or XLID). Currently, it is believed that X-linked gene defects account for about 10-12% of the ID in males (Ropers and Hamel, 2005). Defects of X-linked genes are the most important causes of ID, based on the observation that ID is more common in males than in females (Ropers and Hamel, 2005).

1.5. X-Linked intellectual disability (XLID):

Clinical observations and linkage studies in families revealed that X-linked intellectual disability (XLID) is a highly heterogeneous condition. To date, mutations in 91 X-linked genes have been reported to cause ID (Ropers, 2010). The most common form of XLID is the Fragile X (Fra(X)) mental-retardation syndrome (FRX). X-Linked ID (XLID) is usually divided into two groups: syndromic XLID (SXLID) and non-syndromic (NSXLID). This distinction is made according to the association (SXLID) or not (NSXLID) of other clinical features to ID (Chelly 2000; Ropers, 2010).

SXLID are associated with other clinical disorders, for instance radiological, metabolic, or biological abnormalities (Stevenson and Schwartz, 2009), which may help to determine the diagnosis. Genes leading to SXLID are involved in various cellular functions, such as neurogenesis, neuronal migration, synaptic function, and transcription (Ropers, 2010). Finding molecular causes in NSXLID cases is a great challenges because the genetically distinct subtype are clinically impossible to differentiate and their elucidation is often difficult (Ropers, 2010). NS or non-specific XLID are not progressive and NSXLID cases represent two-thirds of XLID cases (Ropers and Hamel, 2005). But, the development of molecular tools and closer examination of patients (especially after puberty or in adulthood) reveals sometimes that some forms of ID previously classified as non-syndromic are in fact syndromic (Ropers and Hamel, 2005). For example, the OPHN1 gene was originally described as a NSXLID gene. More recently, revisiting some patients carrying mutations in the OPHN1 gene revealed ataxia, epilepsy and cerebellar hypoplasia

(Ropers, 2010) **(Figure 1)**. Thus, it appears that the boundary between S- and NSXLID become increasingly blurred.

A classification of ID, recently suggested, is based on the presence or absence of abnormal brain development (Chelly et al., 2006). Indeed, in some cases, ID appears to be secondary to gross brain abnormalities (microcephaly, lissencephaly ...). In these cases, the mutated gene appears to be necessary for normal brain development. In the second category of ID, where the general organization of the brain is not affected, the anomalies would be at the cellular level. This classification does not take into account the chromosomal location of the causative gene.

I will now do a more detailed description of a form of SXLID, Coffin-Lowry syndrome (CLS); I will present my participation to the investigation of the physiopathological mechanism leading to cognitive dysfunction in the mouse model of CLS.

2. Coffin–Lowry syndrome (CLS):

Coffin-Lowry syndrome (CLS) is an X-linked syndromic form of intellectual disability that was initially and independently reported by Coffin et al. in 1966 and Lowry et al. in 1971. This very disabling disorder is characterized in male patients by facial and digital abnormalities, progressive skeletal deformations and severe psychomotor development impairment (Hanauer and Young, 2002). CLS is caused by heterogeneous loss-of-function mutations in the RSK2 (90-kDa ribosomal S6 kinase) gene (*RPS6KA3*) mapping to Xp22.2. RSK2 acts at the distal end of the (ERK/MAPK) signaling pathway, and is activated by Serine/threonine phosphorylation in response to many growth factors and neurotransmitters (Frodin and Gammeltoft, 1999). This syndrome has been reported in many parts of the world, in America, Europe, Asia and Africa (Zeniou et al., 2004). No exact estimate of the prevalence has been published. However, an incidence of 1 man in 50 000 per year seems a reasonable value as estimated by researchers (Pereira et al., 2010).

Figure 1: Genes implicated in X-Linked Mental Retardation (XLMR) and their position on the human X-chromosome.

Genes associated with syndromic XLMR are grouped on the left of the chromosome, while non-syndromic XLMR are shown on the right. Colors relate to different functional classes (Adapted from Ropers, 2010 and Stevenson and Schwartz, 2009).

2.1. Clinical Features:

The clinical symptoms associated with CLS are highly variable and generally more severe in men than in women. Most of them settled gradually over the life, very few signs being observed at birth.

2.2. CLS and psychomotor retardation:

The main characteristic of CLS is a cognitive impairment of variable severity, with IQ that can range from 15 to 60. However, the majority of male patients have a profound ID (Pereira et al., 2010). Development of speech is always impaired in CLS patients, with most patients mastering only a few words. About 30% of patients have partial or total sensorineural hearing loss. Some patients exhibit also epilepsy and/or cataplexy (drop attacks). In early childhood patient's exhibit generalized hypotonia. Despite limited verbal skills, these individuals are communicative and cheerful. The average age at which affected children do their first steps is usually extended to three years, (Zeniou et al., 2004). Behavioral problems have also been reported in rare cases of female patients (Pereira et al., 2010).

2.3. Morphological abnormalities:

Facial dysmorphism and skeletal abnormalities are the major morphological features of CLS. The typical facial aspect of adult CLS patients includes hypertelorism, prominent forehead and thick lips, palpebral fissures slanted downward and out, ears low and broad, a thick nasal septum, thick lips, and missing teeth and poorly located (Pereira et al., 2010). This feature becomes more pronounced with age.

At birth, the size of affected children is usually normal, growth retardation settling gradually. An analysis of 250 patients showed that the average size of adult male patients was 143cm (Hanauer and Young, 2002). Skeletal malformations include delayed bone formation, spinal scoliosis or kyphosis and chest deformities (Pereira et al., 2010).

Patients have small fleshy hands with tapered fingers **(Figure. 2)**. This is a diagnostic clue because the characteristic shape of the hand is found at birth and is relatively specific to the CLS. Spine deformities may become progressively worse and often require surgery in childhood (Hanauer and Young, 2002; Hanauer, 2008). Radiological defects have been observed in the skull, the spine or hands, including hyperostosis of reduced inter-vertebral spaces and a reduction in size of the distal phalanges (Pereira et al., 2010).

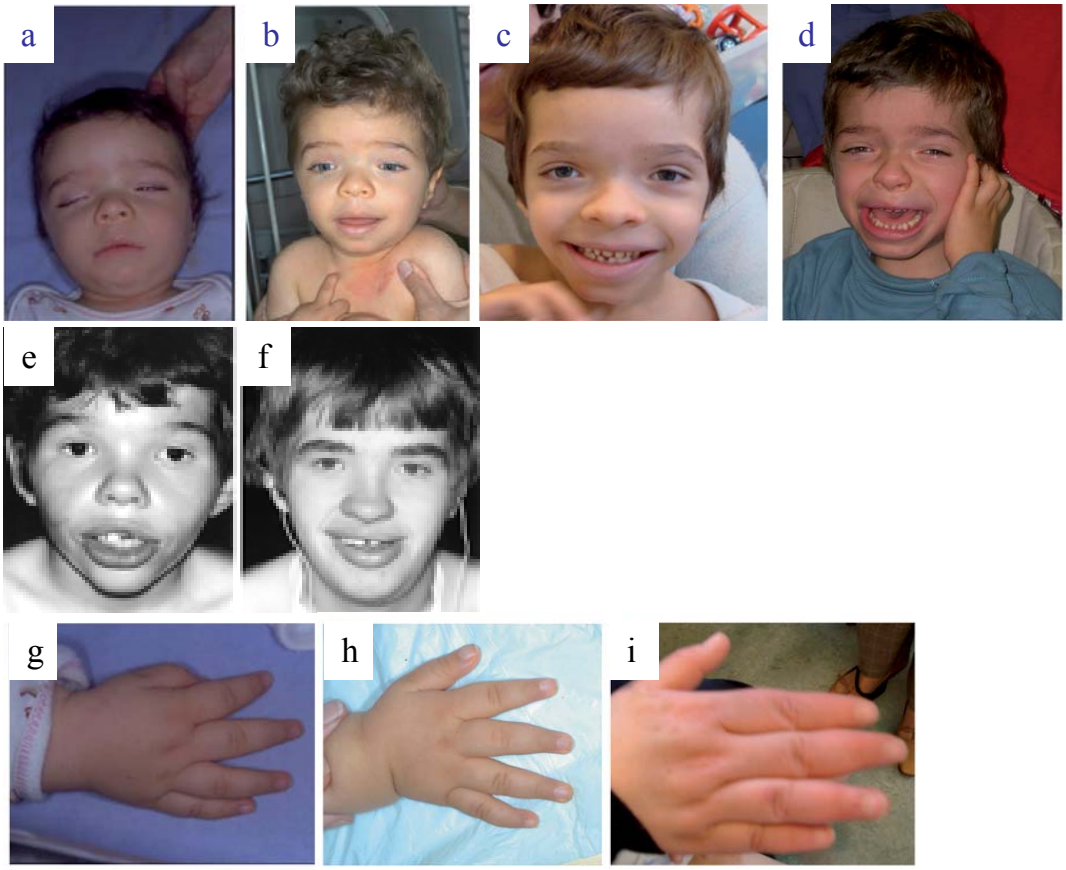


Figure 2: Morphology and Characteristics of the CLS patients.

(a–d) Facial views of a boy with CLS at different ages showing evolution during infancy of facial gestalt. **(a)** At 9 months, **(b)** at 18 months, **(c)** at 3 years, and **(d)** at 6 years. Note the large forehead, hypertelorism, downslanting Palpebral fissure , long philtrum, anteverted nares, and thick lips. This boy carries an RPS6KA3 intra-genic duplication previously reported. **(e–f)** The facial dysmorphism becomes more pronounced with age. **(g-i)** Views of the hands of the same patient. Note the typical broad tapering fingers **(g)** at 9 months, **(h)** at 18 months, and **(i)** at 5 years (Adapted from, Pereira et al., 2010 and Hanauer and Young, 2002).

2.4. Clinical expression in female:

Some of the physical features seen in males may also be present in female. However, they are neither as prominent as in males nor as constant. Carrier females usually exhibit a slight facial dysmorphism, small fleshy hands with tapering fingers and a tendency to obesity **(Figure 3)**. Cognitive impairment may not be present at all. However, some heterozygous women have been reported to have difficulties at school. A few were

suffering from depression, psychotic behavior and schizophrenia. Psychiatric disorder appeared around the age of 20 years and response to therapy was variable (Hanauer and Young, 2002).

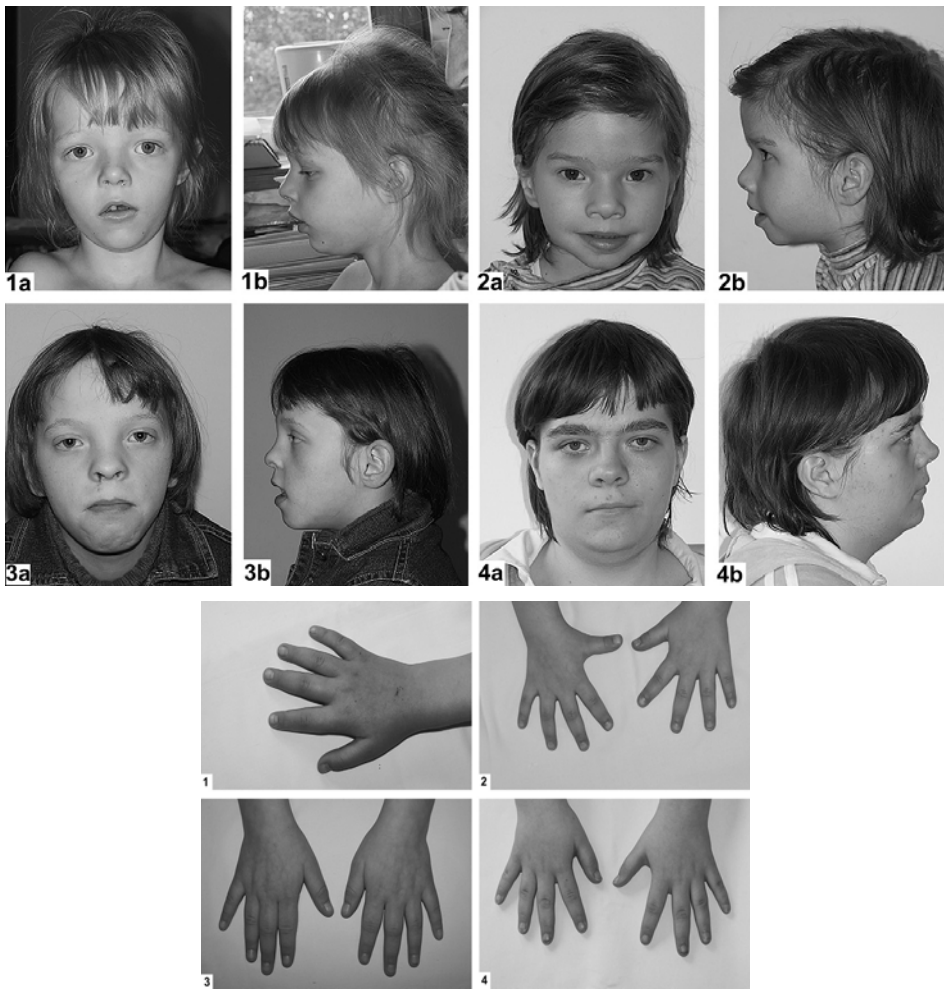


Figure 3: Facial and hand views of CLS patients in women.

Photographs **(1-4)** of four female patients suffering from CLS, showing clinical symptoms of varying severity. A prominent forehead, hypertelorism and small fleshy hands with tapering fingers can be observed. (Adapted from Jurkiewicz et al., 2010).

2.5. Diagnosis of CLS patients:

Since, the clinical findings can be very variable both in severity and in terms of features, the diagnosis based on clinical criteria is often difficult to establish. On the other hand, although some clinical signs are usually quite suggestive in adult patients, they are

much less at birth, in young children or women. There is also a great similarity of the symptoms of CLS with other syndromes, such as alpha-thalassemia with ID (ATR-X) or Williams Syndrome and Pitt-Hopkins syndrome. On the other hand, the high proportion of sporadic cases does not facilitate the diagnosis. In fact, 70-80% of patients have no family history. Thus, in most cases, the diagnosis requires molecular analysis (screening for mutations in the *Rsk2* gene) in order to confirm it by the identification of the genetic defect (Pereira et al., 2010).

2.6. Treatment and Life expectancy:

There is no specific treatment currently available for the CLS. However, rapid diagnosis can allow early regular monitoring of patients. Treatment for individuals with CLS who experience drop attacks includes medication such as volporate and clonazepam or selective serotonin uptake inhibitor. When symptomatic treatment are introduced very early, clinical complications associated with symptoms such as deafness, seizures and heart or/and orthopedic problems can be limited (Pereira et al., 2010).

Life expectancy of CLS patients is lower than the normal population. In 13.5% of CLS male patients and 4.5% of carrier females death occurred between 13 and 34 years. (Mean age of 20.5 years) (Pereira et al., 2010). The reported causes of death are heart abnormalities (cardiomyopathy), pneumonia and surgical complications due to general anesthesia (Hanauer and Young, 2002).

2.7. Molecular and Genetics Basis of CLS:

CLS is caused by the loss of function mutations in the *RPS6KA3* gene, which maps to Xp22.2. The coding region of this gene is split into 22 exons and encodes a serine/threonine kinase: RSK2 (ribosomal S6 Kinase2) (Jacquot et al., 1998a). Over 140 distinct mutations associated with CLS have so far been listed in this gene (Pereira et al., 2010). This number is constantly growing; a list of all these described mutations is available online: (http://www-ulpmed.u-strasbg.fr/chimbio/diag/coffin). Mutations are distributed throughout the gene, with no clustering and the vast majority is unique to a single family. Approximately 30% of mutations are missense mutations, 15% nonsense

mutations, 20% splicing errors, and 30% short deletion or insertion events (Delaunoy et al., 2006) (**Figure 4** also explain the type of mutation). About two-thirds of these mutations lead directly or indirectly to premature translation termination, resulting in complete loss of function of the mutant allele. Misssense mutations often alter the catalytic activity of the RSK2 protein. (Delaunoy et al. 2006; Delaunoy et al. 2006; Jurkiewicz et al., 2010).

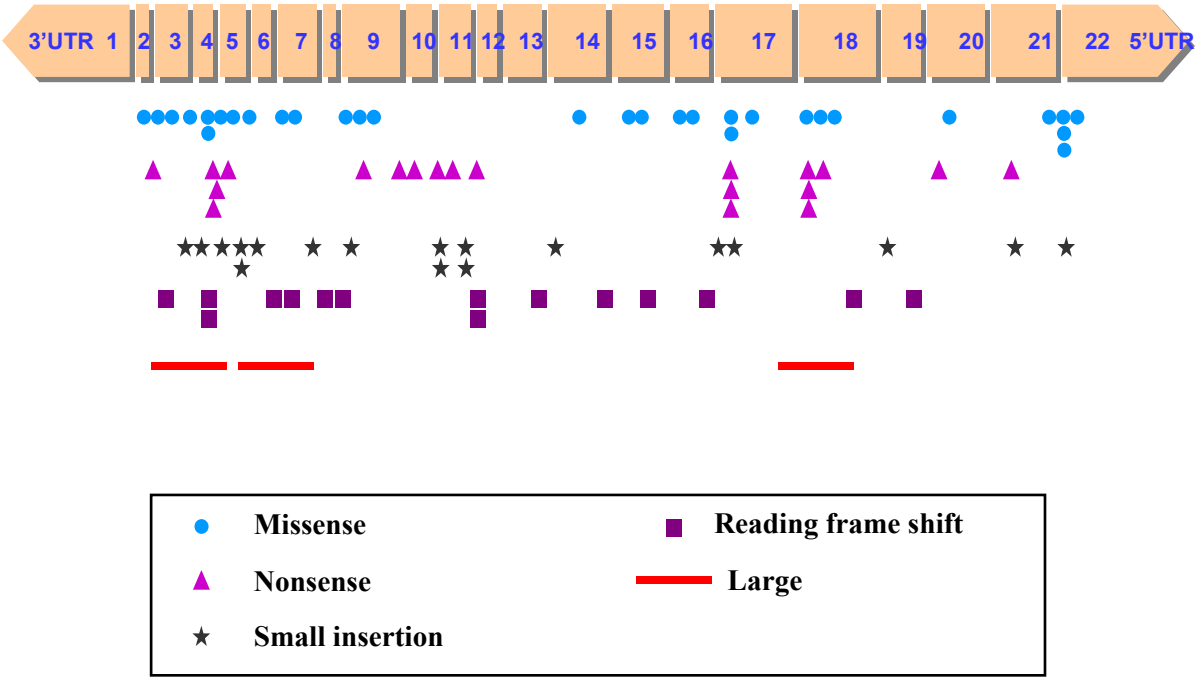


Figure 4: *Rsk2* gene structure and spectrum of mutations.

The *Rps6ka3* gene comprises 22 exons, represented by rectangles numbered 1 to 22. Since the identification of this gene, over 140 mutations associated with CLS have been recorded and this number is constantly growing. The distribution of mutations (as identified in 2002) is shown above (Adapted from Hanauer and Young, 2002).

Strikingly, two thirds of the mutations were de novo. This very high proportion of new mutation is not common in an X-linked disease and is still unexplained (Hanauer and Young, 2002).

2.8. Genotype/Phenotype Relationship:

No obvious correlation exists between phenotype and location or type of *RPS6KA3*

mutation. However, individuals with certain missense mutations, leading only to partial loss of kinase activity, tend to have milder disease expression (Delaunoy et al., 2001). For example in one family classified as having a form of non-syndromic intellectual disability segregated a missense mutation, which caused only a 80% reduction in ribosomal S6 kinase enzyme activity, in contrast the majority of mutations in CLS patients that cause a total loss of ribosomal S6 kinase enzyme activity (Merienne et al., 1999). Some other similar cases were subsequently reported suggesting an essential role of residual enzyme activity in determining severity of symptoms. However, this observation cannot be extended to all known missense mutation. It is also interesting to note that phenotypic expression variability within the same family was found in some families (Hanauer and Young, 2002). Taken together, available data suggest that the mutation is not the only factor leading to the phenotype but that other genes and environment factor are involved in this disease.

It should also be mentioned here that in 2005, a study suggested that truncating mutations, either in, or upstream of the N-terminal kinase domain, might lead to susceptibility to stimulus Induced Drop Attacks (SIDAs). (Nakamura et al., 2005). However, this result could not be confirmed in the series of CLS patients analyzed in Strasbourg.

3. The RSK Proteins:

RSK proteins are serine/threonine kinases, implicated in important and ubiquitous signaling processes. RSKs constitute the protein ribosomal S6 kinase (RSKs) family. RSK proteins are serine/threonine kinases acting at the end of the signaling Ras-ERK/MAPK pathway and are actived by phosphorylation directly by ERK1/2 kinase in response to extracellular stimulation by growth factor or neurotransmitters.

RSK proteins play a key role in several significant cellular processes including cell growth and division, cell differentiation, cell cyclic, gene expression and apoptosis (Kang and Chen, 2011).

3.1. Discovery of RSKs proteins:

The first RSK protein was purified in 1985 in *Xenopus laevis* oocytes and identified as a serine proteins kinase (Erikson and Maller, 1985, 1986). Subsequently, RSKs proteins have been identified in several vertebrate and invertebrate organisms. These include drosophila melanogaster, C. elegan, chickien, rabbit, rat, mouse, and human. Whereas in C. elegans, chicken, and rabbit, only one RSK is expressed (Alcorta et al., 1989; Wassarman et al., 1994), in mammals four distinct RSK proteins have been identified, referred as RSK1-4 (Moller et al., 1994). The size of these protein kinases are very similar, they are made up of 735, 740, 733 and 745 amino acids respectively, and have a molecular weight of about 90kDa. Their amino acid composition is also very similar; they share 75-80% sequence identity. The four RSK family members are encoded by distinct genes (official names *RPS6KA1, 2, 3, 4*) mapping to chromosomes 3 (*Rsk1*), Xp22.2 (*Rsk2*), 6q27 (*Rsk3*), and Xq21 (*Rsk4*) (Hanauer and Young, 2002).

3.2. Expression profile of RSKs proteins:

In humans and mice, RSKs proteins are widely expressed, and different RSK proteins may be co-expressed in some tissues and organs. However, as stated below, their expression profiles may be different, suggesting specific functions for each of these kinases.

3.3. Expression profile in humans:

The expression of *Rsks* mRNAs was investigated in various adult human tissues by Northern blot by Zeniou et al., 2002. RSKs are all widely expressed with, however, variability in the levels. Results revealed a strong expression of *Rsk1* in the kidneys, lungs, pancreas, and in the cerebellum. Expressions of *Rsk2* and *Rsk3* are strong in skeletal muscle, heart and pancreas. While *Rsk2* messenger is strongly detected in the cerebellum, the frontal lobe and the occipital pole, that of *Rsk3* is predominant in the medulla. The transcripts corresponding to *Rsk4* were mainly found at high levels in the kidneys and brain **(Figure 5)**.

Another study examined *Rsk2* expression during embryonic development (Zeniou et al., 2002; Guimiot et al., 2004). It revealed that expression of RSK2 is strictly regulated,

spatially and temporally, during embryogenesis. Indeed, after being highly expressed in many parts of the brain (hippocampus, ventricular zone) in the lungs and liver at nine weeks of fetal life, the expression of *Rsk2* decreases at the thirteenth week of embryogenesis.

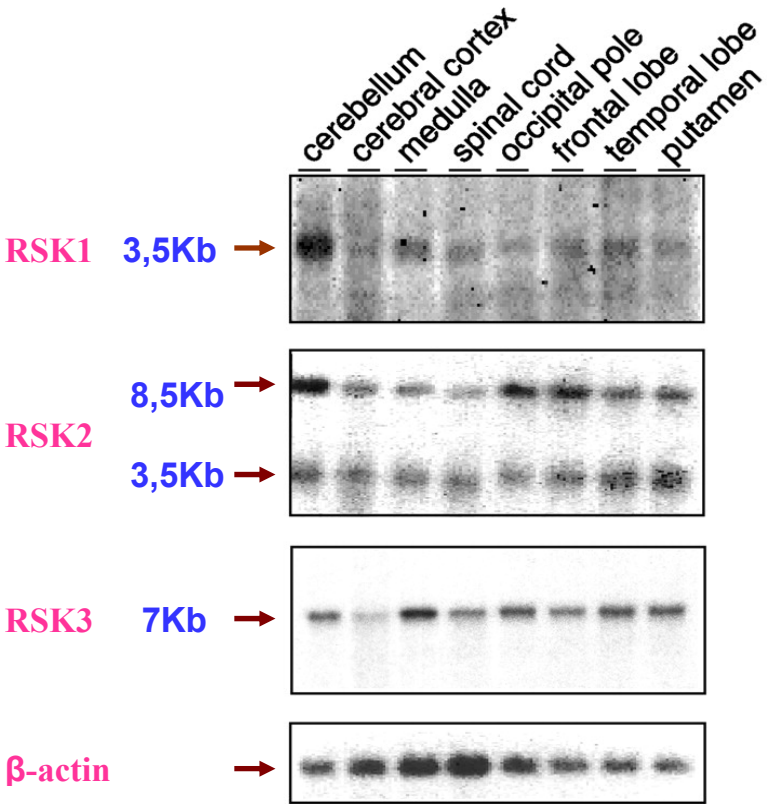


Figure 5: Northern blot analysis of *Rsk1*, *Rsk2* and *Rsk3* expression.

Rsk1, *2* and *3* have been identified in all tissues analyzed by northern blot. However, stronger levels of expression of *Rsk1* and *Rsk2* were found in the cerebellum. In all structures, two *RSK2* transcripts were visible. *RSK3* showed strongest expressed in the medulla (Adapted from Zeniou et al., 2002).

3.4. RSK1, RSK2 and RSK3 expression in adult mouse brain:

In adult mouse brain, RSK1 was primarily detected in the granular cell layer of the cerebellum, whereas RSK2 showed the strongest expression in the hippocampus, an essential structure in learning and memory (C1-C3) (Zeniou et al. 2002). In addition, high

levels of *RSK2* mRNA were also detected in the neocortex, and purkinje cell layer and some deep nuclei of the cerebellum. Darcq et al. (2011) reported also substantial RSK2 expression in the habenula. RSK3 was very highly expressed in the amygdala, the bed nucleus and accumbens nucleus. In the hippocampus, RSK3 was detected in the dentate gyrus (Zeniou et al., 2002), whereas RSK2 is highly expressed in the dentate gyrus and in the CA1-3 areas. *Rsk3* mRNA staining was also seen in external layers of the cortex and several thalamic and hypothalamic nuclei (Zeniou et al., 2002) **(Figure 6)**.

These studies showed that in particular RSK2 is highly expressed in humans and mouse brain regions with high synaptic activity. These regions are key structures in the process of learning and memory. The expression pattern of RSK2 suggested a function of this gene in cognitive processes.

3.5. Sub-cellular localization of RSKs:

Localization of RSK proteins is also regulated at the sub-cellular level. They are localized mainly in the cytoplasm at the baseline state. After mitogenic activation, a portion of phosphorylated RSK molecules translocates into the nuclear compartment (Chen et al., 1992). The exact mechanism of the translocation is not yet defined. A NLS sequence (core: X-Lys-Lys-Leu-Arg-Arg-Lys-Ser-Arg,) has been identified in RSK3 (Zhao et al., 1995), but not in the other RSK family members. However, there is no evidence so far that this putative NLS is used in the transport of RSK3 to the nucleus (Anjum and Blenis, 2008). Recent studies have localized RSK proteins also to the membrane and especially at the presynaptic compartment (Zeniou-Meyer et al., 2008) and post-synaptic densities of dendritic spines (Thomas et al., 2005). Together, the data suggest that RSK kinases play a role in the whole cell.

3.6. Structure-Function Relationship of RSKs Proteins:

Comparison of the RSK structure and sequences to those of other serine/threonine

kinases, revealed that the four RSK family members have an unusual structure that they share only with MSK kinases (Hauge and Frodin, 2006; Jones et al., 1988).

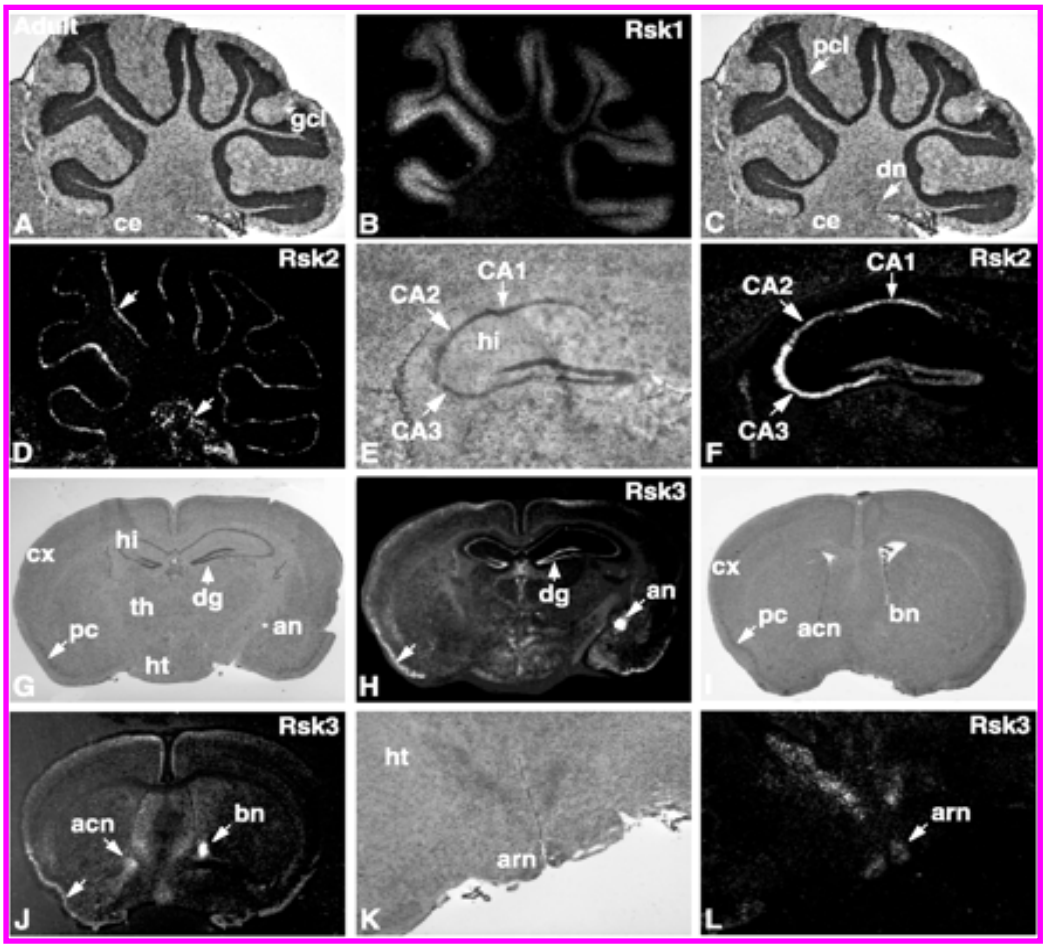


Figure 6: Expression profile of RSKs in the adult mouse brain, obtained by in situ hybridization.

(A–F) Sagittal sections of adult brain. **(B)** *Rsk1* expression was primarily observed in the granular cell layer of the cerebellum. **(D and F)** *Rsk2* expression in the Purkinje cell layer and the deep nuclei of the cerebellum (arrows in **D**) and the hippocampus (arrows in **F**), respectively. The weak staining observed in the dentate gyrus was not specific. **(G–L)** Frontal sections of adult brain. **(H)** Arrows show *Rsk3* expression in the dentate gyrus, the amygdala and the pyriform cortex (Adapted from Zeniou et al., 2002).

In fact, they have two functionally distinct kinase domains separated by a linker region. The N-terminal kinase domain (NTK) belongs to the AGC kinase family and is responsible for phosphorylation of substrates. The C-terminal kinase domain (CTK)

belongs to the CamK family and its only known function is the activation of the NTK.(Anjum and Blenis, 2008; Frodin et al., 2002; Hauge and Frodin, 2006.) **(Figure 7A)**.

The N-terminal kinase domain (NKTD) is highly homologous to other kinases of the AGC family, such as PKG, PKC, PKA or p70S6K (Bjorbaek et al. 1995b; Anjum and Blenis, 2008). The NKTD phosphorylates RSK proteins substrates by recognizing a consensus motif (RxRxxS or RRxS) (Flotow and Thomas, 1992; Leighton et al., 1995). This same pattern is also recognized by the p70S6K. These two families of proteins kinases share several substrates.

The three-dimensional structures of the NTKD of RSK1 and CTKD of RSK2 have been resolved recently by Ikuta et al., (2007) and Malakhova et al. (2008). Each of these two domains contains a binding site for ATP. The N-terminal region of each domain is composed mainly of β sheets, while a majority of α helices form the C-terminal region. The resolution of the structure and CTKD and NTKD allowed the authors to propose a model for the regulation of the RSK enzymatic activity. Malakhova et al. (2008) postulated that the location of the helix between helices αF, αL and G prevents activation of RSK2. Following binding of ERK at the C-terminus of the RSK protein, the hydrogen bond Tyr707-Ser603 (stabilizing the position of the helix αL) is broken, leading to the relocation of this helix. This event allows the activation of CTKD by repositioning the helix αD, alignment of the Glu500 residue with the ATP binding site (this residue is indeed essential for the Binding of these molecules) and rearrangement of the loop T opposite to the catalytic site **(Figure 7C)** NTKD and CTKD of RSK proteins domains are connected by a 100 amino acids called "linker" (Pereira et al., 2010). Phosphorylation of Ser386, in the linker, generates a docking site that recruits 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Hauge and Frodin, 2006). The binding of the PDK1 protein is essential for the activation of the NTKD.

An ERK-docking motif known as the D domain (Leu-Arg-Gln-Arg-Arg) (Roux et al., 2003) is present at the C-terminus. Binding of ERK to this motif is necessary for the activation of RSK proteins. Thomas et al., (2005) identified another region present in all RSKs at the C-terminus of the protein. This sequence (S-T-X-L, where X is an amino

acid) allows the binding of RSKs to the PDZ domain, an interaction domain found, in particular, in many synaptic proteins **(Figure 7A)**.

3.7. Activation mechanism of RSKs proteins:

Activation of RSKs protein is a complex mechanism due to its multiple, sequentially regulated phosphorylation sites, the existence of several interacting proteins and its unique two-kinase domain nature. Following the extracellular stimulation of the cells with a growth factor, or a neurotransmitter and binding of phosphorylated ERK on its docking site, RSK is sequentially phosphorylated at six threonine and serine residues; these phosphorylation events being directly or indirectly initiated by the activation of the ERK/MAPK cascade (Dalby et al., 1998). Four of these sites (Ser221, Ser363, Ser380 and Thr573) are more important in regulating the kinase activity of RSK1-4 **(Figure 7B)** (Chung et al., 1991; Smith et al., 1999). Upon mitogenic signals, the activated ERK is recruited on a docking site at the C-ter of RSK, where it can phosphorylate RSK2 at Thr577 in the CTK domain and also at Ser369 and Thr365 in the linker region. After phosphorylation of Thr577 the activated CTK domain autophosphorylates Ser386 in the linker region, which becomes a docking site for 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Frodin et al., 2000). PDK1 phosphorylates then Ser227 in the activation loop of NTK domain, which triggers transpphosphorylation activity of the NTK towards substrates. Dummler et al, in 2005 reported that RSK4 does not follow the general RSKs activation model. PDK1 is not necessary for the RSK4 activation. In the last step, activated RSK2 is auto-phosphorylated at Ser737 via the NTKD, which leads to disconnection of ERK from RSK.

Recent, studies have suggested that RSK2 needs tyrosine phospholyation to facilitate ERK binding to RSK2 and disruption of an auto-inhibitory region necessary for full activation (Kang et al., 2008; Kang et al., 2009). A diagrammatic model for RSK2 activation is given in **(Figure 8)**. Moreover, some other factors are also involved in RSK protein activation mechanism, such as MK2, MK3, ERK5, FGFR3 as well as some phosphatases (Anjum and Blenis, 2008). Indeed, in dendritic cells, RSK can be activated by

the p38/MAPK signaling pathway via two proteins, MK2/3, which are homologous to the RSK CTKD (Zaru et al., 2007).

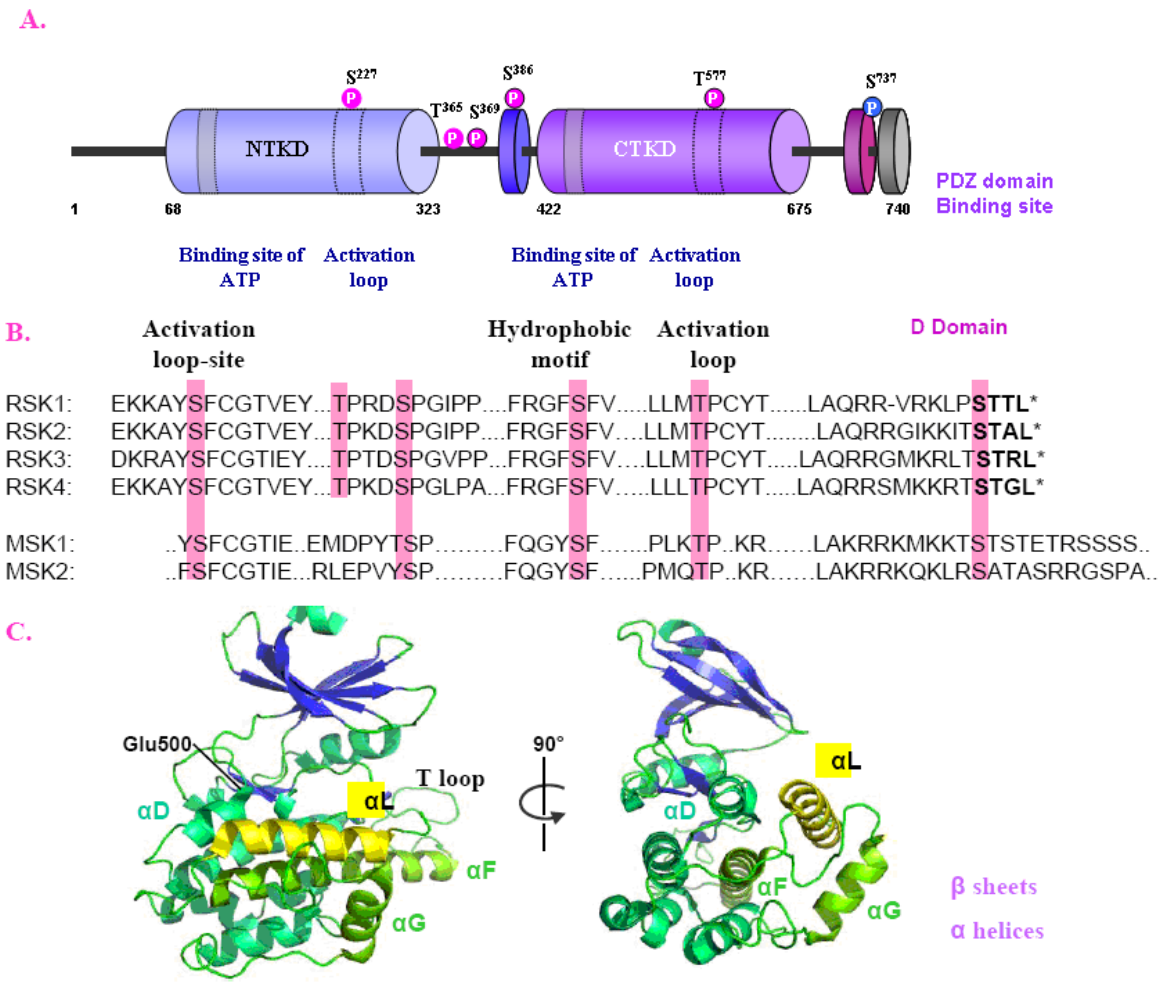


Figure 7: RSKs schematic structure of proteins and identification of its 3D structure.

(A) RSK proteins have two kinase domains (NTKD and CTKD) connected by a linker region. Activation of RSK is associated with increased phosphorylation at six sites (each shown as a coloured circle). The kinase domains have a binding site for ATP and an activation loop. The linker region contains a hydrophobic motif for the binding of PDK1. At the C-terminus, two highly conserved regions are observed, the D domain, the ERK docking site, and a PDZ binding domain. Activation of RSKs is associated with increased phosphorylation of Ser and Thr residues, (pink closed circles). Phosphorylation of Ser737 (light blue) leads to ERK dissociation. **(B)** The different phosphorylation sites are highly conserved among RSKs and MSKS (Rose color). The PDZ binding sequence (S-T-X--L shown in bold) is also conserved in the RSKs. **(C)** Three-dimensional representation of the RSK2 CTDK. A majority of β sheets and α helices compose its N and C-terminus, respectively. The auto-inhibitory helix αL is between helices αG and F (Adapted from (Anjum and Blenis, 2008; Hauge and Frodin, 2006; Malakhova et al., 2008).

Ranganathan et al., (2006) also showed that ERK5 as well is able to phosphorylate RSKs. This activation requires binding of the "common domain" of ERK5 with the domain D of these kinases. A study in the laboratory of Chen has identified Tyr529 as another important site for the activation of RSK. Indeed, phosphorylation of this residue by FGFR3 allows the recruitment of ERK at the domain D and thus the activation of the protein (Kang et al., 2007). In 2009, the same group reported that phosphorylation of Tyr707 by FGFR3 is involved in activation of RSK2 CTKD, probably by disruption of a self-inhibitory left handed helix (αL) (Kang et al., 2009).

In addition to the kinases, the activity of the RSK proteins may also be regulated by protein phosphatases. The combination of PP2Cδ with the N-terminus of RSK2 leads, for example, to a decrease in its kinase activity (Doehn et al., 2004). Another group described an interaction between PP2Ac and RSK1, and observed PP2A phosphatase activity by measuring dephosphorylation (Chaturvedi et al., 2009). RSK activated proteins can phosphorylate various substrates both cytosolic and nuclear, and thus participate in important cellular events such as neuronal development, regulation of transcription, proliferation, gene expression and cell survival (Anjum and Blenis, 2008; Kang and Chen, 2011). MSK1 and -2, two kinases that are highly homologous to RSKs, can be phosphorylated by both the MAPK/ERK and the p38/MAPK pathways (Hauge and Frodin, 2006).

Before detailing the regulation of different cellular processes mediates by RSKs, I am going briefly to discuss the MAPKs signaling pathways, which are responsible to activate the RSKs protein.

4. Signaling molecules involved in activation of RSKs proteins:

4.1. The MAPKs signaling pathways:

To be functional, a cell must be able to receive extracellular signals from other cells and integrate them. To this end, cells have developed sophisticated signaling pathways to

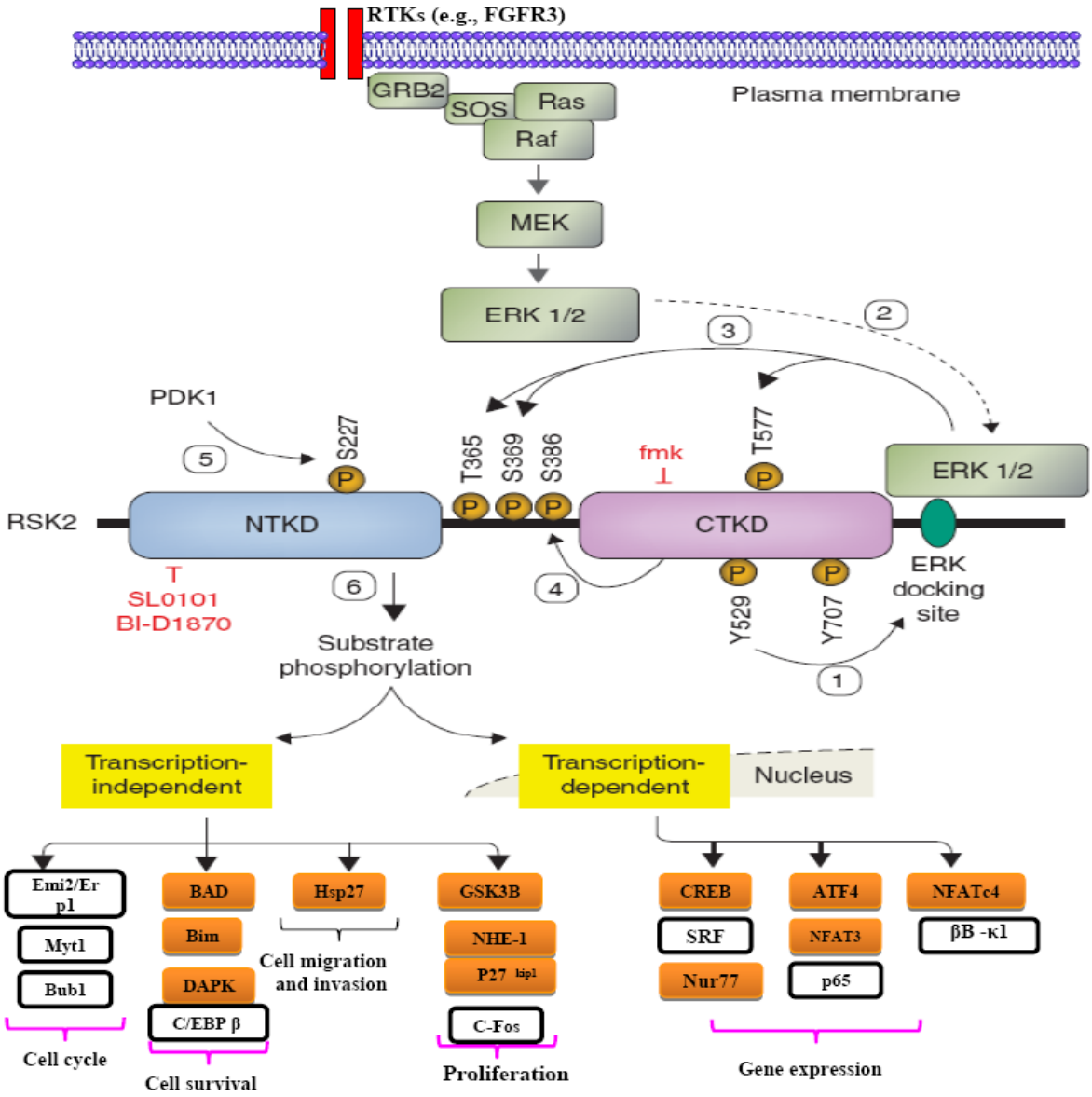


Figure 8: A schematic model for RSK2 activation.

RSK2 have two different kinase domains, the CTKD is responsible for autophosphorylation at Ser386 that is critical for RSK activation, while the NTKD phosphorylates downstream RSK2 substrates. The inactive ERK binds to the C-terminus of RSK2 in quiescent cells, which is necessary for RSK2 activation. Phosphorylation of Y529 and Y707 facilitates inactive ERK binding to RSK2 and disrupts an auto-inhibitory region. When activated, ERK phosphorylates RSK at three sites, T365, S369 and T577 to activate the CTKD, leading to auto-phosphorylation at S386 in the linker region, which provides a docking site for PDK1. PDK1 then phosphorylates S227 in the NTKD and fully activate RSK2. Activated RSK2 remains in the cytosol or translocates to the nucleus, and phosphorylates several substrates to regulate gene expression, cell cycle, cell survival and proliferation. Identified RSK2 substrates are in orange boxes and RSK family substrates are in white boxes. RSK specific inhibitors are marked in red (Adopted Kang and Chen, 2011).

receive extra-cellular signals at the membrane, to transmit the information to various intracellular effector proteins and finally to respond to these stimuli. The vast majority of these signal transduction mechanisms are dependent on post-translational modifications such as phosphorylation. Theses signaling pathways, allowing enzymatic amplification of the signal, provide mechanisms for the integration of different inputs and allow a diverse array of cellular responses to take place. Mitogen-activated protein kinases (MAPKs) are Ser/Thr kinases that convert extra-cellular stimuli into a broad range of cellular responses.

The MAPK pathway is a cascade of proteins in the cell that transmits signals received by a receptor at the surface of the cell to various cytosolic and nuclear effectors, necessary to perform the numerous functions of the cell. The signal initiates when a growth factor binds to a specific receptor on the cell surface and ends when a cytosolic or a nuclear effector is activated and produces some changes in the cell, leading for instance to cell division. The MAPKs phosphorylate many substrates; including members of a family of protein kinases termed MAPK activated protein kinases (MAPKAPKs). This family includes the RSKs, MSKs, MNKs, MK5 and MK2/3. MAPKAPKs (Arthur, 2008; Buxade et al., 2008; Carriere et al., 2008; Gaestel, 2008; Perander et al., 2008; Ronkina et al., 2008; Roux and Blenis, 2004). The MAPKAPK family represents an extra enzymatic and amplification step in MAPK catalytic cascades. MAPKs pathway are ancient and extensively studied signal transduction pathways, which are used in many physiological processes (Widmann et al., 1999). MAPKs are serine/ threonine protein kinases ubiquitously found in eukaryotes. They are involved in important cellular processes such as proliferation, differentiation, cell survival, mitosis, metabolism, motility; regulate gene expression and apoptosis (Cargnello and Roux, 2011; Dhillon et al., 2007; Torii et al., 2006).

The mammalian MAPKs consist of three kinases families, including extracellular signal regulated kinase (ERK), (p38) and c-Jun N-terminal kinase (JNK). Each of these families is represented by several isoforms: eight ERK isoforms, (ERK1-8), four p38 isoforms (p38α, β, γ, δ) and three JNK isoforms (JNK1/2/3) have been reported in the literature (Bogoyevitch and Court, 2004; Bogoyevitch et al., 2010; Cuadrado and Nebreda, 2010; Kim and Choi, 2010). Splicing variants have also been identified, including for example ERK 1b and 1c (Raman et al., 2007).

There are three main kinases acting in the MAPK pathways, including MAPK, MAPK kinases (MAPKK), and MAPKK kinases (MAPKKK). The MAPKKK is activated by a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. The activated MAPKKK phosphorylates and activates then the MAPKK, which in turn phosphorylates and activates the MAPK through dual phosphorylation at Thr and Tyr residues within a conserved Thr-X-Tyr motif situated in the activation loop of the kinase domain. (Kim and Choi, 2010; Cargnello and Roux, 2011). MAPKs can then activate many cytosolic and nuclear substrates, by phosphorylating Ser/Thr residues located in proline rich regions (Raman et al., 2007). Diagrammatic representation of the MAPKs module, which leads to activate downstream targets are shown in **(Figure 9)**.

To be able to transmit the signal, the various MAP Kinases must be close to each other, because the cascade of phosphorylation requires interaction between the different kinases. These interactions are stabilized by formation of a protein complex. The formation of such a complex requires the use of scaffolding proteins (Morrison and Davis, 2003). Activation of the MAPK/ERK pathway is, for example dependent on KSR. Indeed, it has been shown that KSR1 allows the localization of MAPKK/ MEK with the activated form of Raf-1 at the plasma membrane, forming a docking area for MAPK/ERK. KSR1 in this manner facilitates the phosphorylation cascade leading to ERK activation (Cacace et al., 1999, Müller et al., 2000).

The three MAPK families are activated by different intra and extra cellular stimuli. The JNK and p38 signaling pathways are activated by pro-inflammatory cytokines such as tumor necrosis factor (TNFα) and interleukin (IL-1β) or in response to cellular stresses such as genotoxic, osmotic, hypoxic, or oxidative stress, whereas the Ras-ERK pathway is mainly stimulated by growth factors, hormones and neurotransmitters (Raman et al., 2007, Kim and Choi, 2010).

Finally, MAPKs regulate a broad range of biological functions and control a vast array of physiological processes.

4.2. The ERK1/2 MAPKs pathway:

Extracellular signal-regulated kinase 1 (ERK1) was the first mammalian MAPK to be identified, followed by ERK2. ERK1 and ERK2 share 85% amino acid residues identity (Lopez-Bergami, 2011). ERK1 has a molecular weight of 44 kDa and ERK2 42 kDa. ERK1/2 are ubiquitously expressed, with the highest levels in brain, heart, thymus and skeletal muscle (Boulton et al., 1990).

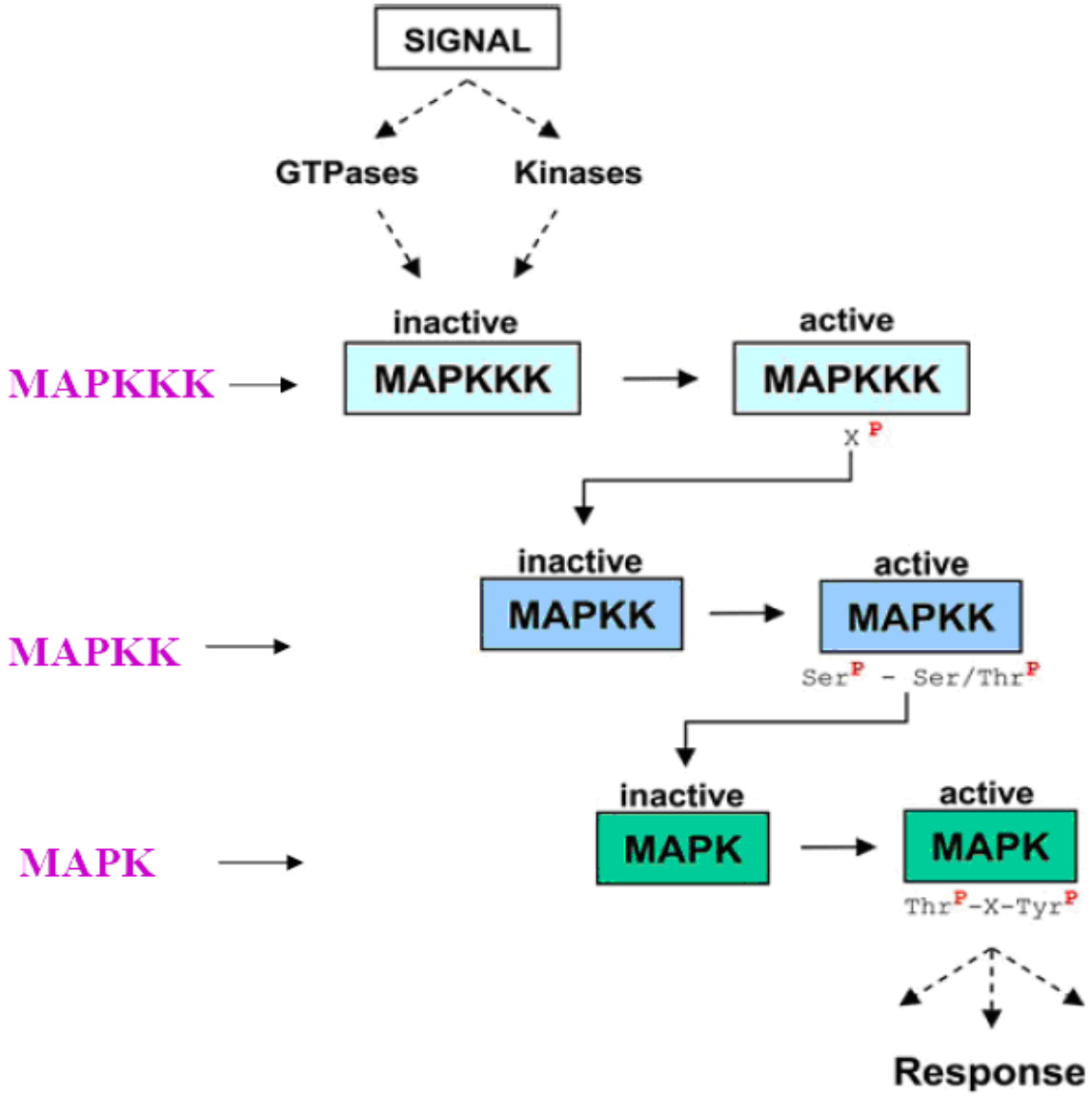


Figure 9: The MAPKs module, leading to activation of downstream targets.

The MAPK unit consists of three kinases: a MAPKKK, a MAPKK and a MAPK. Different MAPK cascades can be activated through various signals. (Adopted from Krens et al., 2006).

ERK1/2 are activated by growth factors, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF) (Boulton et al., 1990).

In addition, ERKs can also be activated by cytokines, osmotic stress, virus infection, and ligands for heterotrimeric G protein-coupled receptors (Raman et al., 2007). Moreover, it was shown that transforming agents and carcinogens could also activate ERK1/2 (Johnson and Lapadat, 2002). The ERK1/2 module in mammals includes A-Raf, B-Raf and Raf-1 (MAPKKKs), MEK1 and MEK2 (MAPKKs) and ERK1 and -2 (MAPKs). Receptor-linked tyrosine kinases such as, for example, the epidermal growth factor receptor (EGFR) are stimulated by extracellular ligands. Binding of epidermal growth factor (EGF) to the EGFR stimulates the tyrosine kinase activity of the cytoplasmic domain of the receptor. The EGFR becomes phosphorylated on tyrosine residues. Docking proteins such as GRB2 contains an SH2 domain that binds to the phosphotyrosine residues of the activated receptor (Schulze et al., 2005). Then GRB2 binds to the guanine nucleotide exchange factor SOS through the two SH3 domains of GRB2. When the GRB2-SOS complex docks to phosphorylated EGFR, SOS turns into its activated form (Karnoub and Weinberg, 2008; Zarich et al., 2006). Activated SOS then promotes the removal of GDP from a member of the Ras subfamily. Ras then binds GTP and becomes active. Ras activates Raf, which in turn binds to and phosphorylates the dual specificity kinases MEK1 and MEK2, which, as a result, phosphorylate ERK1/2. Now activated ERK1/2 can phosphorylate many cytoplasm and nuclear substrate proteins. (Yoon and Seger, 2006), including RSKs. ERK1/2 can phosphorylate more than 160 proteins (Roberts and Der, 2007; Yoon and Seger, 2006). In the nucleus, ERK1/2 regulate gene expression by phosphorylating several transcription factors such as *Sp1*, E2F, Elk-1, AP-1 , NF-AT , STAT3 and c-Fos etc (Murphy and Blenis, 2006; Yoon and Seger, 2006). For example, Elk1 a member of the TCF family is phosphorylated by ERK at Ser383 and 389 in its C-terminal domain. This phosphorylation increases the activity of Elk-1 DNA binding and facilitates its interaction with the co-activator p300. It was also shown (Li et al., 2003) that this modification stimulates the acetyltransferase activity of the Elk-1-p300 complex that is critical for chromatin remodeling and gene activation. Elk-1 is, implicated in the regulation of immediate-early (IE) genes expression, such as c-Fos (Gille et al., 1995). ERK1/2 stabilize also the c-Fos protein through direct phosphorylation (Murphy et al., 2002), thus permitting c-Fos to bind to c-Jun and form transcriptionally dynamic AP-1 complexes. AP-1 activity is required for expression of cyclin D1 (Shaulian and Karin, 2001) and, thus, allow G1/S transition and

cell cycle progression. ERK1/2 plays an essential role in the control of cell proliferation (Katz et al., 2007). Through numerous mechanisms, including induction of positive regulators of the cell cycle (Meloche and Pouyssegur, 2007). Some studies indicated that ERK would act as a negative regulator for cell proliferation and can induce apoptosis when its activity is highly increased (Nakata et al., 2011). However, Campbell et al. in 2010 reported that a secondary metabolite, TLN-4601, inhibits Ras-ERK signaling and decreases cell viability by increasing apoptosis. Ras/ERK has also been implicated in migration and cell motility by regulating cytoskeleton proteins. Erk1/2 activates by phosphorylation the myosin light chain kinase (MLCK), and active myosin promotes the polymerization of actins fibers (Katz et al., 2007) and the formation of membrane extensions necessary for cell migration. The ERK/MAPK pathway and their target substrates are shown in **(Figure 10)**.

4.3. The p38 MAPK pathway:

The p38 protein belongs to an other important MAPK pathway. Initially four groups identified p38 as a 38kDa protein (p38). They observed quick phosphorylation at a tyrosine residue in response to lipopolysaccharide (LPS) stimulation (Han et al., 1994). The p38 is also known under various other names like CSBP, RK, and SAPK2. This MAPK member is generally more responsive to stress stimuli and shows 50% identity with ERK2 (Lee et al., 1994; Rouse et al., 1994). The p38 MAPK has four isoforms (p38α, β, γ, δ), that are approximately 60% identical in their amino acid sequence. Distinct genes encode the different p38 isoforms, which have a different expression pattern in the cell. The first two isoforms (p38α, β) are ubiquitously expressed whereas the last two (p38γ, δ) have more restricted expression patterns and have some specific functions (Jiang et al., 1996).

Because p38α is expressed at higher levels than p38β, the majority of the published literature on p38 MAPKs refers to the former. In mammals, the four-p38 isoforms are activated by stress and inflammatory cytokines, including UV irradiation, ischemia, and interleukin-1. In addition, the p38 isoforms are also activated via G protein-coupled receptors: Rho family GTPases and Cdc42 (Bagrodia et al., 1995). MKK3 and MKK6 are the MAPKK responsible for p38 MAPK activation (Derijard et al., 1995; Stein et al., 1996).

The p38 isoforms are present in the cytoplasm and nucleus and play a significant role in normal immune and inflammatory responses (Cuadrado and Nebreda, 2010).

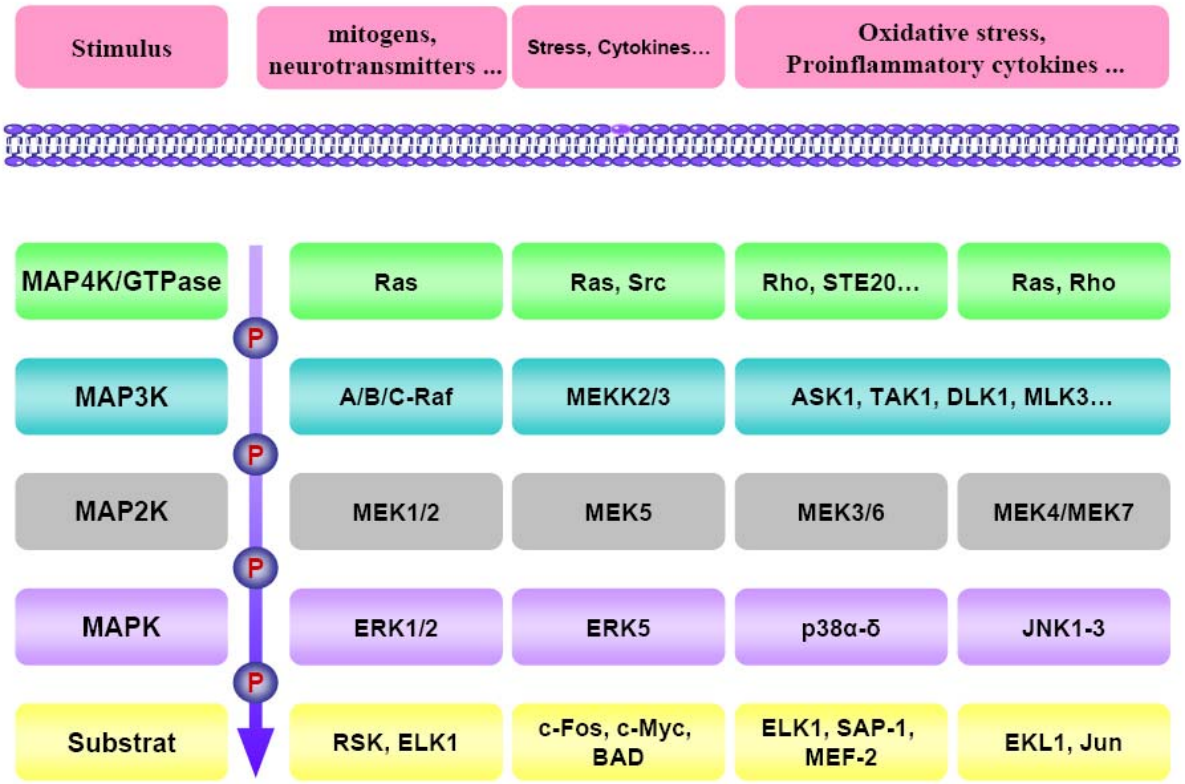


Figure 10: Mitogen-activated protein kinase (MAPK) signaling pathways.

MAPK signaling pathways mediate intracellular signaling initiated by extracellular or intracellular stimuli. MAP3Ks, which are activated by MAP4Ks/GTPases, mediate phosphorylation and activation of MAP2Ks, which in turn phosphorylate and activate MAPKs. The mammalian MAPK family includes ERK, p38, and JNK. In the ERK signaling pathway, ERK1/2 is activated by MEK1/2, which is activated by Raf. Raf is activated by the Ras GTPase, whose activation is induced by RTKs such as the epidermal growth factor receptor. The p38 and JNK pathways consist of a MAP3K such as ASK1, MEKK1, or MLK3 as well as a MAP2K such as MKK3 or MKK6 for the p38 pathway or MKK4 or MKK7 for the JNK pathway (Adapted from Cuadrado and Nebreda, 2010, Kim and Choi, 2010).

The main roles of p38 isoforms is the production of pro-inflammatory cytokines and the regulation of cytokine expression by modulating transcription factor activity, like NF-$_k$B (Karin, 2006). p38 has also a key role in cell proliferation and survival. Activated p38

phosphorylates many substrates in the cytoplasm such as, MNK1/2, MK2/3, phospholipase A2, microtubule associated protein Tau and Bax and nuclear targets including Elk-1, p53, Ets1, ATF2 and NF-$_k$B (Cuadrado and Nebreda, 2010; Kyriakis and Avruch, 2001). Thornton and Rincon in 2009 reported that p38α negatively regulates cell cyclic progression at both G1/S and G2/M transition through a number of mechanisms, including up-regulation of CDK inhibitor and down-regulation of cyclins.

The p38 activity also involves the induction of apoptosis by cellular stresses. These function can be mediated via transcriptional and posttranscriptional modification mechanisms, affecting activity of pro-apoptotic and anti- apoptotic proteins of the Bcl-2 family, and survival pathway (Cuenda and Rousseau, 2007).

Moreover, many reports have established the implication of p38 MAPK in numerous other biological processes, including, chromatin remodeling, protein degradation, mRNA stability, endocytosis, cytoskeleton dynamics or cell migration (Cuadrado and Nebreda, 2010).

4.4. The JNK pathway:

The c-jun N-terminal kinases are also MAPK members. JNKs, also known as stress activated kinase protein (SAKP), were originally purified using c-jun protein bound to beads (Hibi et al., 1993; Kyriakis and Avruch, 1990). It was subsequently found that stress leads to JNK phosphorylation at Thr183 and Tyr185 residues. JNK has three isoforms, JNK1-3.

The three isoforms are more than 85% identical and are encoded by distinct genes, giving rise to more than 10 spliced forms ranging from 46 to 55 kDa (Gupta et al., 1996; Kyriakis et al., 1994). JNK1 and 2 are ubiquitously expressed (Bode and Dong, 2007a), while JNK3 is expressed primarily in neuronal tissues and cardiac myocytes (Bode and Dong, 2007b). JNKs are strongly activated by cellular stress, heat shock, oxidative stress,

UV radition, DNA damming agents, growth factor deprivation and cytokines (Bogoyevitch et al., 2010). Activation of JNK isoforms needs dual phosphorylation at Thr and Tyr residues inside a conserved Thr-Pro-Tyr (TPY) motif in their activation loops. The MAPKKs catalyzing this reaction include MKK4 and MKK7 (Lawler et al., 1998). Following stimulation, activated JNKs relocalize from the cytoplasm to the nucleus (Mizukami et al., 1997). The most important JNK substrate is the transcription factor c-jun. JNK phosphorylates c-jun at Ser63/73 (Weston and Davis, 2002). Some studies revealed differences between JNKs isoforms functions with regard to the regulation of c-jun (Sabapathy et al., 2004). Jaeschke et al., in 2006 and Bogoyevitch et al., in 2010, reported that JNKs are positive regulators of c-jun expression as well as cell proliferation. Several other transcription factors are also phosphosrylated by JNKs, including Elk-1, p53, HSF-1, ATF-2. c-Myc and STAT3. In addition, JNK activity, through c-jun, promotes AP-1 complex formation and transcription of genes including AP-1 binding sites, such as the cyclin D1 gene (Sabapathy et al., 2004). Park et al. in 2007 suggested an anti-apoptotic function for JNK. Indeed, they observed that phosphorylation by JNK of a mitochondrial pro-apoptotic protein, SMAC, prevents the action of the inhibitor of apoptosis, XLAP. This leads to inhibition of the activity of Caspase3 and thus apoptosis. However, the role of the JNK signaling pathway in the processes of cell survival is still controversial. The JNK pathway also regulates molecular mechanisms controlling autophagy (Bogoyevitch et al., 2010). JNKs also participate in the regulation of protein degradation and of the cell cycle, via Mcl-1 and p27kip respectively. Finally, JNKs play a role in other important cellular events including protein degradation, neurodegeneration and cytokines production (Oltmanns et al., 2003; Sherrin et al., 2011).

5. MAPKs cascade signals involved in synaptic plasticity:

5.1. ERK1/2 MAPK and synaptic plasticity:

ERK is the most important neuronal regulator and in the central nervous system, it has a critical role in learning and memory formation. ERK plays a key role in the regulation of neuronal plasticity by direct actions on synaptic effectors or through modifying gene

expression. ERK is also implicated in brain long-term adaptive alteration underlying striatum-dependent behavioral plasticity. In mature neurons, ERK1/2 are activated in synaptic activity, and are crucial for synaptic plasticity associated to learning and memory formation in mammals (Davis and Laroche, 2006). Neuronal ERK1/2 activation in response to direct membrane depolarization or glutamatergic signaling (Zhu et al., 2002), proceeds via a specific way. ERK activation induced via these stimuli is Ras-dependent, but activation of Ras in response to these signals requires calcium influx (Walker et al., 2003). This calcium influx can be made possible by NMDA (N-methyl- D-aspartate) type glutamate receptors or voltage-gated calcium channels. Precisely how high calcium levels trigger Ras activation is yet to be elucidated. Previous studies demonstrated an absolute requirement for ERK1/2 activity in induction of LTP, and more studies that are recent have established its requirement for NMDA dependent and independent forms of LTP induction and maintenance. Thomas and Huganir (2004) have also shown that regulation of AMPA receptor activity is ERK-dependent. Recently Harvey et al., (2008), reported also that glutamate stimulation of a single dendritic spine resulted in continued alteration of the volume of the stimulated spine and subsequent enlargement of adjacent spines that was clearly dependent upon ERK activation. This study argued that such mechanisms might be the basis of the development of LTP. ERKs control synaptic plasticity through their participation to the regulation of transcription and translation. ERK have also newly appreciated functions in memory consolidation. Eckel et al., in 2008 reported, for example, that there is circadian oscillation of ERK activation in the hippocampus, which is necessary for persistent and stable memory formation.

Davis and Laroche, in 2006 reported behavioral analyses on ERK inhibitor treated mice. They demonstrated that ERK activity is crucial for learning and memory tasks (Davis and Laroche, 2006). Mazzucchelli et al (2002) and Selcher et al, (2001) reported ERK1-KO mice showing only a slight behavioral phenotype. However, the mutant animals showed a generalized behavioral excitement phenotype with altered responses to psychostimulant drugs like amphetamine (Engel et al., 2009). On the other hand, ERK1 deficient mice show a paradoxical improvement in a Striatal-based long-term memory task and facilitation of LTP in the accumbens nucleus (Mazzucchelli et al., 2002). Results of another study suggested compensatory action of ERK2 (Kumar et al., 2008). A more extensive behavioral

analysis of these animals is ongoing. Satoh et al., in 2007 reported that ERK2 knockout is embryonically lethal, but studies based on ERK2-hypomorphic mutant mice, in which ERK2 expression was partially (20–40%) reduced, showed a significant deficits in long-term memory. Moreover, in 2008, Samuels and co-worker reported that mice in which ERK2 was deficient in telencephalic radial glial progenitor's, exhibit intense shortage in associative learning in a fear conditioning assay. A number of studies suggested that ERK1/2 play an important role in the genesis of neural progenitors. They also suggested that ERK1/2 have a death promoting apoptotic roles in neuronal cells. Other studies reported that up regulation of ERK1/2 is critically involved in the pathogenesis of autism (Yang et al., 2011).

It is still controversial whether the ERK isoforms, (ERK1 and ERK2), redundantly share their numerous brain functions and compensate for each other or whether they play distinct roles. In the ERK signaling studies, most experiments use inhibitors of the upstream kinase, MEK. ERK1 and 2 are both activated by MEK, and thus, it is difficult to examine the specific contribution of each isoform to physiological functions. All previous reports suggested that even a small dysregulation of ERK function is adequate to result in behavioral impairment. Finally, the various neuro-modulators effects on LTP and LTD induction depend on ERK signaling. Indeed, ERKs are needed for long-term memory formation across a variety of tasks.

6. Regulation of cellular processes by RSKs:

Since initial identification of RSK in xenopus, many proteins have been identified as substrates for RSK kinases. These proteins have diverse biological functions in the cell and may reside and function in different sub-cellular compartments, including the plasma membrane, the cytosol and the nucleus. The substrate specificity of RSKs has been determined with a synthetic peptide library, and is defined by the motifs that are mentioned above in this manuscript. However, these sequences are also recognized by other protein kinases such as AKT/PKB, S6K1/2 and MSKs (Anjum and Blenis, 2008). A number of RSK functions can be assumed from the nature of RSKs substrates. Some reported data

suggest that RSKs are, in particular, implicated in transcription and translation regulation, as well as in synaptic transmission. Overviews of a diagrammatic representation of RSK substrates are shown in **(Figure 11)**.

6.1. RSK mediates the regulation of transcription:

As mentioned earlier in the introduction, activated RSKs phosphorylate numerous transcription factors, some of which contributing to the immediate early genes (IEGs) response or being IEG gene products themselves. Two studies using primary fibroblasts isolated from RSK2 deficient mice and human cells from CLS patients demonstrated that RSK2 mediates mitogen- induced c-Fos transcription (Bruning et al., 2000; De Cesare et al., 1998).

The mechanism proposed included activation of the Elk-1/serum response factor complex and phosphorylation of the cAMP response element-binding protein (CREB) via RSK2. Ginty et al. (1994) and Xing et al. (1996) reported that CREB is phosphorylated by RSK2 at Ser133; However, Wiggin and colleagues in 2002 reported that not RSK2 but MSK is the predominant CREB kinase in somatic cells. However, there data also suggested that RSK and PKA could cooperate with MSK1 and -2 to phosphorylate CREB. The same group reported also that histone H3, previously reported to be phosphorylated by RSK2 at Ser4 (Sassone Corsi et al., 1999) is in fact predominantly phosphorylated by MSK1/2 kinase in response to both mitogenic stimulation and stress. RSK phosphorylates also directly SRF (Rivera et al., 1993), c-Fos (Chen et al., 1993), and Nur77 (Davis et al., 1993; Wingate et al., 2006). In addition, RSK co-operates with the ETS transcription factor ER81 and increases ER81 dependent transcription as a result of the phosphorylation of Ser216 and Ser191 (Wu and Janknecht, 2002). The ER81 factor is implicated in several essential functions including, homeostasis signaling response, and development, potentially connecting RSK with these processes. The transcription initiator factor TIF-1A is also phosphorylated via RSK2 (at two Serine residues) (Zhao et al., 2003), an event which was shown to be necessary for RNA polymerase I transcription and rRNA synthesis. These results suggested that RSK signaling controls growth associated transcription initiation. In addition, RSK phosphorylates ERα at Ser167, thereby enhancing ERα mediated transcription (Yamnik and Holz, 2010).

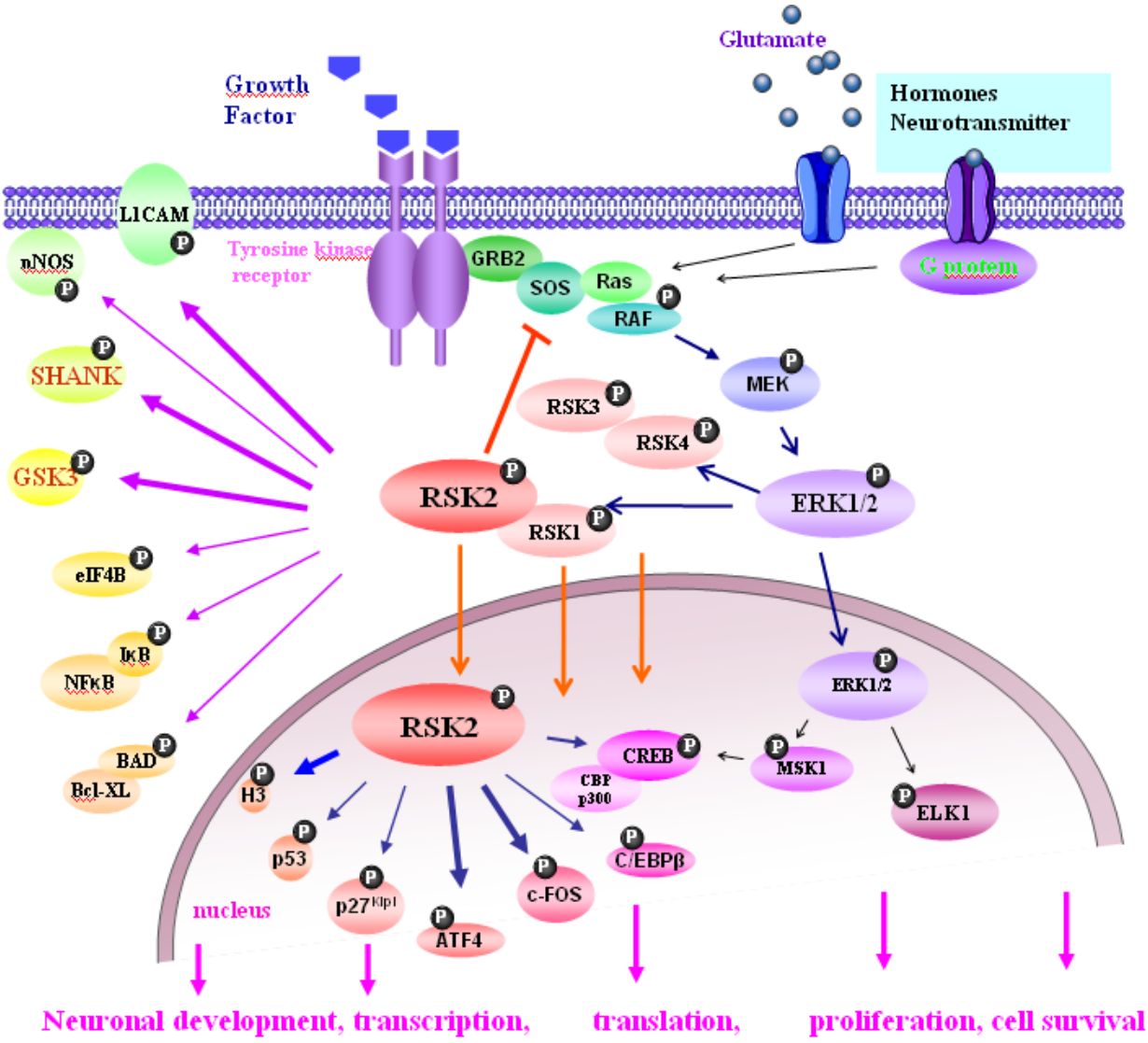


Figure 11: Ras-ERK-RSK/MAPK signaling pathway.

RSKs proteins act at the distal end of the path Ras-ERK/MAPK, which can be activated by growth factors, hormones and neurotransmitters. Once activated, RSKs can phosphorylate various substrates, both cytoplasmic and nuclear, and thus participate in important cellular events such as neuronal development, regulation of transcription, proliferation and cell survival (Adapted from Pereira et al., 2010; Schneider et al., 2011).

Moreover, some studies suggested that other nuclear factors are also regulated by RSKs, including the transcription factor NF-κB. RSK phosphorylates the inhibitory factors IκBα and IκBβ, leading to their degradation and thereby to stimulation of NF-κB activity (Ghoda et al., 1997; Xu et al., 2006H). In addition, the transcription factor, p65, is

phosphorylated at Ser563 by RSK (Bohuslav et al., 2004; Zhang et al., 2005). The role of this phosphorylation event remains still a matter of discussion (Douillette et al., 2006). RSK2 phosphorylates the CREB family member ATF4 (Yang et al., 2004), a transcription factor that is necessary for osteoblast differentiation during development. Indeed, RSK2 protein was found to be required for osteoblast differentiation and function, suggesting a mechanism by which loss of RSK2 activity leads to the CLS related skeletal abnormalities. The transcriptional co-activator CREB binding protein (CBP) has also been recognized as a binding target of RSK1 (Nakajima et al., 1996). Activation of the Ras/ERK pathway promotes interaction between CBP and RSK (Wang et al., 2003), but the precise role of this connection remains to be determined. Some investigations suggested that CBP cooperates with transcription factors that are phosphorylated via RSK1 and RSK2, including CREB, c-Fos, ERα, NF-$_K$B and ER81. These data suggest that RSK may facilitate the recruitment of CBP to promote and regulate these transcription factors.

Together, all the published data support an important implication of RSKs in gene expression. This prompted us to study the transcriptome from *Rsk2*-KO mice and our results further support this implication.

6.2. Regulation of Cell growth and synthesis of protein:

A number of studies revealed the involvement of the Ras/ MAPK signaling pathway in translation regulation, namely by increasing the rates of translation initiation and elongation (Holland et al., 2004). It was also shown that following stimulation of metabotropic receptors in the hippocampus, RSKs translocate into the synaptoneurosomes fraction where they associate with polyribosomes. Once activated, RSK inhibits by phosphorylation the constitutionally active kinase GSK3β. This allows activation of the initiation translation factor eIF2B and thus translation.(Angenstein et al., 1998).

RSK also phosphorylates another important component of the translation machinery, the eukaryotic translation initiation factor eIF4B (Shahbazian et al., 2006). Following phosphorylation of the Ser422 residue, eIF4B is recruited to the eIF3 translation initiation complex **(Figure 11)**. The eIF4B is then able to increase the helicase activity of eIF4E, thereby stimulating the activity of eIF4F. This later protein interacts then with the

initiation complex and facilitates the translation by linking the mRNA and the ribosome. RSK proteins regulate other factors involved in translation, including EF2K and TSC2 (Anjum and Blenis, 2008; Inoki et al., 2006) indicating their crucial role in the mechanisms of protein synthesis (Anjum and Blenis, 2008).

Finally, Ras/MAPK signaling was also found to be involved in the stimulation of activity of mammalian targets of rapamycin (mTOR) via the regulation of the tuberous sclerosis complex. The protein mTOR is a main regulator of cell growth and proteins synthesis, and its activity is controlled through various growth related pathways. RSK can phosphorylate Tuberous sclerosis protein-2 at Ser664 and Ser1798, an event which negatively regulates the Guanine nucleotide activating Protein (GAP) activity of Tuberous sclerosis protein-2 towards the small GTPase Rheb (Rolfe et al., 2005; Roux et al., 2004).

6.3. Cell cycle regulation via RSKs:

The cell cycle is a four-step mechanism (G1, S, G2, and M), leading to division of a mother cell in two daughter cells. All steps in the cell cycle are regulated by many factors; including RSKs. RSK2 was shown to promote G1-phase progression by phosphorylating the Cyclin-dependent kinase 2 (CDK2) inhibitor p^{27kip1} at the Thr198 residue (Fujita et al., 2003; Larrea et al., 2009). RSK mediated phosphorylation of p^{27kip1} promotes its association with protein 14-3-3, which prevents its translocation to the nucleus (Fujita et al., 2003). In addition, RSK2 is an important regulator of cell transformation, since ectopic expression of RSK2 was found to increase proliferation and anchorage independent transformation (Cho et al., 2007). Kang and his group in 2007 reported a role of RSK2 in tumorigenesis, FGFR3 has been shown to promote hematopoietic transformation through activation of RSK2 in a two-step manner: facilitation of ERK-RSK2 interaction and subsequent phosphorylation of RSK2 by ERK (Kang et al., 2007). By using the Xenopus oocyte model system, RSK2 was shown to contribute to the control of the meiotic cell cycle (Schmitt and Nebreda, 2002). RSK2 was also shown to participate in the progression of oocytes through the G2/M phase of meiosis I via inhibition by phosphorylation of the Myt1 kinase (Gross et al., 2001; Ruiz et al., 2010). It remains unknown whether this mechanism is conserved in other species, but in 2002, the Okumura's group demonstrated that Akt can also act as a Myt1 kinase in starfish oocytes (Okumura et al., 2002).

Cho et al., in 2005 reported the involvement of RSK2 in regulating the activity of the p53 protein. The tumour suppressor p53 is a transcription factor acting as a central regulatory switch in networks controlling cell proliferation and apoptosis. RSK2 allows activation of p53 by phosphorylating the protein at Ser15. Activation of p53 regulates the S phase of the cell cycle. It was also shown that RSK2, p53 and histone H3 form a nuclear complex, in which histone H3 activation by RSK2 is p53 dependent (Cho et al., 2005). The RSK2-p53-histone H3 complex may likely contribute to chromatin remodeling and in cell cycle progression.

RSK was also involved in cell cycle control through C/EBPβ, a transcription factor of the bZIP family. RSK activates this protein by phosphorylating Ser273 in the "Leucine Zipper" domain (Lee et al., 2010). It results in homodimerization and increased DNA binding. According to Lee et al. homodimer C/EBPβ-β formation rather than C/EBPβ-γ heterodimers has important consequences for cell growth and proliferation. Homodimers block the passage of the G1-S cell cycle by abolishing the action of the transcription factor E2F (Sebastian et al., 2005). Thus, according to the large amount of data published the cell cycle progression is largely controlled via RSKs signaling.

6.4. RSKs are implicated in synaptic transmission:

RSKs play roles in neuronal development and brain functions, as suggested by their expression patterns. In 1996, Wong and his co-worker showed that these kinases could phosphorylate an adhesion molecule of the immunoglobulin super family, L1CAM. The activity of this protein can be regulated by phosphorylation via the casein kinase II as well as RSKs. Following phosphorylation of L1CAM at Ser1152, a decrease of neurite growth was observed. This study demonstrated that RSKs regulate the cytoskeleton through L1CAM and, thus, can alter synaptic plasticity. The major role of RSK1 in the development of neuronal processes was described eight years later, through analysis of the differentiation of PC12 cells (Silverman et al., 2004).

RSK2/3 are highly expressed in brain regions, such as hippocampus and cortex, that are important for learning and memory (Zeniou et al., 2002). Some findings suggested that RSK2 is required for neuronal development and/or function and that other RSKs cannot

compensate for the loss of RSK2 (Dufresne et al., 2001). Several reports provided also some evidence that RSKs play a role in synaptic transmission and plasticity. The discovery at the RSK C-terminus of a PDZ domain-binding site, suggested that RSKs are able to interact with synaptic proteins and to regulate their activity by phosphorylation. Thomas et al. (2005) described phosphorylation of Shank1 and /3, two PDZ domain containing proteins, by RSK2. This phosphorylation leads to dissociation of Shank with cortactin (a protein involved in synaptic maturation). However, the effect of this dissociation on the structure or synaptic transmission is not yet clear. On the other hand, a decrease of AMPA receptor transmission was observed when the protein RSK2 was inactivated (Thomas et al., 2005). In 1999, a study reported that RSK2 binds to the NMDA receptor, suggesting that it plays a role in NMDA receptor mediated synaptic transmission (Poteet-Smith et al., 1999).

6.5. RSK acts as cell survival signaling and cellular death:

The development and maintenance of healthy tissues is critically dependent on a balance between cell survival and apoptosis. The program of cell death is a very complex mechanism involving many factors in mammalians (mitochondrial proteins, "death receptor pathway" and apoptosis inducing factor (AIF). Deregulation of one of these actors can be pathogenic and lead to neurodegenerative disorders or cancer. There is increasing evidence that RSKs play a role in these processes.

In the early 2000s, studies have revealed the anti-apoptotic function of RSKs (Bonni et al. 1999; She et al., 2002). Indeed, after stimulation with BDNF or UV-B they observed an increase of the level of phosphorylation of Bad (pro-apoptotic protein) at Ser112. This phosphorylation is RSK2 dependent and leads to dissociation of the Bad/Bcl-XL (anti-apoptotic protein complex). The 14-3-3 protein then recruits bad, and Bcl-XL can promote cell survival.(Bonni et al., 1999; Shimamura et al., 2000).

RSK1 and RSK2 also regulate proliferation and cell survival through activation of the transcription factor NF-κB. At baseline, NF-κB is inhibited by sequestration in the cytoplasm through binding to α-IκB. After stimulation of the cells with tetradecanoylphorbol 13-acetate (TPA) or TNF-α, α-IκB is phosphorylated at Ser32 by RSKs, thus, allowing its degradation by ubiquitination (Schouten et al., 1997, Peng et al.,

2010). NF-κB becoming free moves into the nucleus and activates transcription of many genes, including pro-survival genes.

More recently, Anjum et al, in 2005 reported that RSKs phosphorylate and inactivate death related protein kinase (DAPK). Phosphorylation of DAPK at Ser289 inhibits its pro-apoptotic activity and, thus, promotes increased cell survival (Anjum et al., 2005). DAPK acts as a tumor suppressor and its expression is usually silenced in tumors by DNA methylation (Bialik and Kimchi, 2004). RSK2 has also been implicated in the survival of cancer cell lines and in the development of other human cancers like prostate cancer (Eisinger-Mathason et al., 2010).

6.6. Other possible functions of RSK:

RSKs have many functions in various other biological processes, like protein transport and control of the intracellular pH (via RanBP3 and NHE1) (Anjum and Blenis, 2008). RSKs also phosphorylate filamin A (FLNA) a membrane related cytoskeleton protein that crosslinks actin filaments, which are basic need for cell motility (Woo et al., 2004).

RSK2 phosphorylates also the Na^+/ H^+ exchanger isoforms 1 (NHE-1), at Ser703 residue, which was reported to play a role in maintaining cell pH and volume (Takahashi et al., 1997; Takahashi et al., 1999). Another event controlled by RSKs and more particularly by RSK1, is the accumulation of nitric oxide (NO) in the brain. In 2007, a study indicated that phosphorylation of neuronal nitric oxide synthase (nNOS) at Ser847 in cells treated with mitogens, leads to inhibition of NOS activity. It was shown that RSK1 phosporylates this residue in rat hippocampal neurons and cerebellar granule cells (Song et al., 2007), thereby inhibiting the NO production. These findings revealed that the Ras-ERK-RSK1 might have a therapeutic value for neurodegenerative diseases, associated with a NO-dependent toxicity.

Moreover, RSKs phosphorylate the Akt substrate of 160 kDa (AS160), a protein implicated in the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane in response to insulin. Deregulation of glucose transporter type-4 (GLUT4)

translocation occurs early in the pathophysiology of insulin resistance and type 2 diabetes (Geraghty et al., 2007).

A negative feedback function for RSK2 has also been disclosed. Indeed RSK2 regulates the activation of the Ras-ERK through phosphorylation of SOS, a nucleotide exchange factor (guanine nucleotide exchange factor, GEF) (Douville et al., 1997). As already mentioned, the ERK-MAPK pathway is activated by a succession of events leading to the recruitment of Grb2, SOS, and Ras to the activated receptor. Activated Ras then activates the MAPK cascade of phosphorylation. However, the activation of the C-terminal domain of SOS by RSK2 leads either to its dissociation from Grb2 (an adapter molecule) or to the dissociation of the SOS-Grb2 complex from the membrane receptor. This results in a decreased activity of Ras and the, hence, of ERK signaling. The exact mechanism remains controversial (Douville and Downward, 1997).

7. *Rsk2* deficient animals:

In CLS, the pathophysiological mechanisms leading to Intellectual disability and to skeletal abnormality due to *Rsk2* deficiency are still poorly understood. The knowledge of this mechanism might suggest therapeutic approaches. For this study, various animal models have been created.

7.1. *Rsk2*-KO mouse Model 1:

A first mouse model was reported in 2001. It was generated by targeted disruption of the *Rsk2* gene, but details about the construction used to generate the KO were not reported. The KO mice were more or less 10-15 % smaller, 14% shorter and 24% lighter than wild type (WT) littermates and had deficit in learning and motor co-ordination. At the molecular level, increased glycogen synthase activity was found. This led to a decrease of the concentration of muscle glycogen, and resulted from GSK3 inhibition by AKT. AKT activity increased in the mouse model following treatment with insulin (Dufresne et al., 2001). Expression of RSK2 appeared not to be essential for glycogen synthase activity according to the study of this mouse model (Dufresne and Al, 2001). However, another study based on the same mutant mice linked the expression of RSK2 to

the glucose metabolism. In addition, El-Haschimi et al. (2003) reported that the weight loss of the *Rsk2*-KO mice was due to a reduction of white adipose tissue. This reduction was shown to be caused by a decreased concentration of leptin (peptide hormone), a lower glucose tolerance and a resistance to insulin. This later study characterized this mutant mouse as a new model of lipoatrophic diabetes. This symptom is not found in CLS patients. The phenotype suggested that this mouse is not a good model of the human disease. However, it is interesting to note that an increase in the phosphorylation of ERK kinase was also found in the muscles of these mice (Dufresne et al., 2001). This higher activity of ERK was found in many tissues. This mouse model for unknown reasons is no more available.

7.2. *Rsk2*-KO mouse model 2:

The second mouse model was generated in 2004, also by homologous recombination, by the Group of A. Hanauer. The targeting vector was constructed by inserting a neomycine resistance gene, flanked by two loxP sites and followed by three stop codons (in the three forward reading frames), in a BseRI site located in exon 2 of the *Rsk2* gene (Yang et al., 2004). To excise the floxed neo cassette, mice carrying the mutated allele were crossed with a CMV-Cre transgenic mouse. These *Rsk2*-KO mice exhibited behavioral and skeletal abnormalities. Indeed a decrease in bone formation and mass was observed. Yang et al. in 2004 showed that *Rsk2* plays an important role in the differentiation and function of osteoblasts. A delay of osteogenesis was observed during embryogenesis of these mutant mice. A decreased level of phosphorylation and thus of activation of the transcription factor ATF4 was identified in the mutant mice. ATF4 was shown to be a RSK2 substrate, and is essential for the maturation of osteoblasts and bone matrix synthesis. A reduction in its activity would cause the decreased osteogenesis by reducing the synthesis of type I collagen and osteocalcin expression **(Figure 12)**.

Another study showed that adult *Rsk2*- KO mice develop osteopenia due to progressive dysfunction of osteoblasts while osteoclast differentiation is normal (David et al., 2005). Yet such an imbalance of the coupling between resorption and bone formation disrupts its integrity, and may explain some skeletal malformations observed in CLS patients. A mineralization defect was also noted in adult mice. The authors implicated a decreased expression of the Phex endopeptidase in this phenomenon. Moreover, RSK2 was

also shown to be involved in the development of c-Fos-dependent osteosarcoma, by studying H2-c-FosLTR/*Rsk2*-/y mice (David et al., 2005).

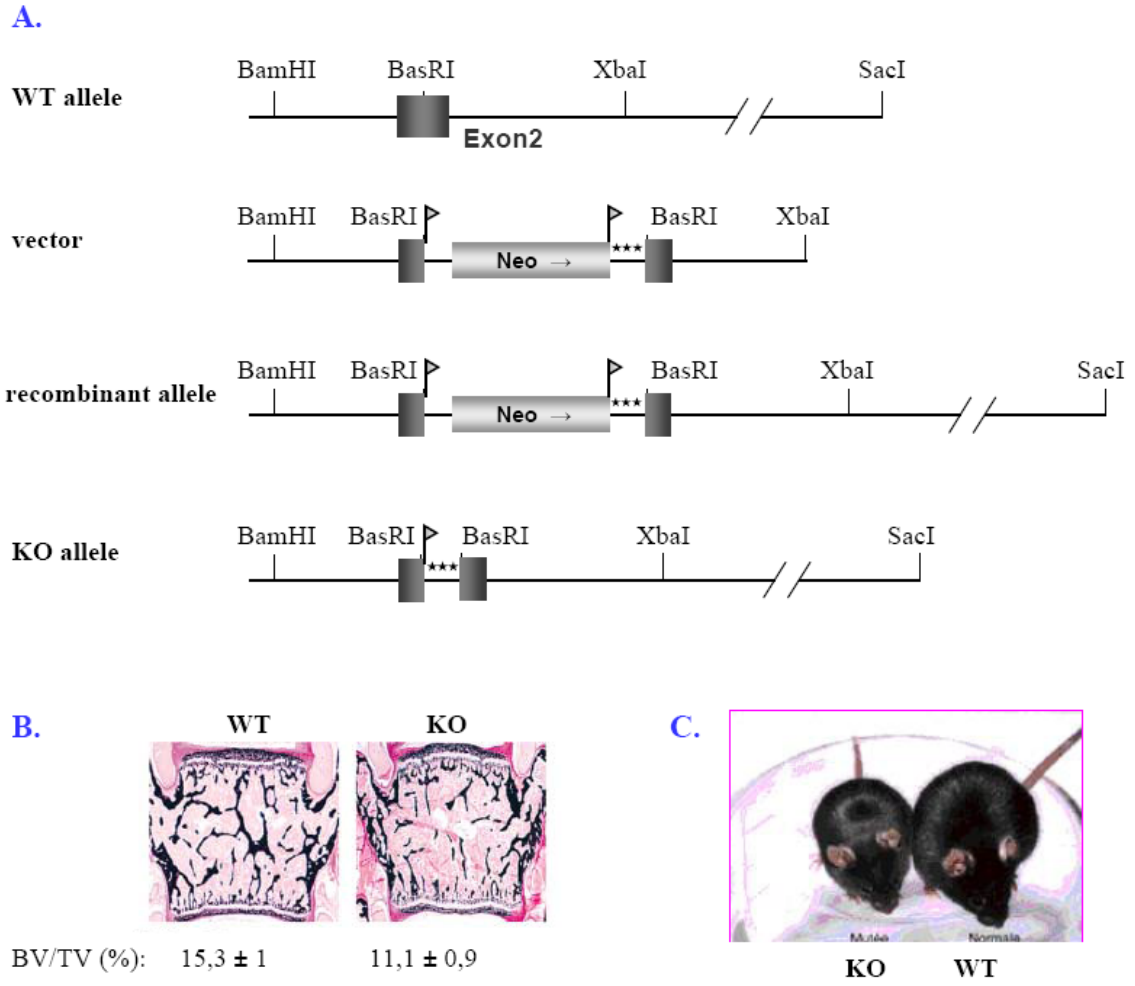


Figure 12: Generation and skeletal phenotype of mice *Rsk2*-KO.

(A) *Rsk2*-KO mice were generated by homologous recombination. A floxed neomycin cassette followed by three stop codons (***) were inserted into exon 2 of the murine Rps6ka3 gene. *Rsk2*-KO mice have skeletal abnormalities, reduced bone formation, bone mass and mineralization. **(B)** Histological observation of a bone in a *Rsk2*-KO and a WT mouse. A decrease in bone matrix mineralization (in black) is observed in KO animals. The ratio of bone volume (BV) on the total tissue volume (TV) is decreased. **(C)** *Rsk2*-KO mice are also about 5% smaller than the non-mutated animal of the same littermates (Adapted from Yang et al., 2004).

Indeed, absence of RSK2 reduced tumor growth by increasing apoptosis and decreasing c-Fos stabilization (David et al., 2005).

Poirier et al. in 2007 showed alterations in long-term memory and spatial learning in these *Rsk2*-KO mice. The phenotypic analysis revealed no deficit of exploratory or locomotors behavior, confirming the suitability of *Rsk2*-deficient mice for behavioral testing. *Rsk2*-deficient mice showed a mild impairment of spatial working memory, whereas deficits in acquisition of a spatial reference memory task and of long-term spatial memory were more severe. No anxiety was found in this model, but the lack of behavioral flexibility could participate in the observed behavioral cognitive problems. In a recent study, Davis et al. (2010) described a lack of long-term memory for spatial recognition of objects after reactivation in the *Rsk2*-KO mice. This points to an involvement of RSK2 in the process of reconsolidation of spatial memory for object recognition after a reminder, rather than to its initial consolidation (Davis et al., 2010) **(Figure 13)**.

This second mouse model of CLS has skeletal and behavioral modification consistent with some of the events described in humans. It is the best current animal model for studying the physiopathological mechanisms leading to the ID and skeletal malformations associated with CLS. While the skeletal phenotype appears to be related, at least in part, to the activity of ATF4, the mechanism leading to cognitive deficits is not yet clear.

7.3. Drosophila:

In contrast to the mouse and human, the drosophila has only one *Rsk* (s6KII) gene, which shows the highest homology with the *RSK2* gene in human. A complete deletion of this gene does not lead to visible morphological defects, but the mutant flies have cognitive impairment, including a short-term memory deficit revealed by operant conditioning (type II) and classical (type I) (thermal and olfactory test, respectively (Putz et al., 2004). Investigation of the mutant flies revealed also involvement of *RSK* in the circadian rhythm as well as in synaptic architecture. RSK regulates by phosphorylation the activity of casein kinase II and thereby the expression of genes involved in circadian rhythm. Akten et al. (2009) described RSK as a new circadian regulator. Fischer et al. (2009b) reported that RSK is involved in the regulation of synaptic boutons of the neuromuscular junction. In their study, they observed an increased number of synaptic boutons in knockout RSK

larvae. The authors suggested that RSK negatively regulates their formation via inhibition of rolled, the homologue of Drosophila ERK.

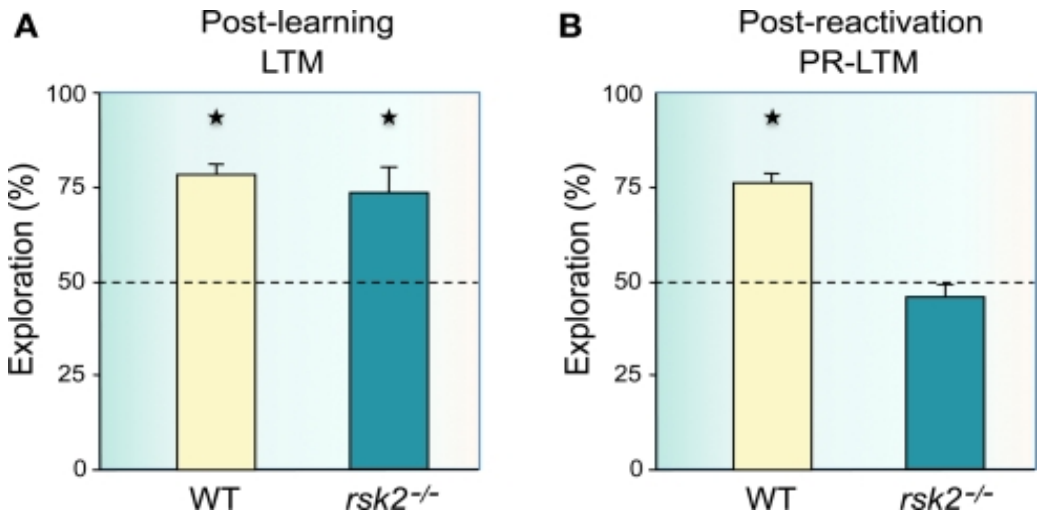


Figure 13: Behavioral phenotype *Rsk2*-KO mice.

Reconsolidation, but not consolidation of spatial, object-place recognition memory is impaired in *Rsk2* mutant mice. **(A)** *Rsk2* mutant mice showed no deficit in long-term spatial recognition memory (LTM) over 48 h as they showed preferential exploration of the displaced object ($n = 13$; $t = 3.53$; $p = 0.0041$) as did wild-type (WT) mice ($n = 13$; $t = 9.83$; $p = 0.0001$), with no significant difference in the amount of time spent exploring the displaced object between WT and mutant mice ($F1,24 = 0.416$; $p = 0.525$). **(B)** In contrast, 24 h after reactivation of the memory, *Rsk2* mutant mice showed a deficit as they displayed no preference for the displaced object ($t = 1.43$; $p = 0.17$) as opposed to the WT mice ($t = 14.61$; $p = 0.0001$); and the level of exploration of the displaced object was significantly greater in WT mice compared with mutant mice ($F1,24 = 70.753$; $p = 0.0001$). Ordinates: percent time spent exploring the displaced object over the mean of the time spent exploring the two non-displaced objects (Adapted from: Davis et al., 2010).

8. Physiopathological mechanism of Intellectual disability:

No morphological abnormalities were observed in *Rsk2*-KO mice, suggesting that RSK2 is not essential for brain development, but rather for more subtle neuronal functions,

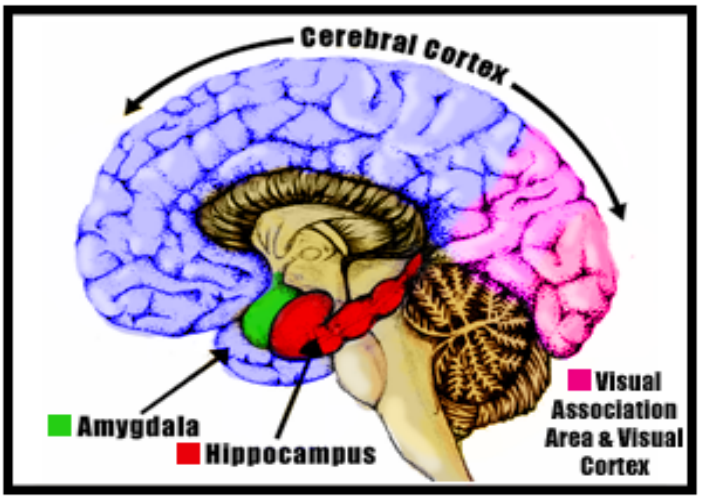
such as neurotransmission and synaptic plasticity. This hypothesis is in part supported by the fact that in human and mouse brain, RSK2 is highly expressed in the hippocampus, in the cortex and in the Purkinje cells of the cerebellum, two brain structures playing a prominent role in cognitive function, in learning, and having a strong synaptic activity (Zeniou et al., 2002). Very little is known about the cellular functions of RSK2 in neurons. The hippocampus plays a critical role in learning and consolidation of spatial long-term memory, is closely related to the cerebral cortex and contains two key interlocking parts, ammon's horn and dentate gyrus (Pearce, 2001). Location and schematic structure of the hippocampus is given in **(Figure 14)**.

In the CLS patients analyzed by Kesler et al. (2007) the cerebellum and the hippocampus volume were particularly impacted by the decreased brain volume observed in some CLS patients. In addition, the *Rsk2*-KO mice showed spatial learning and long-term memory deficits, which are hippocampus dependent functions. Before describing possible causes of ID, I want to introduce some basic information about glutamate receptors, neurotransmission and synaptic plasticity, and its role in learning and memory.

9. Glutamate receptors:

Although more than sixty neurotransmitters have been described, L-Glutamate is the major excitatory neurotransmitter in the mammalian CNS (Santos et al., 2009). It acts via two classes of receptors: ligand gated ion channels, called ionotropic receptors, and G-protein coupled receptors called metabotropic receptors (Palmada and Centelles, 1998). Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD). They have also been involved in memory and learning processes. Glutamate is released from terminal buttons of pre-synaptic neuron, in response to an action potential, crosses the synaptic cleft and acts on the glutamate receptors located on the postsynaptic neuron. The depolarizing effect of glutamate is rapid, suggesting involvement of channel receptors. Previous research showed that glutamate receptors are present in CNS glial cells as well as neurons (Teichberg, 1991).

A.



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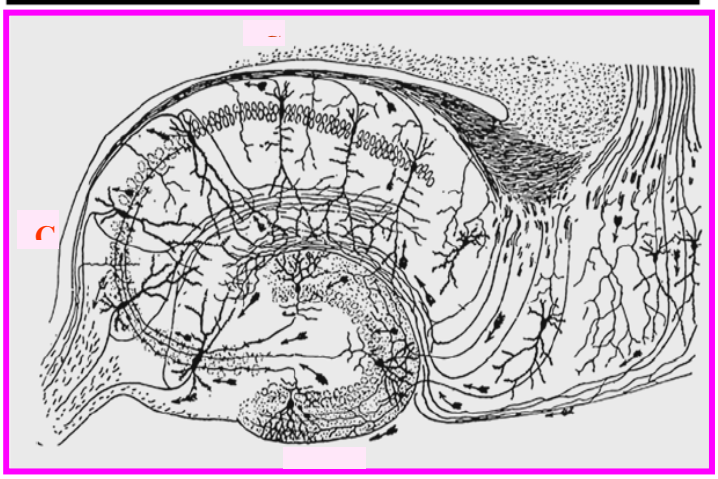


Figure 14: Location and schematic structure of the hippocampus.

(A) Schematic representation of the location of the hippocampus in the brain. In primates, it is located in the temporal lobe, beneath the surface of the cortex. (B) The hippocampus consists of the dentate gyrus (DG) and Ammon's horn, itself divided into three parts (CA1, 2 and 3). Three types of excitatory neurons are found in this structure: the granule cells of the GD project their axons (mossy fibers) to CA3 pyramidal cells. These neurons send their axons (Schaeffer collaterals) to CA1, where they form synaptic contacts with pyramidal cells. These neurons project axons in turn toward the subiculum and entorhinal cortex (Adapted from Kerchner and Nicoll, 2008).

9.1. Metabotropic glutamate Receptors:

Metabotropic glutamate receptors (mGluRs) belong to the family of G protein-

coupled receptors. They allow a “slow” transmission of the signal via second messengers (Ferraguti and Shigemoto, 2006), and consist of seven transmembrane domains, a C-terminal cytosolic domain and an N-terminal large extracellular domain (called "Venus Flytrap Domain"), allowing ligand binding (Niswender and Conn, 2010). Glutamatergic stimulation induces a conformational change of the receptor and the activation of the heterotrimeric G protein, allowing signal transduction **(Figure15)**.

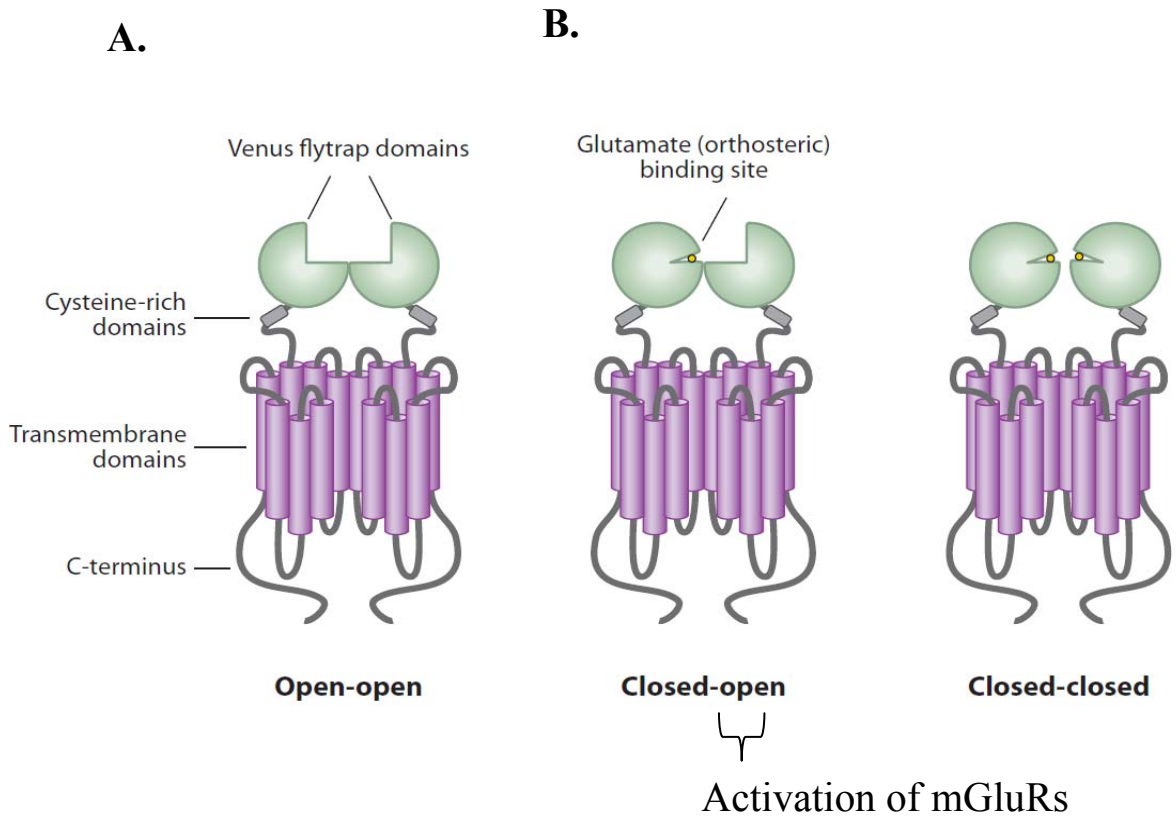


Figure 15: General structure of metabotropic receptors (mGluR).

(A). Schematic representation of a dimeric mGluR, at baseline. These receptors are composed of a C-terminal cytosolic (C-Ter), seven transmembrane domains and an N-terminal extracellular domain, the "Venus Flytrap Domain" (VFD), allowing ligand binding **(B).** the binding of glutamate in one or both VFDs induces a conformational change of these receptors, for signal transduction.(Adapted from (Niswender and Conn, 2010).

Activation of the G protein is produced by the exchange of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) in the α subunit (Niswender and Conn, 2010).

Eight members have been identified in the mGluR family. They are grouped into three classes according to their sequence similarity, the coupled G protein and their agonists (Ferraguti and Shigemoto, 2006). Group I comprises mGluR1 and -5 that can be activated by 3, 5-DHPG, whereas mGluR2 and 3 belong to group II. Group III comprises mGluR4-6-7 and 8. The mGluRs are found in pre-synaptic and postsynaptic neurons in the hippocampus, the cortex and some other parts of brain (Chu and Hablitz, 2000). The mGluRs are found in synaptic and extra-synaptic areas and have diverse functions in synaptic transmission, including regulation of ion channels and neurotransmitter release. (Ferraguti and Shigemoto, 2006). They are implicated in memory, anxiety, learning and perception of pain (Ohashi et al., 2002).

9.2. Ionotropic glutamate Receptor:

Ionotropic receptors (iGluR) are multimeric ion channels that allow "fast" synaptic transmission (Ferraguti and Shigemoto, 2006). They consist of four subunits, each composed of four semi-autonomous areas (Traynelis et al., 2010): two large extracellular domains, the N-terminal domain (NTD) and the ligand-binding domain (LBD). This latter consists of two segments called S1 and S2. They are both parts of the zone of interaction with the ligand. The transmembrane domain (TMD) is involved in the formation of the ion channel and contains 3 helices (M1, M3 and M4) and a stem-loop (M2). The TMD is connected to the intracellular C-terminal domain (CTD), which allows interaction with proteins in the postsynaptic density (PSD) controlling the cytoskeleton and the receptor addressing. This family of glutamate receptors is divided into four groups according to their structural homology and ligand specificity (Traynelis et al., 2010). These groups are designated according to the name of the most selective ligand.

Thus, they include; AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-4-ropionic acid) receptor, NMDA (N-methyl-D-aspartate) receptor, kainite receptor and the δ receptors receptors (Traynelis et al., 2010).

9.3. NMDA receptors:

The NMDA receptor is the predominant molecular tool controlling synaptic

plasticity and memory (Li and Tsien, 2009). NMDA receptors (NMDAR) are tetrameric receptors, formed by the assembly of two GluN1 subunits together with two GluN2 or GluN3 (Traynelis et al., 2010). A single gene encodes the GluN1 subunit, but there are eight splicing variants. The GluN2 and GluN3 ubunits are represented by four and two members respectively (N2A, B, C and D, and N3A and B) (Rebola et al., 2010). The NMDAR differs from other ionotropic receptors, because it requires the attachment of two co-agonists during their activation, and the others not. Glycine binds to GluN1, whereas glutamate binds to the N2 subunit. A number of endogenous and exogenous compounds modulate the NMDA receptor activity, including Mg^{2+} Na+, Ca^{2+}, Zn^{2+} and also CDK5, which regulates the amount of NR2B containing NMDA receptors on the synaptic membrane (Eby and Eby, 2006; Horning and Trombley, 2001; Huggins and Grant, 2005).

Glutamate or NMDA (N-Methyl-D-aspartic acid) binding to NMDARs, leads to activation of the NMDARs and to opening of the ion channel that are nonselective to cations with an equilibrium potential near to zero millivolt (mV). The exceptional property of the NMDAR is its voltage dependent activation. This permits the flow of Ca^{2+} and Na^{+} ions into cell and of K^{+} out of the cell. The response of NMDAR is slower than that of AMPA receptors because its resting potential is blocked by divalent ions (Mg^{2+}). The activity of these receptors is also influenced by the binding of Zn^{2+} ion at the NTD, or by phosphorylation events in the CTD. Several consensus sites for PKA and PKC have been identified in the CTD. It has been shown that these regulatory mechanisms of NMDAR play a central role in LTP and LTD. (Traynelis et al., 2010; Rebola et al., 2010)

9.4. Kinate receptors:

Kinate receptors play only a minor role in signaling at synapses (Song and Huganir, 2002). The kinate receptors (KARs) are composed of five members, grouped into two families who share only 40% sequence homology. The first family contains receptors GluR5, 6 and 7 showing 75 to 80% sequence homology, while the second group contains KA1 and 2, which are 70% homologous. The second family has a better affinity for glutamate. The KARs are found as homo-or heteromers (Bloss and Hunter, 2010).

9.5. The δ receptors:

The δ receptor family contains two members, GluRδ1 and GluRδ2, which are homomers (Traynelis et al., 2010). Their classification in the group of iGluRs comes from their sequence homology with some members of the ionotropic receptor family. Surprisingly, GluRδ1 and GluRδ2 do not bind glutamate and are not ionic channels (MacLean, 2009). However, δ receptors are involved in glutamatergic transmission, the exact mechanism being not yet known.

9.6. AMPA receptors:

The name of AMPA receptor is derived from its ability to be activated by the artificial glutamate analog AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid). This receptor was first discovered in 1982 by Tage Honore and his group at the Copenhagen pharamacy school (Honore et al., 1982). AMPA glutamate receptor channels are expressed ubiquitously in brain neurons and mediate "fast" excitatory neurotransmission in most excitatory synapses. AMPA receptors are composed through combinations of four subunits, GluR1, 2, 3 and/ or GluR4, which are encoded by different genes. In the pyramidal cells of the adult hippocampus GluR1-3 subunits are the most commonly expressed, forming predominantly GluR1/2 and GluR2/3 heterodimers (Malinow and Malenka, 2002). The number, composition and localization, as well as post-transcriptional and translational regulation, of AMPA receptors in neurons are critically significant factors that determine the neuronal response to glutamate (Dingledine et al., 1999). The subunit composition of AMPA receptor will, for example, affect the conductive properties of the receptor. Of all ionotropic receptors, only the AMPA receptors may be impermeable to Ca^{2+}. This property is controlled by the GluR2 subunit and its issue depends on post-transcriptional edition of the Q/R codon at position 607 in the TMD. The presence of an arginine (R) at position 607 gives the channel a linear current-voltage relationship (Boulter et al., 1990) and blocks the Ca^{2+} permeability of GluR2 (Seeburg et al. 1998; Greger et al., 2002).

A second edition site, R/G, at position 743 in the LBD of GluR2 and GluR4 has been described as influencing the kinetics, assembly and membrane expression of AMPA

receptors (Lomeli et al., 1994). The RNA editing process of GluR is catalyzed by adenosine deaminases that act on the mRNA (ADARs).

The properties of AMPA receptors are also controlled by alternative splicing in the LBD (flip and flop variants) (Pei et al, 2009) **(Figure16)**. It determines the speed of desensitization (Mosbacher et al., 1994) of the receptor, the speed at which the receptor is re-sensitized (Sommer et al., 1990), the rate of channel closing and the receptor export (Pei et al., 2007). The function of AMPA receptors can also be regulated by phosphorylation (Santos et al., 2009). While NMDA receptors allow long-term changes of synaptic transmission, AMPA receptor function is to transmit the initial depolarization after glutamate stimulation. AMPA receptors are also critical for the expression of various forms of long-lasting synaptic plasticity, including LTP and LTD (Malenka and Bear, 2004; Luscher and Huber, 2010).

The crucial role of GluR2 in neuronal functions may explain why it is the most tightly regulated of the AMPA receptor subunits. Moderate changes, in GluR2 expression may have severe functional consequences on receptor subunit rearrangement and lead to functional differences in the functioning of synaptic circuits.

I observed an abnormally increased level of GluR2 expression in the hippocampus of *Rsk2*-KO mice.

9.7. Regulation of transcription of Glutamate receptor:

Expression of glutamate receptors is the result of a balance between transcription, translation, protein constancy, assembly of receptor and its arrangement at the cell surface, all of which may be regulated by a number of cellular and environmental stimuli. Therefore, the any particular subunits that every neuron expresses are strong determinants of synaptic phenotype, and this is the basis for understanding how the genetic *cis* elements and *trans* factors control gene transcription in neural cells (Traynelis et al., 2010). Gene encoding glutamate receptors have some common features including several transcriptional starting sites and TATAA- less promoters with a high GC rich content.

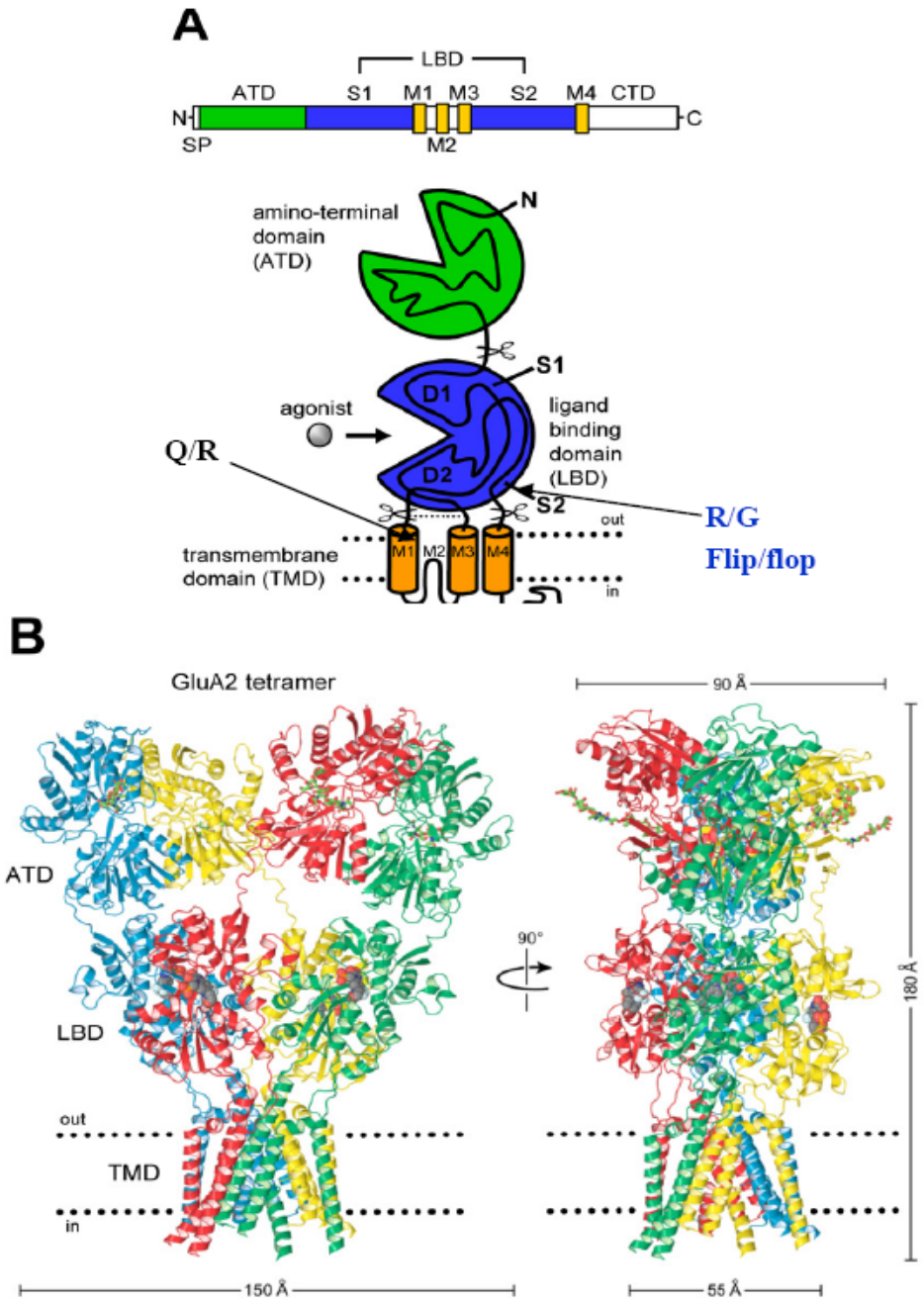


Figure 16: Structure of the GluR2 subunit.

(A). Schematic representation of GluR2 at the plasma membrane. The N-terminal domain (ATD) and ligand binding domain (LBD), consisting of segments S1 and S2 are the extracellular part. The transmembrane domain (TMD) is composed of three helices (M1, M3 and M4) and a stem-loop (M2). It is linked to the intracellular C-terminal domain (CTD), which allows protein interactions. The editing sites Q / R and R / G and the region of alternative splicing (flip / flop) are also represented.

(B) Three-dimensional structure of a GluR2 homotetramer, showing a certain asymmetry between the TMD and the extracellular domains (adapted from Isaac et al., 2007 and Traynelis et al., 2010).

The 5' untranslated region (UTR) ranges between 200bp of the Gria1 (encoding GluR1) genes to more than 1200bp of Grin2a (encoding GRIN2A). Sp1 elements reside close to the major transcriptional start site of all genes involved in expression of glutamate receptor. Many glutamate receptor promoters contain CRE, NRF-1, NF_kB and NRSE sites that are implicated in response to neuronal activity (Traynelis et al., 2010).

We were particularly interested in determining why GluR2 expression is stronger in *Rsk2*-KO as compared to WT littermate. In the following paragraph, I am going to discuss briefly the transcription regulation of the gria2 gene.

9.8. Transcription of the Gria2 gene:

GluR2 is the most critical subunit of the AMPA receptors because it determines in particular, the Ca^{2+} permeability of the receptor. Gria2 expression is influenced strongly at the transcriptional level by two positive (Nrf-1 and Sp1) and one negative (Re1/Nrse-like silencer) regulatory elements in the 5' proximal region of the promoter (Borges and Dingledine, 2001). Myers et al. (1998) showed that the GluR2 Re1/Nrse-like silencer binds the RE1-silencing transcription factor (NRSF/REST), and co-transfection of REST into neurons reduced GluR2 promoter activity in a silencer-dependent manner. The transcription factor Sp1 recruits basal transcription factor TFIID to DNA and induces transcription. It was previously shown that Sp1 activity increases when phosphorylated by Erk 1/2 (Merchant et al., 1999). In 2011, a study confirmed that mutation of Sp1 reduces the expression of the *Gria2* gene. (Ekici et al., 2011). The nuclear respiratory factor 1 (NRF-1) is a transcription factor that was also shown to regulate expression of NMDA receptors subunit genes in neurons (Dhar et al., 2009). NRF-1 has also been associated with the regulation of neurite outgrowth. Dhar et al., 2009 showed that NRF-1 functionally binds to the Gria2 promoter.

I showed that Sp1 up regulated activity is responsible for increased transcription of the *Gria2* gene in RSK2 deficient cells. The schematic organization of the promoter regions of *Gria2* is presented in **(Figure 17)** (Traynelis et al., 2010).

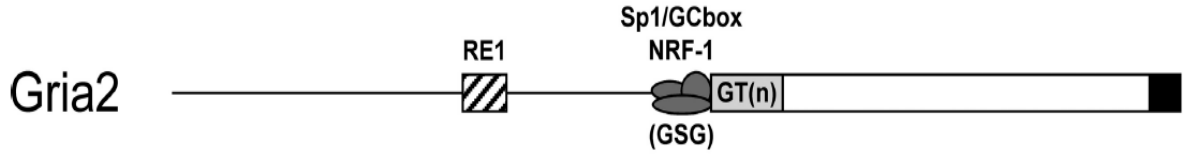


Figure 17: Schematic diagram of the proximal promoter regulatory region of *Gria2*.

The proximal promoter regions of the *Gria2* gene are shown as a thin line. The 5'-untranslated exon sequences is represented by an open bar; the blackened bar designates the protein coding domains (Traynelis et al., 2010).

9.9. Sp1 regulates the GluR2 expression:

Specificity proteins 1 (Sp1) is the prototypic C_2H_2 type zinc finger containing DNA binding proteins. This transcription factor can activate or repress transcription in response to various physiological and pathological stimuli. It binds to the GC rich motif (5′-G/T-GGGCGG-G/A-G/A-C/T-3′ or 5′-G/T-G/A-GGCG-G/T-G/A-G/A-C/T-3′) and can regulate the expression of TATA- containing and TATA less genes via proteins-protein interaction or with other transcription factors (Naar et al., 1998). Sp1 protein contains 785 amino acid and its molecular weight is approximately 110 kDa. It was reported that phosphoraylation events of Sp1 influence its transcription activity (Lin et al., 1997). Various kinases phosphorylate different Ser and Thr sites within the Sp1 proteins. Kinases regulating Sp1 activity include, cyclin-dependent kinase (CDK), extracellular signal-regulated Kinase (ERK) and casein kinase (CKII) (Armstrong et al., 1997; Fojas de Borja et al., 2001; Milanini-Mongiat et al., 2002). Phosphorylation sites are shown in **(Figure. 18)**.

Interestingly Milanini- mongiat and their colleague (2002) demonstrated, that ERK1/2 phosphorylate two Sp1 sites at residues Thr453 and Thr 739. Phosphorylation at both sites is required for maximal Sp1 DNA binding and transcriptional activity (Tan and Khachigian, 2009).

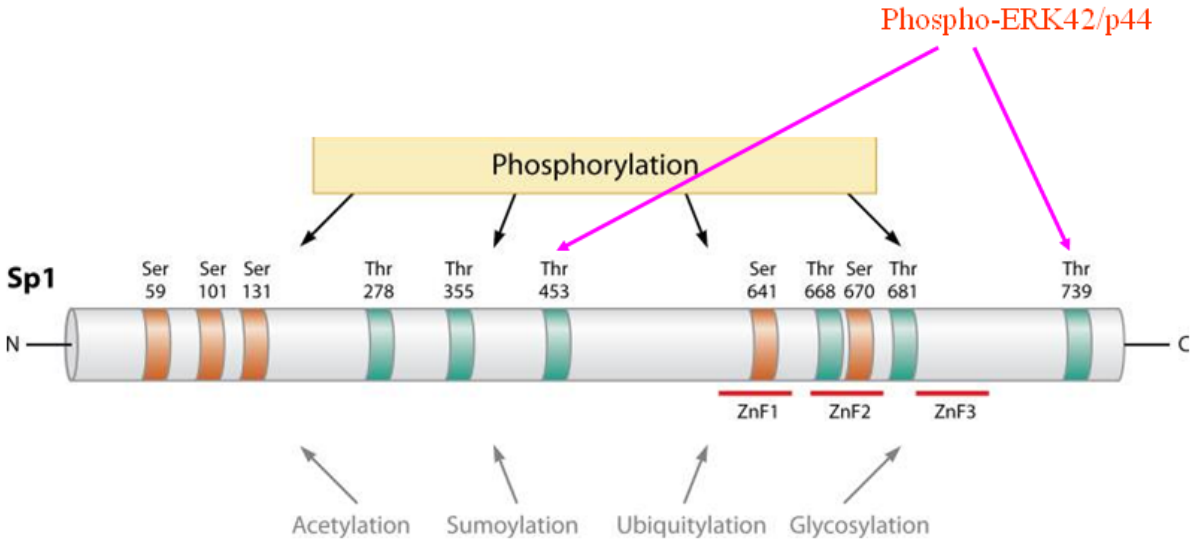


Figure 18: Ser and Thr phosphorylation sites in Sp1.

The Ser and Thr residues are indicated in red and blue, respectively. ZnF, zinc finger domain. Acetylation, sumoylation, ubiquitylation, and glycosylation are among other posttranslational modifications that influence the transcriptional activity and stability of Sp1. Pink color arrows indicate the twos sites that are phosphorylated by phospho-ERK42/44 (Adopted from Tan and khachigian, 2009).

During my thesis, I confirmed that these two sites are phosphorylated, by ERK1/2 kinase activity in PC12 cell line as well as in the hippocampus of adult mice.

9.10. Role of GluR2 subunit in AMPA Receptor:

The AMPA-type glutamate receptor channels are expressed ubiquitously in brain neurons and mediate fast excitatory neurotransmission in most excitatory synapses. AMPA receptors are assembled from combinations of four subunits, GluR1, GluR2, GluR3 and/or GluR4. GluR1 and GluR2 show higher levels of expression in the hippocampus and cortex as compared to GluR3/4. GluR2 exists mainly in AMPARs receptor as GluR1/2 and GluR2/3 heterotetramers. Previous studies reported that 70% of GluR2 is associated with GluR1 (Wenthold et al., 1996). AMPA receptors play also a crucial role in synaptogenesis and formation of neuronal circuitry, as well as in synaptic plasticity (Santos et al., 2009).

There is a huge amount of evidence indicating that the GluR2 subunit plays a pivotal role in AMPA receptors function. The Ca^{2+} permeability, rectification, and single-channel conductance of AMPA receptors are all dominantly influenced by inclusion of an edited GluR2 subunit in the receptor complex (Hollmann et al., 1991; Burnashev et al., 1992; Bowie and Mayer, 1995; Koh et al., 1995; Seeburg, 1996; Swanson et al., 1997; Washburn et al., 1997). Receptors that contain a single edited GluR2 subunit have maximally reduced Ca^{2+} permeability; whereas inward rectification is reduced in a graded manner as the number of GluR2 subunits in a receptor increases (Washburn et al., 1997). Thus, moderate changes in GluR2 expression may have functional consequences on receptor subunit rearrangement and lead to functional differences in the functioning of synaptic circuits. GluR2 expression is governed by neuronal activity (Bai and Wong-Riley, 2003), and its synaptic distribution and levels are modulated as part of the mechanism of synaptic plasticity (Keifer and Zheng, 2010; Sprengel, 2006). GluR2 interacts also with various molecules implicated in receptor trafficking, including the glutamate receptor-interacting protein (GRIP1) containing seven PDZ domains (Dingledine et al., 1999; Collingridge et al., 2004; Isaac et al., 2007). Some studies suggested the GluR2-GRIP1 interaction is important for the synaptic accumulation of the receptor, binding to the cytoskeleton through PSD95 and its insertion into the synaptic membrane.

The *N*-ethylmaleimide-sensitive fusion protein (NSF) is ubiquitously found in the cytoplasm of eukaryotic cells. It is a central component of the cellular machinery in the transfer of membrane vesicles from one membrane compartment to another. The surface expression of AMPAR is inhibited via a peptide (Lys-Arg-Met-Lys-Val-Ala-Lys-asn-Ala-Gln)_that blocks the GluR2-NSF interaction, suggesting that NSF is involved in the insertion of AMPARs into the post-synaptic membrane (Noel et al., 1999). However, whether the NSF-interfering peptide stimulates the loss of AMPARs through blocking their surface delivery or through destabilizing them in the synaptic plasma membrane is not yet clear. The Disruption of this NSF-GluR2 interaction by specific peptides causes a rundown of excitatory post- synaptic currents (EPSC) (Lee et al., 2002; Noel et al., 1999; Song et al., 1998) and the mutated GluR2 in absence of NSF interaction is not delivered to synapses in hippocampal slice cultures (Shi et al., 2001). Moreover, GluR2-NSF also co-assembles with α and β- Synaptosomal-associated protein **(**SNAPs) to form a multi molecular complex that can be reversible subsequent to ATP hydrolysis. In addition, α-SNAP dissociates the GluR2–PICK1 interaction and stabilizes GluR2 expression on the cell surface, which as a

result affects PICK1 mediated endocytosis (Hanley et al., 2002; Osten et al., 1998). PICK1 is a peripheral membrane protein containing a PDZ domain that binds a large number of membrane proteins. In addition another protein, the Activating Protein 2 (AP2) also cooperates with GluR2, and its binding site in the GluR2 C-terminal region overlaps, but is not the same as the NSF binding site (Lee et al., 2002; Kastning et al., 2007). The AP2 is a clathrin adaptor complex involved in endocytosis processes, and its interaction with GluR2 seems to be involved in clathrin-mediated endocytosis during NMDAR mediated LTD. Kasting et al. (2007) demonstrated that blocking AP2–GluR2 binding increases AMPA receptor-mediated transmission (Kastning et al., 2007). An earlier study established that blocking this interaction has no effect on basal transmission but selectively stops the LTD (Lee et al., 2002) **(Figure 19)**.

The extra-cellular domain of GluR2 has a protein-protein interaction site that is significant for the growth, formation and maintenance of dendritic spines. There is suggestion that over-expression of GluR2 in cultures of primary hippocampal neurons increases the length, width and density of spine (Bassani et al., 2009). Passafaro and his colleague in 2003 showed that silencing of GluR2 inhibits spine morphogenesis. **(Figure 19)** shows the mechanism that directs the interaction of the N-terminal region of GluR2 with N-cadherin, and believed to stimulate the development and formation of synaptic and dendritic spines via novel structural communication at the synaptic junction (Saglietti et al., 2007).

Now in the following chapters, I will present the synaptic plasticity and its role in memory formation.

10. Synaptic plasticity (SP):

One of the most important and fascinating properties of the mammalian brain is its plasticity; the capacity of the neural activity generated by an experience to modify neural circuit function and thereby modify subsequent thoughts, feelings, and behavior.

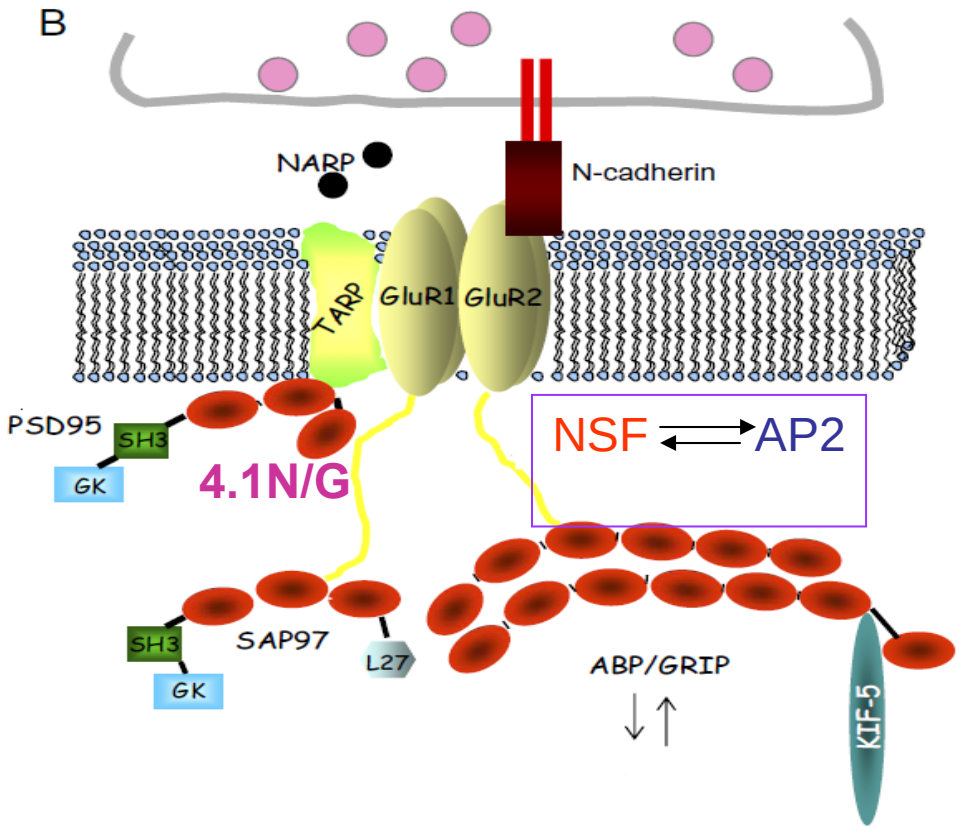


Figure 19: The proteins that regulate clustering of AMPA receptors at the synapse.

GluR1 binds 4.1 N/G, MYOSIN 5 and SAP97. The C-terminal region of GluR2 binds directly NSF or AP2, ABP/GRIP or PICK1. N-cadherinbinds directly to the extracellular domain of GluR2 to influence their clustering. TARPscooperate with AMPA receptor ligandbinding domain (S1 and S2) and PSD-95, regulating their clustering and retention at the synapse.(Adopted from Bassaniet al., 2009).

Synaptic plasticity specifically refers to the activity-dependent modification of the strength or efficacy of synaptic transmission at preexisting synapses, and which has been proposed to play a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces. Synaptic plasticity is also thought to play key roles in the early development of neural circuitry and evidence is accumulating that impairment in synaptic plasticity mechanisms contribute to several prominent neuropsychiatric disorders. Thus, elucidating the detailed molecular mechanisms underlying synaptic plasticity is critical for understanding the neural basis of many aspects of normal and pathological brain function.

The brain is composed of hundreds of trillions of neurons, forming functional neural networks (Hotulainen and Hoogenraad, 2010). Functioning of these networks is related to the connections of the neurons through highly specialized cell junctions called synapses. A chemical synapse allows the passage of electrical information from a presynaptic neuron to its postsynaptic partner. The majority of excitatory synapses in the hippocampus are located on small outgrowths on the dendrite called dendritic spines (Hotulainen and Hoogenraad, 2010). Synaptic plasticity in excitatory synapses is mainly dependent upon calcium (Gerrow and Triller, 2010). Formation of memory requires modification of the strength of synaptic transmission and remodeling of neuronal networks, and these modifications span temporal domains ranging from milliseconds to enduring modifications that might continue for days or weeks and maybe even longer. This refers to synaptic plasticity (SP). This is possible because the synapse can integrate a large number of signals and adapt the cellular response (Laroche, 2010). At excitatory synapses, plasticity is mediated through molecular machinery that detects local Ca^{2+} signals in the dendritic spines. For yet unknown reasons, spine size is strongly correlated with synaptic strength. Short forms of synaptic plasticity have been associated with short-term adaptations to sensory inputs, fleeting changes in behavioral states, and short-lasting forms of memory. Lasting changes are believed to play crucial roles in the construction of neural circuits during development and in long-term forms of memory in the adult nervous system. The relationship between synaptic plasticity and memory has emerged following the study of Bliss and Lomo in 1973. They showed that a sustainable form of SP, called the long-term potentiation (LTP) is obtained by repeated stimulation of the dentate gyrus granule cells. Sah et al. in 2008 confirmed the link between LTP and learning, with tests of fear conditioning in the amygdala.

Other types of SP have been described (habituation, facilitation ...). However, we will focus in our manuscript on two of them, the long-term potentiation (LTP) and long-term depression (LTD) and their activation mechanism. The mechanisms of two forms of synaptic plasticity involve both the AMPA and NMDA glutamate receptors. In the following paragraphs, I will discuss the roles of AMPA and NMDA receptors in synaptic plasticity.

10.1. AMPA receptor trafficking:

AMPA receptors are found throughout neurons including, at synaptic, intracellular and extra-cellular sites **(Figure 20)**. AMPA receptor can move in and out of synapses, which is called receptor trafficking. Receptor are removed from synapses through lateral diffusion or endocytosis and introduced into synapses via exocytosis or lateral diffusion from intracellular as well as extra-synaptic sites respectively (Newpher and Ehlers, 2008; Triller and Choquet, 2008). AMPA receptors are highly clustered at the postsynaptic density (PSD) of a synapse, which is the protein-rich domain in the postsynaptic membrane that is directly apposed to the pre-synaptic active zone. Receptors are localized within the PSD through interactions with various scaffolding proteins and cytoskeletal elements. There is a large body of experimental evidence suggesting that fast trafficking of AMPA receptors into and out of the PSD contributes to activity-dependent long-lasting changes in synaptic strength (Malinow and Malenka, 2002; Sheng and Kim, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003; Triller and Choquet, 2003, 2005; Collingridge et al., 2004; Kennedy and Ehlers, 2006). Such changes are thought to be a necessary sub-cellular component of certain forms of learning and memory (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). AMPA receptor trafficking appears to occur through a combination of two major mechanisms: exocytic/endocytic exchange of surface receptors with intracellular receptor pools, and lateral diffusion of receptors between the PSD and the surrounding extra-synaptic membrane (ESM). It follows that, under basal conditions, the steady-state receptor concentration within a synapse is determined by a dynamical equilibrium in which the various receptor fluxes, into and out of the PSD, are balanced. Activity-dependent changes of one or more of these fluxes can then modify the number of receptors in the PSD and thus alter the strength of a synapse.

The precise mechanisms underlying the activity-dependent regulation of AMPA receptor trafficking are currently not known. However, they are likely to involve one or more of the following processes: changes in the interaction between receptors and scaffolding proteins within the PSD, changes in the rates of exocytosis/endocytosis, and modifications in membrane or receptor structure that alter the surface transport of receptors.

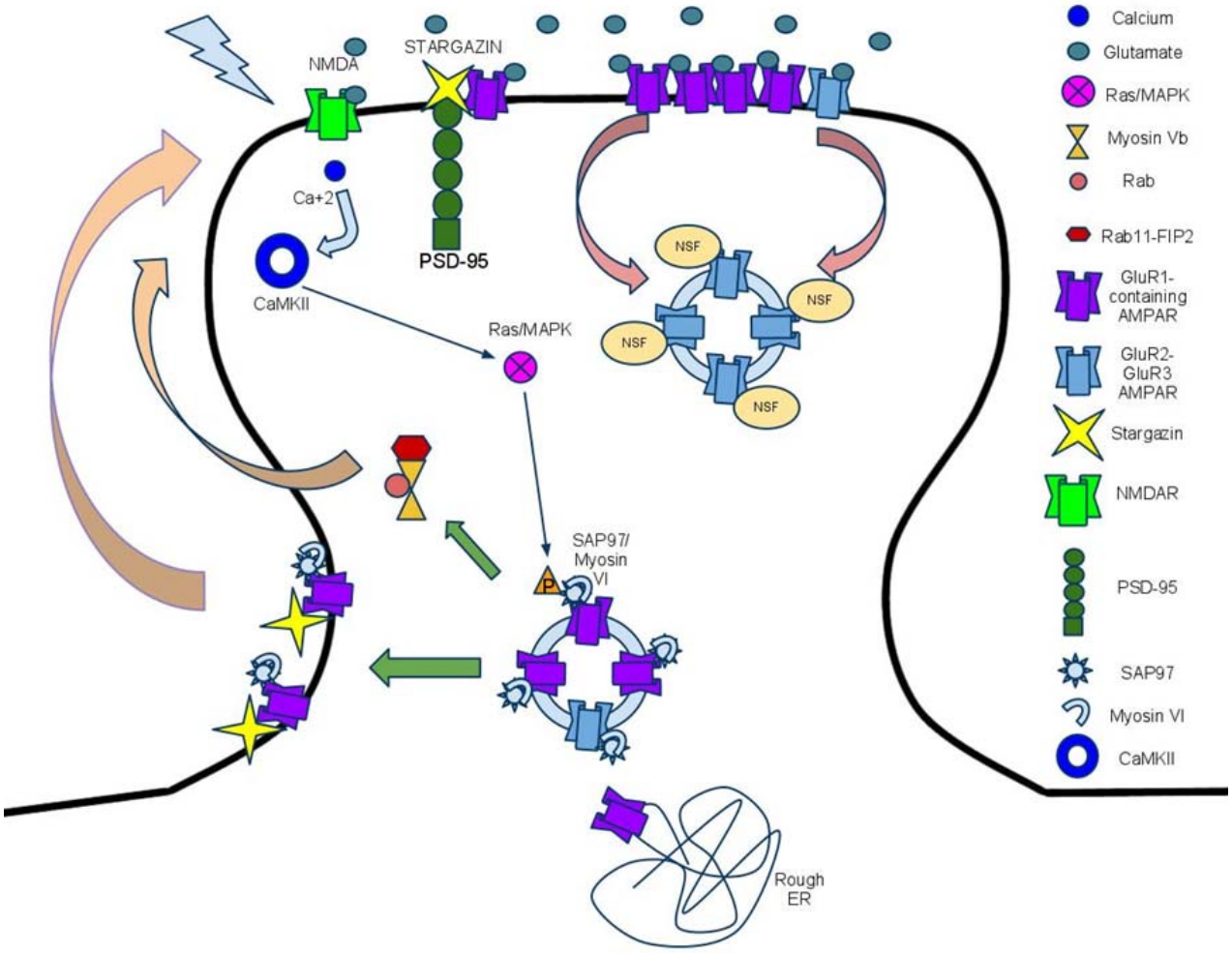


Figure 20: Regulation of AMPAR trafficking in response to LTP-inducing stimuli.

After post-ER processing in the golgi apparatus, AMPARs are released into the peri-synaptic membrane as a reserve waiting for the LTP process to be initiated. Glutamate binding to NMDARs leads to the influx of Ca^{2+} through the NMDAR receptors and consequently the activation of CaMKII. CaMKII has two known modes of activation to trigger the incorporation of AMPA receptors into the peri-synaptic membrane. The first is by direct phosphorylation of the synaptic-associated protein 97 (SAP97), which leads to binding of the complex SAP97 / Myosin-VI to the C-terminus of AMPARs. The AMPAR/Stargazin/SAP97/Myosin-VI complex in then inserted into the peri-synaptic membrane. The second mode of activation is via the MAPK pathway. CaMKII activates the Ras proteins, leading to MAPK/ERK activation, which drives AMPAR insertion directly into the peri-synaptic membrane (Shepherd and Huganir, 2007).

10.2. AMPA receptors and Synaptic Plasticity:

Synaptic plasticity refers to an extraordinary ability of the communication between

two neurons to change in strength. These changes in the strength of synaptic transmission affect the activity of neuronal networks and, finally the behavior of the whole animal. It is supposed that synaptic plasticity represents the cellular correlate of information storage and motivates vital physiological and pathophysiological processes (Citri and Malenka, 2008; Kessels and Malinow, 2009).

In 1949 D.O. Hebb, for the first time reported the basic mechanism of activity dependent synaptic plasticity. It was described as "When an axon of cell X is near enough to excite a cell Y and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that X's efficiency, as one of the cells firing Y, is increased." (Hebb, 1949). In 1973, the Hebb's theory achieved the experimental support with seminal observation that repetitive activation of excitatory synapses into the rabbit hippocampus prompted a persistent increase in synaptic transmission that can last hours and even days (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). This long lasting enhancement in synaptic strength has been known as LTP, and a persistent weakening of synaptic strength relative to baseline has been known as long-term depression (LTD). In last decades, several advances in studies of learning and memory have been done, and a huge literature suggested that LTP and LTD are involved in mechanism of learning and memory (Fleming and England, 2010; Neves et al., 2008; Pastalkova et al., 2006). In 2008, Citri and Malenka reported that two molecular mechanisms are involved in the change of synaptic strength: i) the amount of neurotransmitter released by the pre-synaptic neuron onto postsynaptic neuron and ii) changes in the number and function of receptor on the postsynaptic neuron that respond to that neurotransmitter (Citri and Malenka, 2008).

AMPAR is the most important mediator of excitatory synaptic transmission (Traynelis et al., 2010), and regulation of its function is vital for modulating different forms of SP (Turrigiano and Nelson, 2004). GluR2 expression is governed by neuronal activity (Bai and Wong-Riley, 2003) and its synaptic distribution and levels are modulated as part of the mechanism of synaptic plasticity (Keifer and Zheng, 2010). Indeed, GluR2 interacts with various molecules and plays a critical role in AMPA Receptor trafficking (Isaac et al., 2007). The role of GluR2 in AMPAR trafficking has attracted special attention because it

provides a key mechanism for regulating the number of synaptic AMPA receptors and plasticity (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Collingridge et al., 2004; Shepherd and Huganir, 2007; Kerchner and Nicoll, 2008). The trafficking of post-synaptic AMPA receptors has been shown to be an important mechanism underlying activity-induced alterations in synaptic transmission (Bredt and Nicoll, 2003; Kessels and Malinow, 2009). However, the exact mechanisms by which GluR2 regulates AMPA receptor trafficking and synaptic plasticity are not yet clear. Moreover, the previous studies suggested that AMPA receptors in CNS are complexed with auxiliary subunits: transmembrane AMPA receptor regulatory proteins (TARPs) (Kato et al., 2008; Milstein et al., 2007). TARPs have a profound impact on AMPA receptors, by affecting the trafficking of the receptor, gating ions permeability and pharmacology of the receptors (Bats et al., 2007; Menuz et al., 2009; Tomita et al., 2005).

As above stated, the main function of AMPA receptor is to mediate fast excitatory synaptic transmission to ensuring the rapid responses to glutamate. There is strong evidence that alteration in the number of AMPA receptors localized at synapses triggers changes in the strength of synaptic transmission at a variety of synapses (Citri and Malenka, 2008; Derkach et al., 2007; Kauer and Malenka, 2007).

10.3. NMDA receptor and its role in synaptic plasticity:

The N-methyl-D- aspartate receptors (NMDARs) are glutamate-gated ion channels extensively expressed in the CNS and playing also crucial roles in excitatory synaptic transmission, memory function (Tsien, 2000). The NMDAR is a specific type of ionotropic glutamate receptor. The NMDA receptor forms a heterotetramer between two NR1 (NR1A and NR1B) and two NR2 (NR2A and NR2B) subunits; two obligatory NR1 subunits and two regionally localized NR2 subunits. The structure of NMDA receptors includes: an extracellular domain containing two globular structures, a modulatory domain and a ligand-binding domain. NR1 subunits bind to the co-agonist glycine and NR2 subunits bind the neurotransmitter glutamate. An agonist-binding module links to a membrane domain, which consists of three trans-membrane segments and a re-entrant loop reminiscent of the

selectivity filter of potassium channels. A membrane domain contributes residues to the channel pore and is responsible for the receptor's high-unitary conductance, high-calcium permeability, and voltage-dependent magnesium block.

Each subunit has a large cytoplasmic domain, which contain residues that can be directly modified by a series of protein kinases and protein phosphatases, as well as residues that interact with a large number of structural, adaptor, and scaffolding proteins. Multiple isoforms with distinct brain distributions and functional properties arise by selective splicing of the NR1 transcripts and differential expression of the NR2 subunits.

The NMDAR is a non-specific cation channel, which can allow Ca^{2+}, Na^{+}, and K^{+} to pass into the cell. The excitatory postsynaptic potential (EPSP) produced by activation of an NMDAR increases the concentration of Ca^{2+} in the cell. The Ca^{2+} can in turn function as a second messenger in various signaling pathways. However, the NMDA receptor cation channel is blocked by Mg^{2+} at resting membrane potential. To unblock the channel, the postsynaptic cell must be depolarized (Purves et al., 2008a). The NMDA receptor consequently functions as a "molecular coincidence detector". Its ion channel only opens when the following two conditions are assembled simultaneously: glutamate is bound to the receptor, and the post-synaptic cell is depolarized (which removes the Mg^{2+} blocking the channel). This property of the NMDA receptor explains many aspects of LTP and synaptic plasticity (Purves et al., 2008b).

NMDA receptors are modulated by a number of endogenous and exogenous compounds and play a key role in a wide range of physiological (e.g. memory) and pathological processes (e.g. excitotoxicity). NMDA receptors constitute a subfamily that has exceptional pharmacological and functional properties (Cull-Candy and Leszkiewicz, 2004; Dingledine et al., 1999). Of particular significance is the high permeability to Ca^{2+} ions, which confers the NMDA receptors an essential role in synaptic plasticity under physiological conditions and in neuronal death under excitotoxic pathological conditions. NMDA receptors are heteromers formed from a relatively large pool of subunits, so NMDARs exist as various subtypes with characteristic pattern of expression and functional properties (Yamakura and Shimoji, 1999). Previous studies showed that blocking NMDAR in the mouse leads to impairment of synaptic plasticity and learning and memory. On the other hand, genetics enhancement of NMDAR function improves memory in adult mice

(Cao et al., 2007; Tsien, 2000) . The NR2 subunit directs the properties of NMDA receptor channels and the degree of synaptic plasticity; a relative abundance of NR2 confers a greater plasticity (Li and Tsien, 2009). In 2009, Ng et al. demonstrated that the Neto1 protein, a synaptic transmembrane protein, interacts with the NMDA receptor (Ng et al., 2009). The intercellular domain of Neto1 binds the postsynaptic density protein 95 (PSD-95), Ng et al., showed, on the basis of Neto1 deficient mice, that the synaptic NR2A was reduced in the mutant mice whereas the level of NR2B was unchanged. This discriminating decrease in synaptic NR2A suggests that Neto1 might be obligatory for achieving a normal abundance of NR2A-containing synaptic NMDA receptors. The NR2B subunits are present in the brains of Neto1 mutant mice and are the rate-limiting factors in controlling NMDA receptor mediated synaptic plasticity and memory formation (Tsien, 2000).

Our preliminary findings suggest that NMDAR based neurotransmission is also significantly decreased in RSK2 deficient mice.

10.4. Long-term potentiation (LTP):

Long-term potentiation is a type of activity dependent plasticity, which results in a constant enhancement of synaptic transmission. LTP has been a source of large attraction to neuroscientists since its discovery in the early 1970s. LTP is a persistent increase of the efficiency of synaptic transmission following high-frequency stimulation (Bliss and Lomo, 1973). The synapse has the ability to maintain these changes for weeks, months or even years (Laroche, 2010). LTP is a synaptic strengthening, which is the basis of information storage and learning (Malinow and Malenka, 2002). Several phases have been identified in this process, early LTP (E-LTP) and lasting LTP (L-LTP). E-LTP is based on post-translational modifications, whiles the consolidation of memory and synaptic changes, observed in L-LTP, requires expression of various genes and protein synthesis (Laroche, 2010). LTP is believed to account for various type of learning, including classical conditioning and higher-level cognition observed in human (Cooke and Bliss, 2006).

10.5. The activation mechanisms of LTP:

LTP can be induced by several mechanisms, but it seems that the critical event is the increase of intracellular Ca^{2+} concentration. In the following text, I am going to discuss the involvement of AMPA and NMDA receptors in the process of hippocampal LTP.

The prevailing view is that NMDARs play an essential function in the induction of several forms of activity-dependent LTP and LTD, by acting as a coincidence detector of pre-synaptic and postsynaptic firing (Malenka and Bear, 2004). The use of aminophosphonovalerique, (APV), a specific inhibitor of the NMDAR, blocks the induction of LTP. The entry of Ca^{2+} in the postsynaptic neuron is in fact dependent on the activation of NMDAR (Rebola et al., 2010). However as previously mentioned Mg^{2+} blocks that ion channel in a voltage "voltage-dependent". Opening of this channel is only possible if a ligand (glutamate) binds to the receptor in parallel with a depolarization of the post-synaptic membrane (Humeau et al., 2009). Previous studies indicated that AMPA receptors are involved in the process of LTP in hippocampus. Activation of AMPAR on the surface induces the initial depolarization necessary for NMDA receptor activation. However, some synapses called silent, do not possess AMPA receptors, and the mechanism of NMDA receptor activation is then AMPAR independent. The calcium channels voltage-dependent or the mGluR might in these cases induce the initial depolarization (Rebola et al., 2010). The synapses called silent may be matured via the insertion of AMPAR (Kerchner and Nicoll, 2008).

The entry of Ca^{2+} in the postsynaptic compartment allows the maintenance of LTP through the activation of many protein kinases such as CaMKII, PKC or PKA and ERK1/2 (Rebola et al., 2010). Maintaining this type of long-term plasticity requires the expression of several genes, protein synthesis but also the recruitment of AMPA receptors at the surface. Indeed, recycling of AMPA receptors after activation of the NMDA receptor represents a key step in strengthening synaptic plasticity (Malinow and Malenka, 2002). Shi et al. (1999) showed that the insertion of GluR1 at surface is regulated through the activation of the NMDA receptor after a tetanic stimulation. GluR1 traffic was inhibited by APV, an antagonist of the NMDAR. On the other hand, many researchers have demonstrated the role of GluR1 subunits in the expression of LTP. Zamanillo et al., in 1999 have shown that the absence of this subunit in the GluR1-KO mice leads to a loss of LTP. The mechanism of insertion of AMPA receptors into the synapse involves two steps (Isaac et al., 2007; Humeau et al., 2009). Initially, during the E-LTP, the homomeric GluR1/1

(permeable to Ca^{2+}) arrives at the membrane in a PKA dependent manner, they allow entry of Ca^{2+} into the neuron and an increase in its concentration. An increase in the proportion of GluR1/GluR2 receptors, impermeable to Ca^{2+}, is then observed at the surface, which allows the transition to L-LTP. This biphasic mechanism remains, however, controversial.

It is interesting to note that the recycling activity of AMPA receptors is regulated by phosphorylation; many phosphorylation sites have been reported for all AMPA receptors (Santos et al., 2009). This phosphorylation permits recycling and contributes to the maintenance of LTP. Oh et al., in 2006, for example, have identified Ser845 of GluR1 as one of the sites recognized by PKA. Following forskolin stimulaztion they observed that phosphorylation of this residue leads to the anchoring of GluR1 at the cell surface. The activation of NMDA receptors allows parallel expression and synaptic induction of LTP. In 2005, Qin and his group described the insertion of GluR1 and GluR2 at the synapse, following the activation of the Ras/MAPK pathway. The stimulation of the NMDA receptors induces the phosphorylation of GluR2 at Ser841 through ERK/MAPK and its synaptic expression. The authors proposed the intervention of other kinases, such as the CaMKII, PKA but also RSKs in this process (Qin et al., 2005). These studies confirmed the link between the activation of the NMDA receptor, the stimulation of a channel and recycling of the AMPA receptor in the establishment of the L-LTP. Other studies have also involved PKC in this process (Santos et al., 2009).

The transport of the AMPA receptor to the synaptic surface requires a process of membrane fusion, involving the SNARE protein, and a mechanism for the lateral diffusion, depending on post-synaptic protein connections (Santos et al., 2009). In brief, an induction of LTP leads to the fusion of endosomes containing the AMPA receptor with the cell membrane, usually in extra-synaptic regions. This fusion is in fact a process of exocytosis whose main actors are the SNARE proteins. Their importance has been described by the work of Lledo et al. in 1998. A decrease in LTP induced by tetanic stimulation was observed after injection of botulinum toxin in hippocampal slices. This toxin inhibits the formation of the SNARE anchoring proteins and therefore their action. The authors also showed that injection of SNARE increases synaptic transmission. Once anchored at the plasma membrane, AMPA receptors diffuse laterally, moving to the PSD. It seems that this mechanism is dependent on Stargazin and PSD95. Stargazin, belonging to the TARPs protein family, interacts and regulates the AMPA receptors. PSD95 is a major postsynaptic

protein, interacting with Stargazin through PDZ domains. Through the use of mutants of compensation (alteration of the interaction between the PDZ and ligand), Bats et al. in 2007 showed that the interaction of these two proteins is necessary for the accumulation of AMPA receptor in the synapse by lateral diffusion (Schnell et al., 2002).

During LTP, the fusion of endosomes with the plasma membrane allows the recycling of AMPA receptors but it also regulates the growth of dendritic spines. These small protrusions rich in actin are the major sites of glutamatergic synapses. Analysis and electron microscopy imaging of living cells showed that the fusion of vesicle recycling induced during LTP, enhances the membrane surface and thus contribute to the expansion and growth of dendritic spines (Park et al., 2006). In addition, constitutive recycling of AMPA receptors was also observed at baseline, especially for heteromeric GluR2 /3. This is much slower than the previous one and does not lead to a change in synaptic activity. AMPA receptor traffic in this case represents a way to maintain basal synaptic transmission and synaptic concentration of AMPA receptors (Hu et al., 2007). The C-terminal cytoplasmic end of the subunits GluR2/3 can interact with different PDZ proteins and thus regulates the trafficking and stability of AMPAR in the synapse (Passafaro et al., 2001).

Maintain of LTP, requires the synthesis of new proteins. The local translation in dendritic spines is possible because the molecular machinery of translation is found in the spines and dendrites. In 2002, Ostroff and his group reported translocation of polyribosomes in these structures following a tetanic stimulation, confirming the establishment of a local translation. Recent studies have shown the importance of local synthesis of Arc, an IEG, in the LTP. Activation of NMDA receptors, inducing the LTP, activates the Rho kinase, which in turn regulates the polymerization of actin and the activation of the ERK kinase. These two mechanisms facilitate the transport of the mRNA of Arc into the dendrites, and its local translation (Huang et al., 2007). Another study suggested that early synthesis of this protein contributes to the expression of LTP, while the maintenance of LTP requires a more sustained synthesis of Arc. These results have been achieved through the inhibition of Arc by injection of antisense oligonucleotides in the dentate gyrus (Messaoudi et al., 2007). Study of the Arc-KO mouse showed the importance of this protein in the late phase of the LTP, but also in the formation of the LTD (Bramham et al., 2008). A diagrammatic representation showing the activation mechanism of NMDARs and AMPARs dependent LTP is shown in **(Figure 21)**.

10.6. Long-term depression (LTD):

Low frequency stimulations leads to a lasting reduction in synaptic efficacy, a phenomenon called Long Term Depression (LTD) (Santos et al., 2009). LTD occurs in various areas of the central nervous system with different mechanism depending upon developmental progress and brain region (Massey and Bashir, 2007; Santos et al., 2009). LTD and LTP are two complementary mechanisms: the first allowing storage of information whereas the second would be a synaptic mechanism of forgetting, allowing decrease of synaptic activity at its base level, and thus storage of new information. These mechanisms of PS can be regulated at the pre-synaptic level, depending on the release of neurotransmitters in the synaptic cleft, but also at the level of the post-synaptic compartment depending on the number, type, or even the properties of membrane receptors (kessels et al., 2009; Humeau et al., 2009).

10.7. The activation mechanisms of LTD:

Long-term depression (LTD) may be experimentally induced by prolonged low frequency stimulation of excitatory synapses (Dudek and Bear, 1992). Induction of LTD is also NMDAR-dependent in the hippocampal CA1 region and, like LTP induction, requires Ca^{2+} influx through NMDARs (Bear and Malenka, 1994). In contrast to LTP, low levels of synaptic stimulation can stimulate NMDARs to produce NMDAR-dependent LTD of glutamatergic synaptic transmission, or to produce mGluR dependent LTD or to activate mGluRs (Santos et al., 2009). These two forms of LTD are thought to result from internalization of surface AMPA receptors. It seems that one is preferred to another according to the stage of development. Thanks to measures of LTD in the CA1 of the hippocampus, Lee et al. in 2005 suggested that NMDAR-dependent LTD is involved in cognitive processes of young rats, whereas non-NMDAR dependent LTD seems more important in adults. NMDAR-dependent LTD requires low Ca^{2+} entry in the post-synaptic neuron, activation of phosphatases and internalization of AMPAR (Santos et al., 2009). Several studies have shown the importance of calcineurin and PP1, two phosphatases, in the induction of LTD. In 2007, Hu et al. reported that PP1 is involved in endocytosis of the GluR1 receptor.

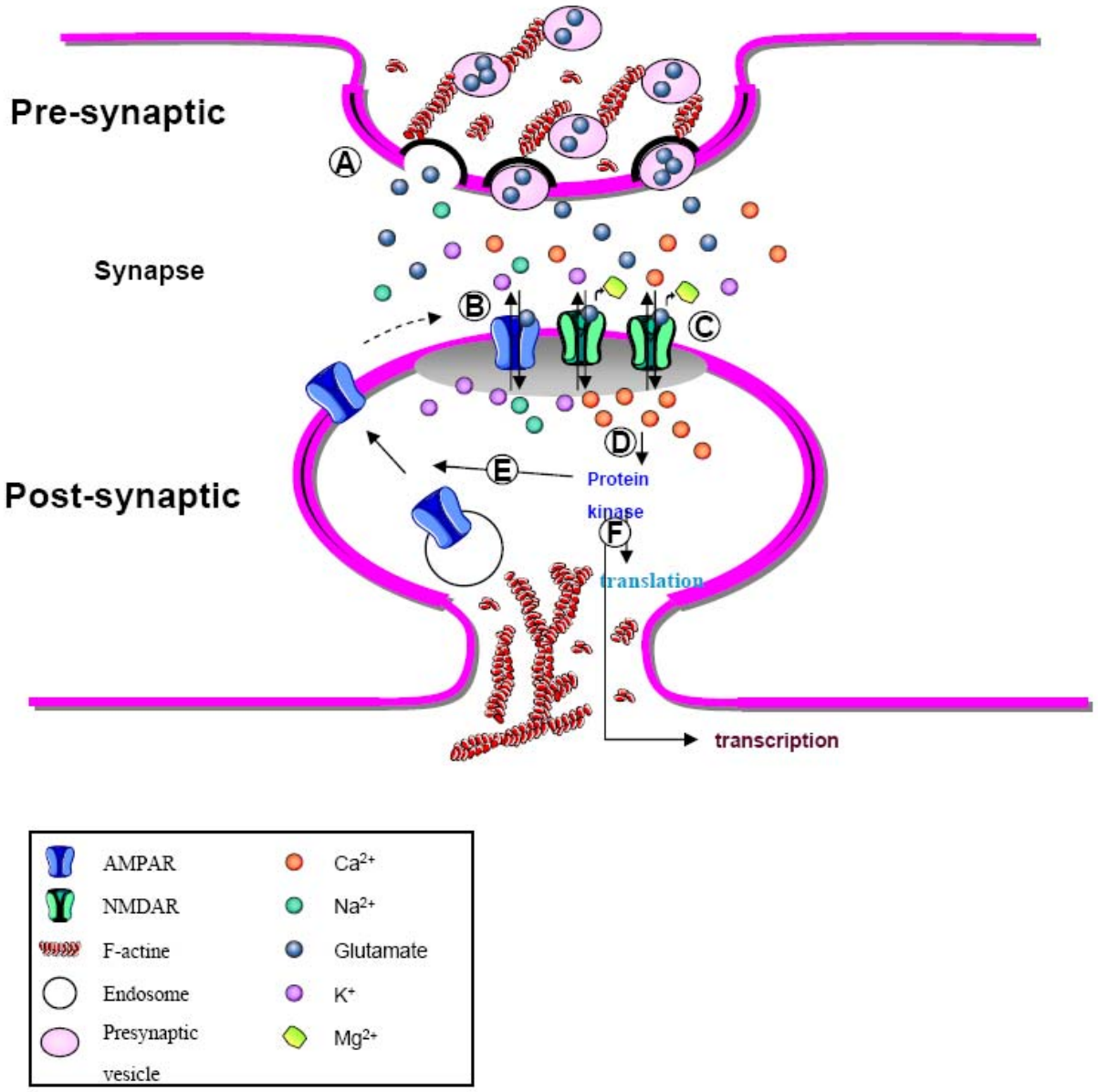


Figure 21: Mechanism of activation of the LTP dependent on NMDARsand AMPARs.

(A) The presynapticneuron releases glutamate in the synaptic cleft. **(B)** It is captured by the post-synaptic AMPARsand NMDARs, allowing their opening. **(C)**The depolarization of the cell induced by the entry of Na+through AMPARs, allows opening of NMDARsand the massive influx of Ca^{2+} in the postsynaptic compartment. **(D)**The entry of Ca^{2+} in the post-synaptic compartment leads to the activation of multiple proteinkinases, promoting the recycling of theAMPARsto the membrane by Phosphorylation. **(E),** but also the transcription and translation necessary for maintaining the LTP **(F)** K+, potassium) (adapted from Kerchnerand Nicoll, 2008).

PP1 is a phosphatase that interacts with a protein of the actin cytoskeleton, Neurabine I. This interaction allows the localization of synaptic PP1 and its action on membrane receptors. PP1 participates in the organization of LTD by dephosphorylating GlR1 at Ser831 and Ser845. Moreover, the mechanism of phosphorylation and dephosphorylation, involved in the endocytosis of AMPAR is clathrin-dependent. In 2002, Lee et al. described that AP2, an adapter of clathrins, is required for internalization of GluR2. By using GluR2 and AP2 inhibiting peptides, the authors blocked the interaction of these two proteins. It resulted in a decrease of GluR2 endocytosis associated with a reduction of NMDAR-dependent LTD. Another important factor in this process was identified in 2005: Rab5, a member of the small GTPases family. Brown et al. showed that induction of LTD triggers the activation of this factor. Once activated Rab5 is involved in clathrin-dependent internalization of GluR1 and GluR2, as indicated by the use of a mutant GluR2 unable to bind the clathrin adapter AP2 (Brown et al., 2005). Following recycling, the authors also identified a decrease in GluR1 phosphorylation at Ser845 and Ser831. This dephosphorylation appears to be important for the expression of LTD as mentioned previously (Hu et al., 2007) **(Figure 22)**.

In the next section, I will discuss briefly dendritic spines their functions and pathology of spines.

11. Dendritic spines:

The dendritic spine is a small membranous protrusion from a neuron's dendrite that typically receives input from a single synapse of an axon. The dendritic spines serve as a storage site for synaptic strength and assist to transmit signals to the neuron's cell body. The human brain consists of hundred billion neurons interconnected into useful neuronal circuits that cause all our emotions, dreams, behaviors and memories. The chemical synapses control the electrical communication in neuronal system and pass information from pre-synaptic axons terminals to postsynaptic dendritic regions (Hotulainen and Hoogenraad, 2010). Previous studies suggested that alteration in spine morphology account for functional differences at the synaptic level (Kasai et al., 2003; Yuste and Bonhoeffer, 2001).

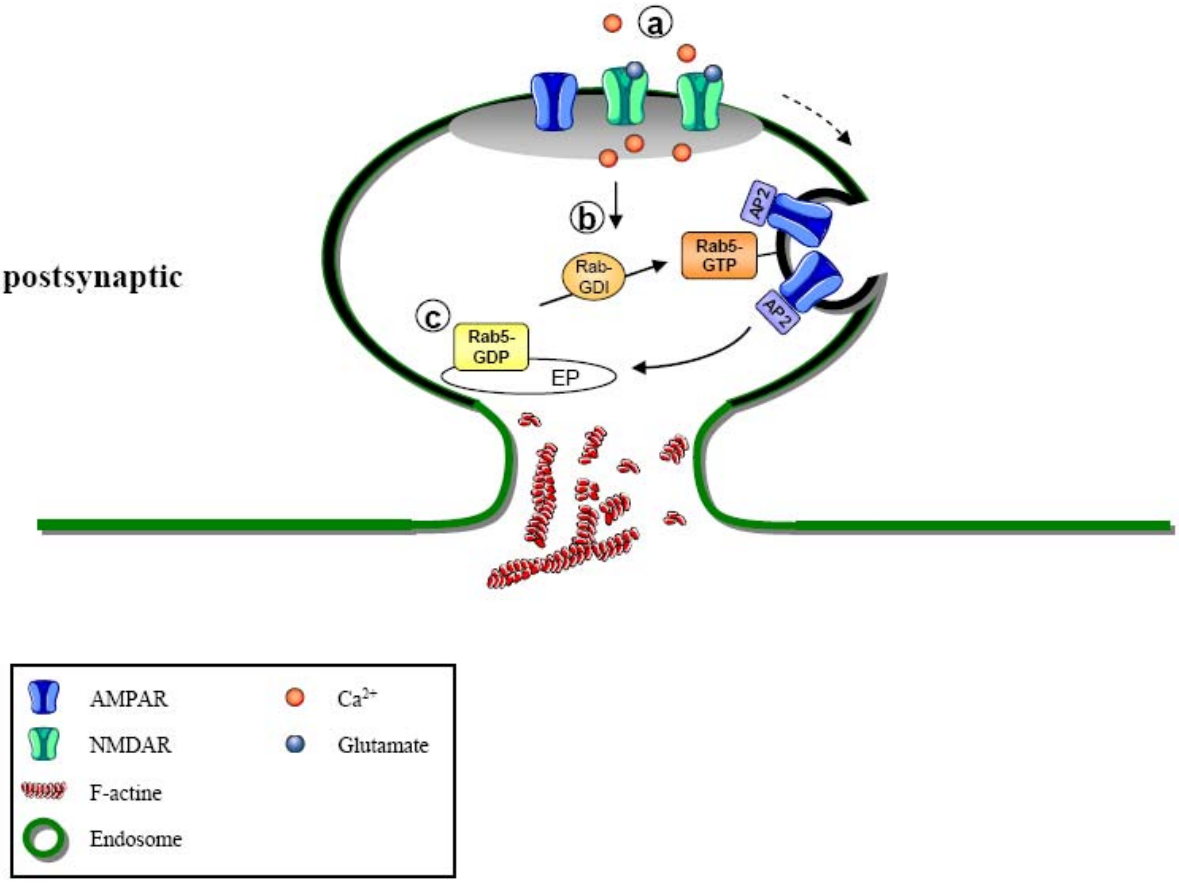


Figure 22: Mechanism of Rab5 dependent AMPAR endocytosis during Long Term Depression (LTD).

(a). A small entry of Ca^{2+} in the postsynaptic neuron induces LTD, by phosphorylation of Rab-GDI, an inhibitor of Rab-GDP dissociation **(b).** Its activation leads to translocation of Rab5-GDP from early endosome (EP) to the plasma membrane **(c),** where Rab5 is activated (to Rab-GTP) and is involved in clathrin-dependent internalization of GluR1 and GluR2 (Adapted from Brown et al., 2005).

Now it is widely accepted by scientists that the functional and structural changes at spines and synapses are the basis of learning and memory in human brain (Holtmaat and Svoboda, 2009; Kasai et al., 2010).

11.1. Dendritic spine structure and function:

The spines occur at a density of 1-10 spines/ μm of dendrite length, and some neurons, like a hippocampal neurons, have thousand of spines all over the dendritic arbors

(Sorra and Harris, 2000). A bulbous head, allowing contact with a presynaptic partner, represents the morphology of a typical spine. It is attached to the dendrite through the spine neck (Hotulainen and Hoogenraad, 2010). Although this classic description, the morphology of dendritic spines is extremely variable, depending on the development but also of synaptic activity (Hering and Sheng, 2001; von Bohlen und Halbach, 2009). The spines can be grouped into categories according to their shape: mature spines, and immature spines (Boda et al., 2004, Grossman et al., 2010). On the other hand, the electron microscopic studies suggested that spines can be categorized into three groups, based on morphology; thin, flopodia-like protrusions, short spines without a well defined spine neck (also called stubby spines), and spines with a large bulbous head (mushroom spines) (Bourne and Harris, 2008). The most interesting characteristic of the spine structures is that they change morphology continuously, even through adulthood, which reflects the plastic nature of synaptic connections (Grutzendler et al., 2002; Trachtenberg et al., 2002). The volume of a spine can vary from 0.001 to 1 μm^3. A difference in length has also been reported, spines from 0.2 to 2μm were observed (Hotulainen and Hoogenraad, 2010).

The proposed mechanism of synaptogenesis is as follows (von Bohlen und Halbach, 2009. In general, a newly synthesized dendrite does not yet have synapses or dendritic protrusions. In the early stages of synaptogenesis, filopodia appear, and can grow and retract rapidly to meet a pre-synaptic partner. Following this meeting, the filopodium retracts to approximate the continuation pre-synaptic dendrite of its own, allowing the synapse formation and maturation of by stabilizing the spine. The explanation is shown in **(Figure23)**.

A large number of studies revealed that morphology of spines could be modified by a neuronal activity in vitro as well as in vivo (Holtmaat et al., 2006; Matsuzaki et al., 2004; Roberts et al., 2010). For example, LTP causes the enlargement of spine heads (Kasai et al., 2003; Yuste and Bonhoeffer, 2001). An attractive mechanism for regulating the modification in spine morphology is the local addition or removal of synaptic membrane and turnover of postsynaptic receptors. Indeed, synaptic stimulation assembles AMPA receptors in recycling endosomal compartment into spines, an event necessary for subsequent spine enlargement (Park et al., 2004; Park et al., 2006).

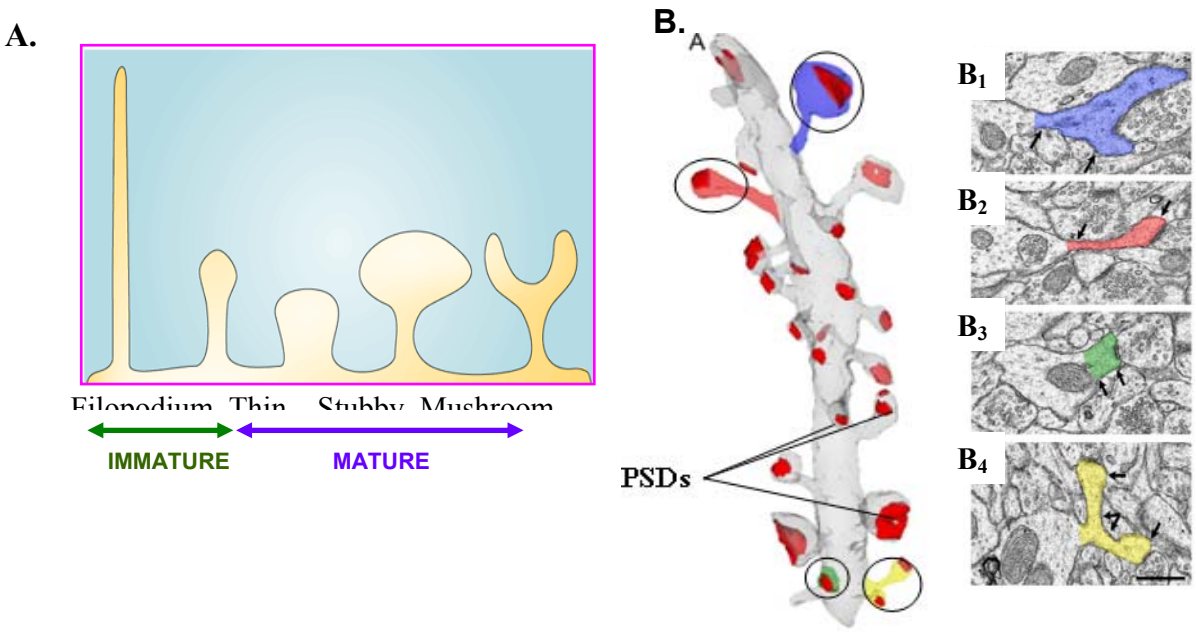


Figure 23: Morphological classification of dendritic spines.

The morphology of dendritic spines is very variable according to the development but also to synaptic activity. They can be grouped into categories according to their shape. **(A)** Immature spines include filopodia (long and thin, without a head) and the so-called "thin" spines (shorter, with a small head). The mature spines include mushroom-shaped, "branched" spines (multi-headed spine) and spines without neck without (stubby spines). **(B)** Three-dimensional reconstruction of a dendrite in the hippocampus with spines of different sizes and morphologies. A "mushroom" spine is represented in blue **(B1),** a thin spine in red **(B2),** a stubby spine in green **(B3)** and a multi-headed spine in yellow **(B4)** (Adapted from Hering and Sheng, 2001 and Bourne and Harris, 2008).

11.2. Organization:

The dendritic spines are highly specialized cell compartments, which contains many molecules modulating both their activity and morphology. They contain the postsynaptic machinery, including glutamate receptor, actin cytoskeleton and postsynaptic density (PSD). Many other organelles are also located in this compartment and contribute to its function including the smooth endoplasmic reticulum, mitochondria and endosomes (Hotulainen and Hoogenraad, 2010). The PSD is a highly organized structure, found at the top of the head of the spine. It contains membrane receptors, ion channels, transmembrane adhesion molecules and scaffolding proteins. It also contains many signaling proteins (Feng and Zhang, 2009; Hotulainen and Hoogenraad, 2010) **(Figure 24)**. It is recognized that the

size of the PSD is proportional to that of the head of the spines, the "mushroom" having the broadest and more complex PSD (Bourne and Harris, 2008). On the other hand, the size of the PSD is also correlated to the number of synaptic glutamate receptors. Thus, the size of the head of the spine can be correlated with synaptic efficacy (Cingolani and Goda, 2008).

As I mentioned previously in this manuscript, an induction of LTP leads to the expression of AMPARs at the surface, which can contribute to the increased size of the dendritic spine (Park et al. 2006). Different proteins found in the PSD have been involved in the activity and morphology of spines. PSD95 is a postsynaptic protein that interacts with the major NMDARs thus influencing their distribution (von Bohlen und Halbach, 2009). It also interacts with Homer and Shank proteins, which are also involved in synaptic maturation of spines. El-Husseini et al., in 2000 showed that over-expression of PSD95 in hippocampal neurons leads to the maturation of glutamatergic synapses. They also observed an increase in the number and size of dendritic spines. In 2007, Kopec and his group reported that the function of AMPAR is not necessary for the enlargement of the spines. They observed that a decrease in the AMPAR transmission does not prevent the increase in the size of spine after induction of LTP. Thus, the synaptic localization of the AMPA receptor rather than its function appears to be involved in the morphological maturation of dendritic protrusions. The morphology of dendritic spines can also be controlled by the regulation of the actin cytoskeleton. The dendritic spines are indeed very rich in filamentous actin and the level of actin polymerization influence the formation and morphology of spines (Hotulainen and Hoogenraad, 2010). The actin cytoskeleton of a spine is composed of two isoforms, monomeric actin (G-actin) and polymerized or filamentous actin (F-actin) (Fukazawa et al., 2003**)**. The actin skeleton dynamic can be regulated by many factors. The actin related protein (Arp2 /3) complex and Cofilin plays an important role in the organization of the actin cytoskeleton. The Arp2/3 complex is probably the most thoroughly characterized actin regulator of spine morphogenesis. Arp2/3 binds to actin filaments and allows network formation by nucleation of new filaments. Inhibition of this complex by siRNA increases the proportion of "mushroom" or "stubby" like spines. This result indicates that Arp2/3 participates in the formation of the head spines (Hotulainen et al., 2009).

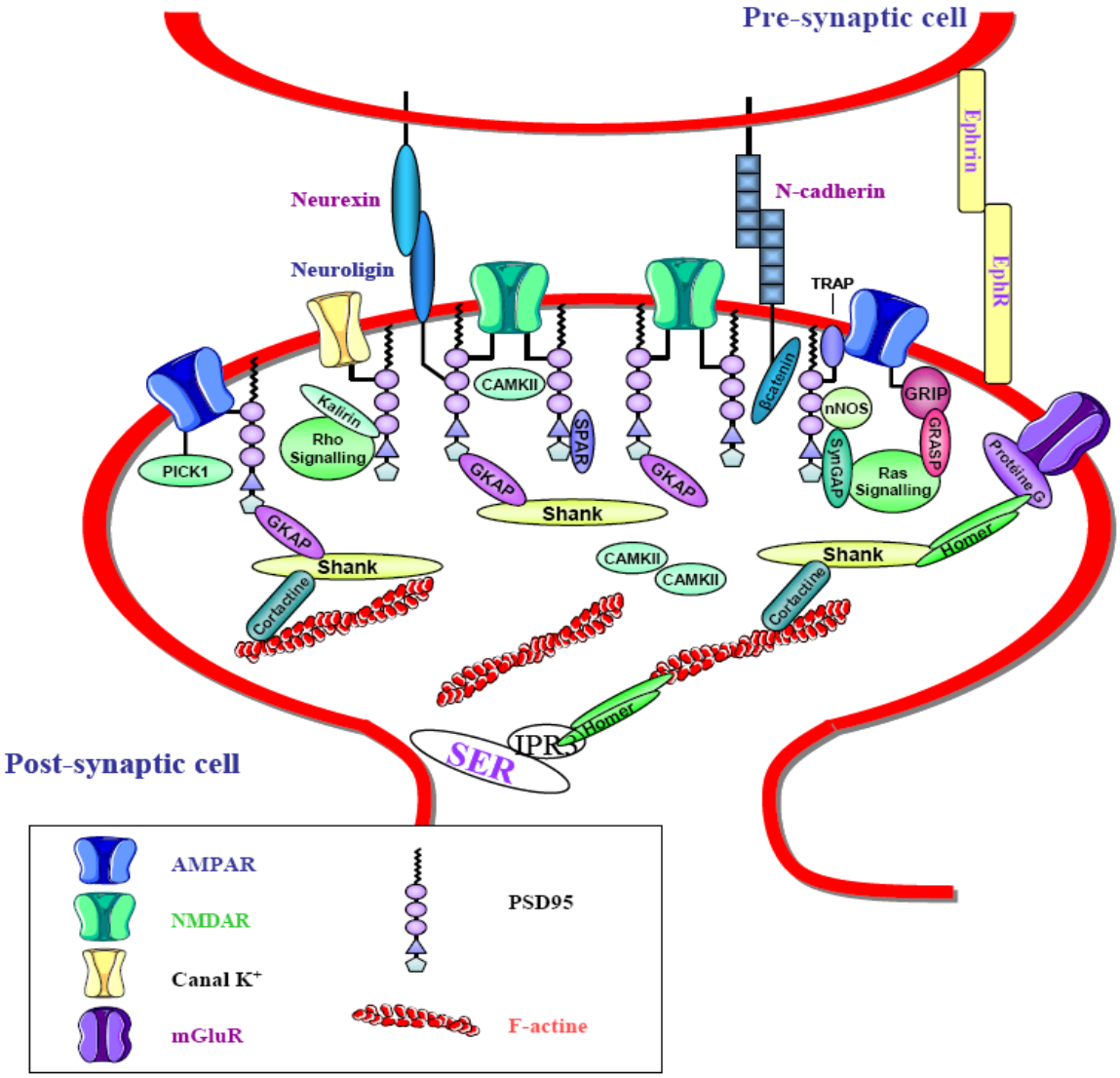


Figure 24: Protein organization in the postsynaptic density (PSD).
The PSD is an electron-dense structure found at the top of the head of the spines. The PSD can be divided into three layers: the first layer contains membrane receptors AMPARs, NMDARs, mGluR), ion channels (K + channels) and transmembrane adhesion molecule (N-cadherin), the second layer consists of scaffolding proteins arranged perpendicularly to the membrane (PSD95), the last layer is formed of Shank proteins and proteins of the GKAP family. They are organized parallel to the membrane and are connected to the actin filaments. Many other signaling proteins are found including AKAP, adenylate-kinase anchoring protein; EphR, ephrin receptor; GKAP, guanylate kinase-associated protein; GRASP, GRIP-associated protein; GRIP, glutamate receptor interacting protein; IP3R, inositol-1,4,5-trisphosphate receptor; MAP1A, microtubule-associated protein 1A; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase; PICK1, protein interacting with PRKCA1; SER, smooth endoplasmic reticulum; SPAR, spine-associated RAPGAP; SV, synaptic vesicle; SyNGAP, synaptic Ras GTPase-activating protein; TIAM1, T-cell lymphoma invasion and metastasis 1; TRAP, C-terminal receptor-binding region (Adapted from Cingolani and Goda, 2008 and Carlisle et al., 2008).

Cofilin regulates the dynamics of the actin cytoskeleton by binding and depolymerizating F-actin. Hotulainen et al. showed in their study in 2009 that inhibition by

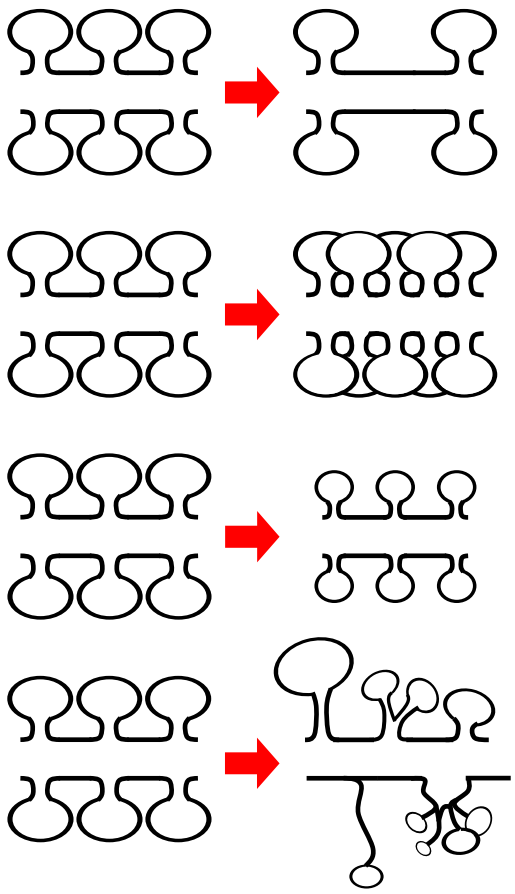
siRNA of cofilin causes an increase of "branched" spines. In addition, it is interesting to note that the activity of cofilin is inhibited by phosphorylation at Ser3. Several factors may control its activity, including the LIM kinase and phosphatases such as SSH or calcineurin (Kurita et al., 2007, Meng et al., 2002, Carlisle et al., 2008). In 2007, Zeng et al. described an alteration of dendritic spines after injection of kainate. A decreased level of phospho-Cofilin and thereby polymerized actin was the cause. The authors succeeded in improving the dendritic phenotype by using a calcineurin inhibitor. Gu et al., in 2010 reported that the state of phosphorylation of Cofilin regulates the morphology of spines.

11.3. Dendritic spine pathologies:

Some neurological diseases or cognitive impairment disorders are due to a loss of spines or spine morphological disturbance (Blanpied and Ehlers, 2004). For example, spines become abnormal or are lost in stroke, epilepsy, major depression, normal aging and chronic substance abuse (Fiala et al., 2002; Nimchinsky et al., 2002; Swann et al., 2000). The spine morphology being linked to synaptic function, spine abnormalities are likely to have diverse functional effects (Calabrese et al., 2006) **(Figure 25)**. They are, as already mentioned, the support of the glutamatergic synapses in the hippocampus and their structure is related to this synaptic activity.

Altered density and morphology of dendritic spines has been identified in patients with Fragile X Syndrome (XFRA). Indeed the post-mortem observation of the neo-cortex of these patients showed an increase in the density of these spines, including the proportion of immature spines. The study of FID1-KO mouse model confirmed the dendritic phenotype in the neo-cortex (von Bohlen und Halbach, 2009). Grossman et al., in 2010 described an increase in the amount of "thin" immature spines and a decline of mature spines ("mushroom" and "stubby") in the dentate gyrus of these mice (Grossman et al., 2006). Mutation in the PAK3, a serine/thréonine kinase of the family of the “p21-activated kinase” (PAK1-3), cause a nonsyndromic ID. Boda et al., in 2004 described that inactivation of PAK3 (by siRNA or antisense oligonucleotides) leads to alterations in the function and morphology of dendritic spines. Indeed, following PAK3 gene silencing in hippocampal organotypic cultures, they observed a reduction in the number of mature spines and of LTP. The molecular mechanism is not yet defined, but it seems that activation

of PAK3 by phosphorylation (by Rac or Cdc42) is necessary for activation of the LIMK and Cofilin.



Decrease in density:
MR, epilepsy, Alzheimer's malnutrition

Increase in density:
X syndrome, Fragile, sudden death syndrome of the newborn

Decrease in size:
Schizophrenia, Down syndrome

Change in morphology:
MR, epilepsy, malnutrition

Figure 25: Pathologies associated with an alteration of the density and morphology of spines.

Morphological defects of dendritic spines have been identified in many diseases, such as MR (Fragile X Syndrome and Down syndrome), schizophrenia, epilepsy or malnutrition. Alterations in the density, the size and morphology of spines have particularly been reported (Adapted from Fiala et al., 2002).

Boda et al., in 2008 showed that a constitutionally active form of PAK1, another member of this family, could restore the dendritic phenotype caused by the inhibition of PAK3. It is important to note that Meng et al. in 2005 did not observe these spine abnormalities in their PAK3-KO mouse model. However, these mice showed a reduction in L-LTP. The authors suggested that it might be due to a decrease in the level of phosphorylation of CREB and thus of gene transcription. The role of PAK3 in the maturation of dendritic spines remains

controversial. More recently, a study in the laboratory of JC Lauterborn has demonstrated a role for PAK kinases in the dendritic phenotype of FID1-KO mice. Indeed, a decreased activity of PAKs as well as their activator Rac1 was observed in the hippocampus of KO animals after "theta burst" stimulation. The authors suggested the involvement of these proteins in the stabilization of newly formed actin filaments (Chen et al., 2010).

A Ph.D. student, Anne Scneider, in our laboratory has shown that the spine morphology in *Rsk2*-KO neurons is altered. Anne preliminary results showed that there is no difference in total spine density between WT and *Rsk2*-KO neurons, whereas the proportion of mature spine was significantly higher in *Rsk2*-KO neurons.

12. The RSK2 protein and cognitive deficit:

The development of animal models for *Rsk2* mutation and shRNA or siRNA technology are major advances to study the disease mechanisms of CLS and cognitive impairment. The main goal of our team is to explain the cognitive and behavioral alterations of CLS, based on our *Rsk2*-KO mouse model. A previous study described a deregulation of the dopaminergic system in the m*rsk2*_KO mouse. Based on HPLC analysis, an increased level of dopamine expression (neurotransmitter monoamines was identified in the cortex of *Rsk2*-KO mice **(Figure 26A1)**. It was shown that absence of RSK2 leads to increased phosphorylation of ERK1/2. This higher activity of ERK in parallel with a decreased Protein phosphatase 2 (PP2A) activities resulted in an increased Tyrosine hydroxylase (TH) activity (Marques Pereira et al., 2008) **(Figure 26A2)**. Indeed, this deregulation may contribute to the Cognitive impairment. It is interesting to note that a deregulation of the dopaminergic system was also found in other pathologies such as Parkinson's or schizophrenia.

A gene silencing study of the *Rsk2* gene by siRNA in neuroendocrine cells, (Zeniou-Meyer et al., 2008) showed the importance of RSK2 in the process of exocytosis and in neurotransmission. It was shown that RSK2 regulates the activity, by phosphorylation, of PDL1, a phospholipase required for membrane fusion process. Phosphorylation of PDL1 at Thr147 activates PDL1, allowing biosynthesis of phosphatidic acid (PA) required for exocytosis (Zeniou-Meyer et al., 2008) **(Figure 26C1-C2)**.

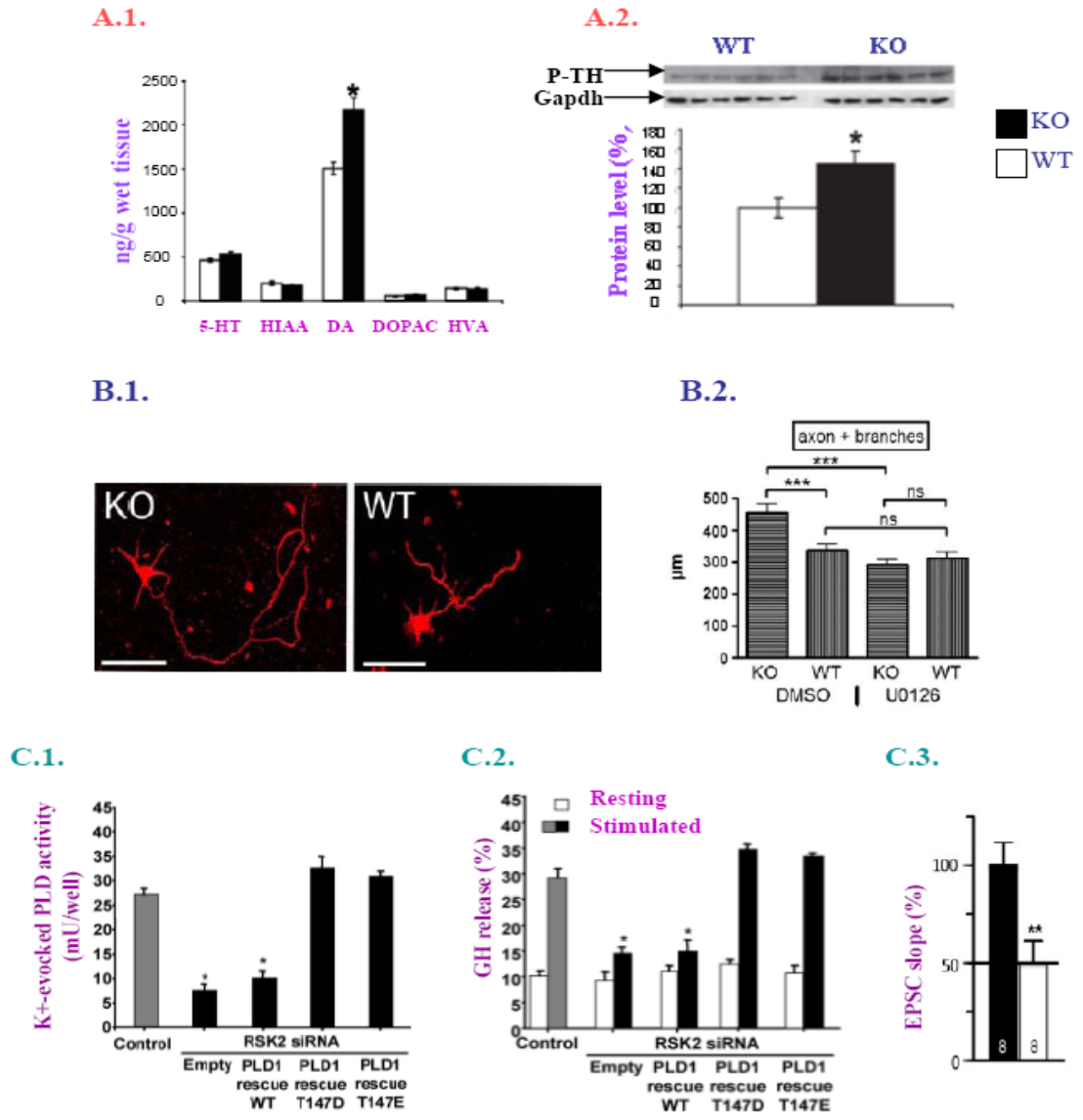


Figure 26: Recent advances in the study of the pathophysiology of MR associated with CLS.

(A) In 2008, Marques Pereira et al. described a dysregulation of the dopaminergic system in the cortex of *Rsk2*-KO mice. An increase in dopamine was identified by HPLC **(A.1.)**Data showed that it was due to increased tyrosine hydroxylase (TH) activity **(A.2.). (B)** In 2009 Fischer et al. identified, in motoneurone cultures, increased axonal length and of the number of their connections in the absence of RSK2 **(B.1.).** The authors rescued the phenotype by inhibiting the activity of ERK kinase **(B.2.). (C)** Zeniou-Meyer et al. in 2008 and 2009 describe the importance of RSK2 in exocytosis. Inhibition of RSK2 in endocrine cells led to reduced PDL1 activity **(C.1.)** and to the release of growth hormone **(C.2.).** These observations may explain the decreased expression of presynaptic LTP that was observed in the lateral amygdala by Zeniou-Meyer et al.in2010. **(C3).** (Adapted from Marques Pereira et al., 2008, Fischer et al., 2009a; Zeniou-Meyer et al. 2008-2009-2010).

This production is further enhanced by the recruitment of membrane Spo20p-GFP (a sensor of the synthesis of PA, homologous to SNAP25). The mobilization of this sensor is also regulated by RSK2 (Zeniou-Meyer et al., 2009). Moreover, it seems that another critical factor in the process of exocytosis, Scribble, allows the membrane localization of RSK2 (due to its PDZ domain) and its action on the biosynthesis of PA (unpublished but cited by Zeniou-Meyer and al., 2008). This study suggested that growth retardation and cognitive impairment associated with CLS could partially be explained by a defect in release of hormones or neurotransmitters.

Other studies involved RSK2 in axonal growth and branching, and in synapse formation. Fischer et al. observed in 2009 that axonal growth of *Rsk2* deficient motor neurons is affected. An increase in the length of axons and the number of connections has been identified in cultures of isolated neurons derived from KO mice **(Figure 26B1)**.

This effect is dependent on ERK activity because inhibition of ERK activity reversed this phenotype **(Figure26B2)**. The authors proposed that the increased level of ERK activity observed in RSK2 deficient neurons leads to a stronger activation of GSK3β and MAP1B and their substrate. Up regulation of the activity of these factors induces increased growth of microtubules as well as of axons.

In addition, recent data confirmed the role in the pre-synaptic function assigned to RSK2. A decrease in the expression of pre-synaptic LTP was identified in the lateral amygdala, whereas neither the release of neurotransmitters nor the probability or the recycling of synaptic vesicles was altered in mice *Rsk2*-KO (Zeniou-Meyer et al., 2010).

Results

Results:

The RSK2 protein is member of the RSK serine/threonine protein kinase. Although many cellular functions have been known through the RSK2 kinase. RSK2 belongs to a family of four highly homologous (Ser/Thr kinases, RSK1-4) members in human, RSK2 encoded by *Rps6ka3* gene and phosphorylated by ERK kinase. In order to understand the molecular and cellular consequences of *Rsk2* gene mutation, the physiopathological mechanisms of Intellectual disability associated with CLS patients and to develop a therapeutic strategy, our laboratory developed a *Rsk2*-KO mouse in 2004. This mouse displays learning and long-term spatial memory deficits (Poirier et al., 2007). In addition, although ubiquitously expressed, RSK2 shows, in the adult and embryonic brain, its highest levels of expression in the hippocampus (Zeniou et al., 2002) a structure involved in these cognitive processes. Some studies suggested that RSKs are involved in synaptic function, and especially in the synthesis and release of hormones and neurotransmitters. Together all the data suggested that RSK2 dysfunction might lead to deficiencies in synaptic transmission and plasticity, and especially in the hippocampus.

The objectives of my thesis were to further investigate the role of RSK2 in the hippocampus. My study was focused initially on identifying target gene of RSK2 by comparing global gene expression profiles of hippocampi from *Rsk2*-KO and WT littermate mice. My subsequent goal was to try to unravel the increase of ERK1/2 signaling in hippocampal neurons of *Rsk2*-KO mice. The third objective was to explore the signaling mechanism involved in the increased expression of one gene identified by transcriptome profiling, Gria2, encoding for the GluR2 protein. This GluR2 protein is important for the function of glutamergic AMPA receptors and play and important role in synaptic plasticity and neurotransmission.

We conducted most of the studies on hippocampal tissues or on hippocampal derived primary neuronal. Moreover, to understand the molecular mechanism involved in the alteration of expression of GluR2 in RSK2 deficient cells, we used RNA interference technology to knockdown *Rsk2* gene in the PC12 cell line.

Our findings may be helpful, to develop new therapeutic strategies for Coffin-

Lowry syndrome, e.g. by modulating the glutamatergic neurotransmission and probably synaptic plasticity. The studies were done in the laboratory of Dr. Andre Hanauer.

Publication-1

2.1. Transcriptome profile reveals AMPA receptor dysfunction in the hippocampus of the *Rsk2*-knockout mice, an animal model of Coffin Lowry syndrome

Tahir Mehmood, Anne Schneider, Jérémie Sibillec, Patricia Marques Pereira, Solange Pannetier, Mohamed Raafet Ammar, Doulaye Dembele, Christelle Thibault-Carpentier, Nathalie Rouach and André Hanauer. (2011).
Hum Genet *129*, 255-269.

The first aim of my PhD project was to identify new target genes of *Rsk2* in the hippocampus of *Rsk2*-KO mice, with the hope that they may help us to identify cellular processes affected by the absence of the RSK2 kinase. To this end we performed a comparison of the hippocampal gene expression profiles from *Rsk2*-KO and WT littermate. There was increasing evidence that RSK2 plays a role in regulating gene expression at the transcription level. As discussed in detail in the introduction, RSK2 is implicated in the control of the activity of many transcription factors by direct phosphorylation (CREB, ATF4, ER, p53….) (Cho et al., 2005; Sutherland et al., 1993) or via phosphorylation of an intermediate molecule, as for GSK3 and NFκB (Peng et al., 2010a). Furthermore, RSK2 deficiency leads to decreased expression of immediate early genes, including the transcription factors c-Fos in fibroblasts of patients or in osteoblasts (David et al., 2005; De Cesare et al., 1998). Importantly, there is a huge body of evidence showing that the ERK pathway plays a pivotal role in neuronal and cognitive functions. Therefore, we speculated that deregulation of gene expression in the hippocampus might play a role in the mechanism leading to cognitive deficits.

By microarray analysis we have identified differential expression of 100 genes (75 increased and 25 decreased in *Rsk2*-KO mice) encoding proteins acting in various biological pathways. The genes identified by the study are involved in various biological pathways, including cell cycle, cellular development, growth and proliferation apoptosis, and contain molecule transporters, enzymes, transcription or translation factors and some are also involved in different metabolic pathways, such as the metabolism of iron, calcium or in various genetic diseases. Deregulation of many of these genes was confirmed at RNA level by QRT-PCR and protein level by Western blot and immunohistochemistry.

We focused on functional studies for one of these genes, which showed an abnormal two-fold increased expression at the protein and RNA levels, Gria2. The Gria2 gene encodes the GluR2 proteins, a subunit of the glutamate ionotropic AMPA receptor. We showed that *Gria2* expression is up regulated in the hippocampus of *Rsk2*-KO mice by in situ hybridization and that GluR2 subunit is over represented at the surface of synapses in primary neuronal cultures. Electrophysiology studies conducted in collaboration with N. Rouach (College de France, Paris), showed a 25% reduction of basal AMPA receptor-mediated transmission in the hippocampus of *Rsk2*-KO mice. Furthermore, analysis of the coding sequence of *Gria2* showed a modification of splicing and editing of gria2 in *Rsk2*-KO animals. An increase in the flop variant and a decrease in editing R/G were actually observed.

In this study, we identified a number of genes whose expression is altered in the hippocampus of mouse *Rsk2*-KO as compared to WT mice littermate. This definitively confirms that RSK2 is implicated in regulating many cellular processes. The main conclusion of this study was that deregulation of the expression, splicing and editing *Gria2* in *Rsk2*-KO mice, and leading to altered AMPA neurotransmission, may contribute to the cognitive dysfunction in *Rsk2*-KO mice and in CLS patients. However, many other interesting genes identified by the transcriptional study remain to be explored for their implication in neuronal and cognitive dysfunction in *Rsk2*-KO mice.

A more detailed description and illustration of these results are found in publication- 1.

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DOI 10.1007/s00439-010-0918-0

ORIGINAL INVESTIGATION

Transcriptome profile reveals AMPA receptor dysfunction in the hippocampus of the *Rsk2*-knockout mice, an animal model of Coffin–Lowry syndrome

Tahir Mehmood · Anne Schneider · Jérémie Sibillec · Patricia Marques Pereira · Solange Pannetier · Mohamed Raafet Ammar · Doulaye Dembele · Christelle Thibault-Carpentier · Nathalie Rouach · André Hanauer

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Abstract Coffin–Lowry syndrome (CLS) is a syndromic form of mental retardation caused by loss of function mutations in the X-linked *RPS6KA3* gene, which encodes RSK2, a serine/threonine kinase acting in the MAPK/ERK pathway. The mouse invalidated for the *Rps6ka3* (*Rsk2*-KO) gene displays learning and long-term spatial memory deficits. In the current study, we compared hippocampal gene expression profiles from *Rsk2*-KO and normal littermate mice to identify changes in molecular pathways. Differential expression was observed for 100 genes encoding proteins acting in various biological pathways, including cell growth and proliferation, cell death and higher brain function. The twofold up-regulated gene (*Gria2*) was of particular interest because it encodes the subunit GLUR2 of the AMPA glutamate receptor. AMPA receptors mediate most fast excitatory synaptic transmission in the central nervous system. We provide evidence that in the hippocampus of *Rsk2*-KO mice, expression of GLUR2 at the mRNA and at the protein levels is significantly increased, whereas basal AMPA receptor-mediated transmission in the hippocampus of *Rsk2*-KO mice is significantly decreased. This is the first time that such deregulations have been demonstrated in the mouse model of the Coffin–Lowry syndrome. Our findings suggest that a defect in AMPA neurotransmission and plasticity contribute to mental retardation in CLS patients.

T. Mehmood and A. Schneider contributed equally to this work.

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Introduction

Coffin–Lowry syndrome (CLS; MIM#303600) is a rare syndromic form of mental retardation that is characterized by moderate to severe psychomotor retardation, growth retardation, facial and digital dysmorphisms, as well as progressive skeletal deformations (Hanauer and Young 2002). The gene mutated in CLS patients (*RPS6KA3*) encodes a protein of 740 amino acids, RSK2 (alternative names: $p90^{RSK2}$, MAPKAPK1B), which belongs to a family of four highly homologous proteins (RSK1–4), encoded by distinct genes. RSKs are Ser/Thr protein kinases that act at the distal end of the mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) signaling pathway. RSKs are directly phosphorylated and activated by ERK1/2 in response to many growth factors and neurotransmitters (Frödin and

Gammeltoft 1999). RSK2 phosphorylates a wide range of cytosolic substrates, such as GSK3 and IκB, and nuclear substrates including ATF4, c-FOS and NUR77, CREB and histone H3 (De Cesare et al. 1998; Sassone-Corsi et al. 1999). Activation of RSK2 is, therefore, thought to influence gene expression and to be involved in cell proliferation and survival. Numerous studies implicate the MAPK/ERK signaling cascade and CREB-mediated gene transcription in synaptic plasticity and memory (Davis and Laroche 2006). In human and mouse brain, RSK2 is highly expressed in the hippocampus, that is, an essential brain structure in cognitive function and learning (Zeniou et al. 2002; Guimiot et al. 2004). RSK2-deficient mice show delayed acquisition of a spatial memory reference task and long-term spatial memory deficits (Poirier et al. 2007). Thus, together the data suggest that RSK2 plays an important role in cognitive function in human and in mice.

To gain greater insight into the molecular mechanisms leading to learning and memory impairments in the *Rsk2*-KO mice and to mental retardation in CLS, we examined in the present study global gene expression profiles in hippocampus from KO mice. The data revealed significant alteration of 100 genes acting in a great variety of biological pathway in *Rsk2*-KO hippocampi. We further investigated the function of one of these genes, *Gria2*, which showed a twofold up-regulation in mutant mice. *Gria2* encodes the subunit GLUR2 of the AMPA receptor (AMPAR). AMPARs are ligand-activated cation channels that mediate the fast component of excitatory postsynaptic currents in neurons of the central nervous system. The GLUR2 subunit controls AMPAR Ca^{++} permeability, and is involved in several forms of long-term synaptic plasticity. Our results show that the expression of GLUR2 is increased at the mRNA and at the protein level in the hippocampus, as well as at the surface of synapses in hippocampal primary cell cultures. Furthermore, basal excitatory synaptic transmission through AMPARs is impaired in the hippocampus of *Rsk2* mutants.

Materials and methods

Animals and tissue dissection

Male *Rsk2*-KO and WT mice with a C57Bl/6x genetic background were killed by cervical dislocation. Brains were rapidly dissected and the hippocampus was isolated using a standard dissection procedure. Tissue samples were immediately frozen in liquid nitrogen and kept at −80°C until use. All experiments were carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC). Every effort was made to minimize the number of animals used and their suffering.

Microarray hybridization

To reduce variability, but also to obtain enough RNA for the hybridization of each DNA chip, all probes for the gene array experiments consisted of pooled RNA samples from either two WT or two KO animals. In brief, total RNA was extracted from hippocampi of six KO and six WT 5-month-old male mice and purified using the TRIzol reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions. The quality of total RNA was monitored by Agilent 2100 Bioanalyzer (LabChip, Agilent technologies, Massy, France). Two RNA samples for each genotype were then pooled in equal quantities (thus, resulting in a total of three arrays for each genotype). Generation of double-stranded cDNA from 2.5 μg of total RNA of each pooled RNA sample, preparation and labeling of cRNA, hybridization to 430A 2.0 mouse genome arrays (Affymetrix, Santa Clara, CA), washing, and scanning were performed according to the protocols recommended by Affymetrix in their GeneChip® Expression Analysis Technical Manual (Affymetrix). The data of the expression arrays produced for this report have been deposited in the Gene Expression Omnibus (GEO) databank.

Microarray data analysis

Data were processed using the Affymetrix GeneChip Operating Software [GCOS v1.4; Microarray Suite (MAS 5.0) algorithm]. Genes differentially expressed were selected using the following steps: (1) selection of probesets having a signal value above 15 (35th percentile of all expression values) in at least one array out the 6, (2) selection of probesets called present in at least two out of three arrays for one of the two genotypes, (3) selection of probesets with a *t* test $p < 0.03$, (4) selection of probesets having a fold change greater than 1.5. We finally verified that selected probes have acceptable false discovery rate (10%) (Benjamini and Hochberg 1995).

Ingenuity pathways analysis

Biologically relevant networks were created using the ingenuity pathways analysis program (http://www.Ingenuity.com), using the default parameters. Based on the algorithmically generated connectivity between gene–gene, gene–protein, and protein–protein interactions, the program develops functional molecular networks that overlay genes in the dataset. This program calculated *p* values for each network by comparing the number of focus genes that were mapped in a given network, relative to the total number of occurrences of those genes in all networks. The score for each network is shown as the negative log of the *p* value, which indicates the likelihood of finding a set of genes in the

network by random chance. A score of 20 indicates that there is a 10^{-20} chance that the focus genes would be in a network because of random chance.

Real-time QRT-PCR analysis

QRT-PCR assays were performed on hippocampal RNA samples obtained from WT and KO mice different from those used for transcriptional profiling. RNA extraction and QRT-PCR was performed as previously described (Marques Pereira et al. 2008). A probe set for detection of mouse *Gapdh* was used as an endogenous control gene. The sequences of primers of the tested genes are listed in Supplemental Table 1.

Western blot analysis

Protein extractions and Western blot analyses were performed as previously described (Marques Pereira et al. 2008). Quantifications were carried out with the GeneTool software of the Chemigenius apparatus (Syngene, Frederick, MD, USA). Data were normalized either to GAPDH or to β-TUBULIN. Student's t test (two-tailed) was used to determine the significance between the control and *Rsk2*-KO samples, and $p \leq 0.05$ was considered significant. Antibodies against GLUR2 (Millipore Corporation), CACNG8 and VAMP4 (Abcam), EIF3A (Cell Signaling Technology), DIABLO (Calbiochem), GAPDH (Chemicon) and β TUBULIN (Millipore) were used.

Immunohistochemistry

Frozen brain section was left 10 min at room temperature, fixed for 10 min with 4% PFA and washed twice (1× PBS). Endogenous peroxidase was inhibited by a treatment with 0.3% H_2O_2 solution. After washing, slides were incubated in 10% normal goat serum in 1× PBS for 1 h. Primary antibodies were added to the sections in 10% normal goat serum and incubated overnight at 4°C. Antibody dilutions were as follows: rabbit anti-GLUR2 (1:1000, Millipore Corporation), rabbit anti-VAMP4 (1:1,000, Abcam), and rabbit anti-IGF-1 (1:100, Abcam). Slides were subsequently washed four times for 10 min in 1× PBS and incubated with biotinylated secondary antibody for 2 h. After washing, sections were incubated for 30 min in Vectastain elite ABC reagent and treated with peroxidase substrate solution until desired stain intensity. After washing, samples were mounted with KAISER's glycerol gelatin (Merck).

In situ hybridization

Plasmids containing 3′UTR regions of mouse *Gria1* (encoding GLUR1) or *Gria2* (encoding GLUR2) cDNAs were amplified by PCR using vector-specific primers and PCR reactions were purified using Montage 96 (Millipore Corporation, Bedford, USA). Amplicons were then used as template for in vitro transcription of sense and anti-sense Dig-labeled riboprobes. To this aim, 1 µg linearized DNA was transcribed using T7, T3 or Sp6 polymerases and the 10× DIG RNA labeling mix (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. Brain sections 25-µm thick were processed for ISH using GenePaint robotic equipment and procedures (http://www.genepaint.org) as previously described (Nakamoto et al. 2007).

Primary hippocampal cultures

Hippocampi dissected from WT and *Rsk2*-KO male mice at embryonic day 17 were triturated and plated into wells of 24-well plates containing poly-D-lysine-coated coverslips (Sigma), at a density of ~1,00,000 neurons/well. Growth media consisted of NeuroBasal (GIBCO, Invitrogen) supplemented with 1× B27 (GIBCO, Invitrogen), 0.5 mM L-glutamine and 1× penicillin/streptomycin. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and cultivated for 14 DIV prior to experimentation.

Immunocytochemistry

To label surface GLUR1 (sGLUR1) and GLUR2 (sGLUR2)-containing AMPARs, 14 DIV live neurons were treated as previously described (Ghate et al. 2007) with minor modifications. Cells were incubated with rabbit anti-N-terminal GLUR1 (Calbiochem) or mouse anti-N-terminal GLUR2 (Millipore) and mouse anti-PSD95 (NeuroMab).

Microscopy and data analysis

All images acquisitions and quantifications were performed using standardized settings on a microscope (model DM4000 B, Leica) equipped with CCD camera (CoolSnap CF, color) with a ×63 objective. Obtained Tiff files were subjected to quantification with ImageJ software (http://www.rsb.info.nih.gov/ij/). For sGLURs quantification, the three thickest dendrites per pyramidal neuron and five neurons per sample were blindly chosen and the dendritic branches were manually traced and measured. AMPA receptors clusters were counted and the number of clusters was normalized with the dendritic length. Student's t tests were used for comparison between WT and *Rsk2*-KO cultures.

Determination of relative *Gria2* Q/R and R/G editing, and flip/flop splice levels

Total RNA was extracted (as above) from five *Rsk2*-KO and five WT hippocampi, the *Gria2* mRNA amplified by

RT–PCR (three times each from independent RNA preparations) and the product sequenced to determine the relative levels of editing and splicing (Lee et al. 1998).

Electrophysiology

Standard techniques were used to prepare transverse acute hippocampal slices (400-μm thick) from 4-week-old mice. Slices were maintained at room temperature in a storage chamber that was perfused with an artificial cerebrospinal fluid (ACSF) (mM: 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$ and 11 glucose and equilibrated with 95% O_2 and 5% CO_2) for at least 1 h prior to recording. For synaptic recordings, a cut was made between the CA3 and CA1 region to prevent bursting, and the slices were bathed in a modified ACSF containing 100-μm picrotoxin to block GABAAreceptor- mediated inhibitory postsynaptic currents. Field excitatory postsynaptic potentials (fEPSPs) were recorded with glass pipettes (2–5 MΩ) filled with ACSF, by stimulating Schaffer collaterals in stratum radiatum (0.1 Hz) with a monopolar stimulating electrode. Responses were collected with Axopatch-1D amplifier (Axon Instruments), filtered at 2 kHz, digitized at 10 kHz, and analyzed online using Clampfit software (Molecular Devices).

Results

Expression profiling of wild-type and *Rsk2*-KO mice

To identify molecular changes potentially responsible for the phenotype associated with *RSPS6KA3* gene mutation in the hippocampus, we performed a detailed comparison of the transcriptional profiles of hippocampi isolated from six KO and six WT 5-month-old male mice. To reduce variability, equivalent amounts of RNA from two mice with the same genotype were pooled and processed for hybridization to the genome wide oligonucleotide microarray (thus, three arrays per genotype). Out of the 22,690 probesets represented on the microarray, filter A selected 16,865 probesets that were restricted to 14,348 probesets by filter b and to 635 probesets by filter c. Filter d, selected a final number of 109 probesets. Eight of these 109 differentially expressed genes were verified by two or, in one case, three distinct probe sets. These multiple probe sets of eight genes displayed consistent direction and similar extent of changes in abundances of corresponding mRNAs. The final list of 100 significant non-redundant genes is shown in Table 1. Genes are tabulated according to functional category and degree of over-expression/repression.

Most of them have a recognized function and can be assigned to functional categories and subcategories. Among them, 75 genes were transcriptionally up-regulated, whereas 25 genes were down-regulated. The most upregulated genes were thymosin beta 10 (*Tmsb10*, fold change of 3.51), followed by establishment of cohesion 1 homolog A (*Esco 1*, fold change of 2.91) and thyroid hormone receptor interactor 11 (*Trip 11*, fold change of 2.27). The most downregulated genes were erythroid differentiation regulator 1 (*Erdr1*) and serpin peptidase inhibitor, clade C, member 1 (*Serpin1*) (−2.9 and −2.4 fold downregulations, respectively). Major functional categories include enzymes with various activities (17 up-regulated/2 down-regulated), growth factors (2/0), ion channels (3/0), kinases (1/3), peptidases (2/1), transcription regulators (6/1), transmembrane receptors (1/0), transporters (7/2) and molecules with other functions (33/14).

Validation of microarray data by QRT-PCR

Twenty-four of these differentially expressed genes were selected for validation, by QRT-PCR, based on the known or putative neuronal functional roles (Table 1). These genes represent different categories: genes implicated in exocytosis (*Stxbp3* and *Vamp4*), in mental retardation (*Cul4b*, *Lamp2*, *Vldlr, Igf1*), in apoptosis (*Stk3*, *Rasl10a*, *Diablo*), in cell differentiation and cytoskeleton organization (*Phkg1*, *Pdlim5*, *Timsb10*, *Enc1*, *Nptxr*, *Ptpn2*, *Carhsp1*, *Phip*, *Plek*, *Arhgap12*, *Cfl1*), in translation regulation (*Eif3A*) and finally genes encoding ion channel sub-units (*Cacnb4*, *Cacng8*, *Gria2*). The results of the microarray findings were validated in all the genes tested by real-time PCR, although the fold change was not always accurately replicated. We also confirmed by QRT-PCR unaltered expression of some genes (including cFos, CREB) that had similar levels of expression in KO and WT animals on microarrays (results not shown).

Identification of biologically relevant networks

To gain insight into interactions among the differentially expressed genes, we constructed biologically relevant networks using the ingenuity pathway analysis software. From the 100 differentially expressed genes, 78 genes were mapped and assembled into five biological networks with a score of $\geq$20. The network with the most significant score (of 45) contains 23 of the differentially expressed genes. This network contains genes involved in cell cycle, cellular development, growth and proliferation and centers on the NFκB complex. This network contains also Rb1 and Sod2, both important actors in cellular growth and apoptosis, and over expressed in *Rsk2*-KO neurons. The network with the second highest score (of 34) is centered on TGFβ1, which controls proliferation, cellular differentiation, but also various other functions. Fourteen genes with altered

Table 1 List of genes differentially expressed in the hippocampus of Rsk2-KO mice

Functional category	Probe set	Genbank	Gene symbol	Gene name	Microarray		qRT-PCR			
					F C KO/WT	*p*	WT mean Ct ± SD	KO mean Ct ± SD	FC	*p*
Genes showing decreased expression										
Kinases	1422315_x_at	NM_011079	*Phkg1*	Phosphorylase kinase gamma 1	0.54	0.0162	1 ± 0.03	0.68 ± 0.11	0.68	0.003
	1418052_at	NM_023556	*Mvk*	Mevalonate kinase	0.55	0.0190				
	1427282_a_at	NM_008044	*Fxn*	Frataxin	0.61	0.0074				
Enzymes	1448330_at	NM_010358	*Gstm5*	Glutathione *S*-transferase M5	0.61	0.023				
	1427975_at	NM_145216	*Rasl10a*	Ras-like, family 10, member A	0.62	0.015	1 ± 0.05	0.70 ± 0.059	0.70	0.004
Peptidase	1433918_at	NM_034941	*Atg4d*		0.59	0.0057				
Cytokine	1418345_at	NM_023517	*Tnfsf13*	Tumor necrosis factor (ligand) superfamily, member 13	0.60	0.0057				
Transcription regulator	1418636_at	NM_001083318	*Etv3*	Ets variant gene 3	0.55	0.026				
Transporters	1423927_at	NM_028662	*Slc35b2*	Solute carrier family 35, member B2	0.59	0.011				
	1450073_at	NM_008444	*Kif3b*	Kinesin family, member 3B	0.64	0.00093				
Transmembrane receptor	1450147_at	NM_030689	*Nptxr*	Neuronal pentraxin receptor	0.62	0.013	1 ± 0.008	0.87 ± 0.35	0.87	0.035
Other functions	1452406_x_at	NM_133362	*Erdr1*	Erythroid differentiation regulator 1	0.35	0.029				
	1417909_at	NM_080844	*Serpinc1*	Serpin peptidase inhibitor, clade C, member 1	0.41	0.023				
	1415976_a_at	NM_025821	*Carhsp1*	Calcium regulated head stable protein 1,24 kDa	0.50	0.00036	1 ± 0.07	0.63 ± 0.07	0.63	0.034
	1452652_a_at	NM_001002267	*Tmem158*	Transmembrane protein 158	0.55	0.015				
	1448577_x_at	NM_009304	*Syngr2*	Synaptogyrin 2	0.56	0.021				
	1455098_a_at	NM_011707	*Vtn*	Vitronectin	0.57	0.0022				
	1453111_a_at	NM_026542	*Slc25a39*	Solute carrier family 25, member 39	0.61	0.011				
	1418396_at	NM_134116	*Gpsm3*	G-protein signaling modulator 3	0.63	0.016				
	1423940_at	NM_026553	*Yif1A*	Yip1 interacting factor homolog A (*S. cerevisiae*)	0.63	0.016				
	1418123_at	NM_011676	*Unc119*	Unc-119 homolog (*C. elegans*)	0.64	0.0016				
	1450468_at	NM_010865	*Myoc*	Myocilin	0.64	0.0078				
	1415886_at	NM_013781	*Sh2d3c*	SH2 domain containing 3C	0.64	0.001				
	1422596_at	NM_021426	*Nkain4*	Na+/K+ transporting ATPase interacting 4	0.65	0.022				
	1450255_at	NM_020260	*Cdgap*	Cdc42 GTPase-activating protein	0.65	0.009				
Genes showing increased expression										
Kinases	1418513_at	NM_019635	*Stk3*	Serine/threonine kinase 3	1.76	0.023	1 ± 0.05	1.45 ± 0.08	1.45	0.042
Phosphatase	1438562_a_at	NM_001127177	*Ptpn2*	Protein tyrosine phosphatase, non-receptor type 2	1.95	0.016	1 ± 0.091	1.85 ± 0.28	1.83	0.045
Growth factors	1437401_at	NM_010512	*Igf1*	Insulin-like growth factor 1	1.65	0.0093	1 ± 0.09	2.24 ± 0.32	2.24	0.042
	1417069_a_at	NM_022023	*Gmfb*	Maturation factor, beta	1.54	0.0055				

Table 1 continued

Functional category	Probe set	Genbank	Gene symbol	Gene name	Microarray		qRT-PCR			
					F C KO/WT	*p*	WT mean Ct ± SD	KO mean Ct ± SD	FC	*p*
Enzymes	1448734_at	NM_001042611	*Cp*	Ceruloplasmin	2.29	0.029				
	1417262_at	NM_011198	*Ptgs2*	Prostaglandin-endoperoxidase synthase 2	2.28	0.0047				
	1452484_at	NM_053070	*Ca7*	Carbonic anhydrase VII	2.23	0.028				
	1417194_at	NM_013671	*Sod2*	Superoxide dismutase 2	1.98	0.0089				
	1452158_at	NM_029735	*Eprs*	Glutamyl-prolyl-tRNA synthetase	1.95	0.015				
	1453928_a_at	NM_009278	*Ssb*	Sjogren syndrome antigen B	1.86	0.020				
	1451436_at	NM_001081203	*Sbno1*	sno, strawberry notch homolog 1 (*Drosophila*)	1.78	0.022				
	1454696_at	NM_008142	*Gnb1*	Guanine nucleotide binding protein, beta polypeptide 1	1.70	0.0016	1 ± 0.033	1.36 ± 0.061	1.36	0.0005
	1419064_a_at	NM_011674	*Ugt8*	UDP-glycosyltransferase 8	1.69	0.0059				
	1416497_at	NM_009787	*Pdia4*	Protein disulfide isomerase family A, member 4	1.68	0.0061				
	1423033_at	NM_008408	*Stt3a*	Subunit of the oligosacchayltransferase complex, homolog A	1.66	0.028				
	1435164_s_at	NM_011666	*Ube1c*	Ubiquitin-activating enzyme E1C	1.65	0.0062				
	145388_at	NM_183028	*Pcmtd1*	protein-L-isoaspartate *O*-methyltransferase domain containing 1	1.60	0.026				
	1416343_a_at	NM_010685	*Lamp2*	Lysosomal-associated membrane protein 2	1.58	0.023	1 ± 0.05	1.65 ± 0.19	1.65	0.038
	1418908_at	NM_013626	*Pam*	Peptidylglycine alpha-amidating monooxygenase	1.56	0.021				
	1451828_a_at	NM_207625	*Acsl4*	Acyl-CoA synthetase long chain family member 4	1.54	0.0035				
	1417697_at	NM_009230	*Soat1*	Sterol *O*-acyltransferase	1.51	0.015				
Peptidase	1420964_at	NM_007930	*Enc1*	Ectodermal-neural cortex	2.07	0.015	1 ± 0.05	1.77 ± 0.08	1.77	0.002
	1449718_s_at	NM_026273	*C3orf38*	Chromosome 3 open reading frame 38 homolog (human)	1.54	0.019				
Transcription regulators	1427406_at	NM_028446	*Trip11*	Thyroid hormone receptor interactor 11	2.27	0.0045				
	1423501_at	NM_008558	*Max*	MYC-associated factor X	1.91	0.023				
	1417187_at	NM_016786	*Hip2*	Huntington interacting protein 2	1.76	0.0071				
	1424704_at	NM_001145920	*Runx2*	Runt-related transcription factor 2	1.67	0.0068				
	1418265_s_at	NM_008391	*Irf2*	Interferon regulatory factor	1.60	0.023				
	1417850_at	NM_009029	*Rb1*	Retinoblastoma 1	1.58	0.0053				
Translation regulator	1416661_at	NM_010123	*Eif3a*	Eukaryotic translation initiation factor 3, subunit A	1.74	0.0087	1 ± 0.05	1.84 ± 0.11	1.84	0.005
Nuclear receptors	1422631_at	NM_013464	*Ahr*	Aryl hydrocarbon receptor	1.61	0.028				
	1416958_at	NM_011584	*NriD2*	Nuclear receptor subfamily 1, group D, member 2	1.54	0.0064				
Transporters	1422966_a_at	NM_011638	*Tfrc*	Transferrin receptor	2.10	0.0010				
	1416653_at	NM_011504	*Stxbp3*	Syntaxin-binding protein 3	1.92	0.013	1 ± 0.03	2.19 ± 0.6	2.19	0.038
	1442169_at	NM_013703	*Vldlr*	Very low density lipoprotein receptor	1.79	0.0068	1 ± 0.05	1.44 ± 0.055	1.44	0.0007
	1424924_at	NM_153055	*Sec63*	SEC63 homolog (*S. cerevisiae*)	1.74	0.015				
	1419975_at	NM_011327	*Scp2*	Sterol carrier protein 2	1.66	0.023				
	1416374_at	NM_018829	*Ap3m1*	Adaptor-related protein complex 3 mu 1 subunit	1.59	0.0032				
	1434513_at	NM_001128096	*Atp13a3*	ATPase type 13A3	1.52	0.0054				
Ion channels	1453098_at	NM_013540	*Gria2*	Glutamate receptor, ionotropic, AMPA2	2.01	0.024	1 ± 0.032	1.87 ± 0.09	1.87	0.0003
	1452089_at	NM_001037099	*Cacnb4*	Calcium channel, voltage-dependent, beta 4 subunit	1.85	0.0031	1 ± 0.022	1.34 ± 0.02	1.34	0.022
	1451864_at	NM_133190	*Cacng8*	Calcium channel, voltage-dependent, gamma subunit 8	1.79	0.012	1 ± 0.08	1.84 ± 0.26	1.84	0.005

Table 1 continued

Functional category	Probe set	Genbank	Gene symbol	Gene name	Microarray		qRT-PCR			
					F C KO/WT	*p*	WT mean Ct ± SD	KO mean Ct ± SD	FC	*p*
Other functions	1455946_x_at	NM_001039392	*Tmsb10*	Thymosin, beta 10	3.51	0.0052	1 ± 0.04	2.56 ± 0.05	2.56	0.028
	1424325_at	NM_001081222	*Esco1*	Establishment of cohesion 1 homolog 1 (*S. cerevisiae*)	2.91	0.024				
	1425768_at	NM_023232	*Diablo*	Diablo homolog (*Drosophila*)	2.05	0.023	1 ± 0.032	2.16 ± 0.05	2.16	0.0002
	1455475_at	NM_026622	*C4orf29*	Chromosome 4 open reading frame 29 homolog (human)	2.00	0.023				
	1423198_a_at	NM_134034	*Smek2*	SMEK homolog 2, suppressor of mek1 (Dictyostelium)	1.93	0.014				
	1424074_at	NM_027453	*Btf3l4*	Basic transcription factor 3-like 4	1.88	0.018				
	1420485_at	NM_023554	*Nol7*	Nucleolar protein 7	1.78	0.016				
	1438418_at	NM_144535	*Mudeng*	MU-2/AP1M2 domain containing, death-inducing	1.77	0.014				
	1421945_a_at	NM_001042556	*Bxdc1*	Brix domain containing 1	1.77	0.0077				
	1450418_a_at	NM_026553	Yif1a	Yip1-interacting factor homolog A (*S. cerevisiae*)	1.74	0.029				
	1449884_at	NM_026626	Efcab2	EF-hand calcium binding domain 2	1.71	0.020				
	1418008_at	NM_026110	C21orf66	Chromosome 21 open reading frame 66 homolog (human)	1.68	0.023				
	1438535_at	NM_001081216	Phip	Pleckstrin homology domain interacting protein	1.65	0.011	1 ± 0.19	2.08 ± 0.09	2.08	0.03
	1451525_at	NM_001039692	Arhgap12	Rho GTPase-activating protein 12	1.64	0.020	1 ± 0.09	1.54 ± 0.09	1.54	0.033
	1426271_at	NM_153808	Smc5	Structural maintenance of chromosome 5	1.63	0.015				
	1417170_at	NM_033322	Lztfl1	Leucine zipper transcription factor-like 1	1.62	0.029				
	1452247_at	NM_001113188	Fxr1	Fragile X mental retardation, autosomal homolog 1	1.62	0.0047				
	1451325_at	NM_027226	Fyttd1	Forty-two–three domain containing 1	1.61	0.0050				
	1425913_a_at	NM_144882	Dnaptp6	Viral DNA polymerase-trans-activated protein 6	1.60	0.012				
	1421940_at	NM_009282	Stag1	Stromal antigen 1	1.59	0.011				
	1448748_at	NM_019549	Plek	Pleckstrin	1.58	0.021	1 ± 0.05	1.12 ± 0.09	1.12	0.004
	1451830_a_at	NM_009260	Spnb2	Spectrin, beta, non-erythrocytic 1	1.58	0.019				
	1437463_x_at	NM_011577	TgfbI	Transforming growth factor, beta-induced	1.57	0.016				
	1423841_at	NM_026396	Bxdc2	Brix domain containing 2	1.57	0.026				
	1427104_at	NM_175480	Znf23	Zinc finger protein 612	1.56	0.015				
	1426806_at	NM_028696	Obfc2a	Oligonucleotide/oligosaccharide-binding fold containing 2A	1.55	0.023				
	1422896_at	NM_016796	Vamp4	Vesicle-associated membrane, protein 4	1.55	0.016	1 ± 0.07	1.48 ± 0.16	1.48	0.034
	1437790_at	NM_001081191	Eml5	Echinoderm microtubule associated protein like 5	1.54	0.020				
	1417453_at	NM_001110142	Cul4b	Cullin 4B	1.53	0.028	1 ± 0.03	1.12 ± 0.09	1.12	0.018
	1418066_at	NM_007688	Cfl2	Cofilin 2	1.53	0.019				
	1450407_a_at	NM_009672	Anp32a	Acidic nuclear phosphoprotein 32 family, member A	1.53	0.013				
	1450786_at	NM_019808	Pdlim5	PDZ and LIM domain 5	1.53	0.0057	1 ± 0.11	1.44 ± 0.059	1.44	0.047
	1425597_a_at	NM_021881	Qk	Quaking homolog, KH domain RNA binding	1.52	0.029				

Real-time quantitative RT–PCR was performed for 24 genes in hippocampi of *Rsk2*-KO ($n = 6$) and WT ($n = 6$) mice, using the primer pairs shown in Table S1. Relative expression levels are shown with the average expression value of the WT group set to 1

Ct cycle threshold values, *SD* standard deviation, *FC* fold change, *p p* value

expression in *Rsk2*-KO mice are associated with this second network, among which Nptxr, Cacng8, Pdlim5 and Soat1. The third network centered on MYC is mainly implicated in lipid metabolism and cell death. Thirteen focus genes are associated with this network, including Acsl4, Eif3a, Ca7, Cfl2 and Fxr1. The last two networks are mainly implicated in molecular transport and lipid metabolism (4th network) and amino acid metabolism and protein synthesis (5th network). The fourth network is centered on Ptgs2 and includes 12 focus genes, among which Igf1, Vamp4, Mvk, Stxbp3 and Gnb1. The fifth network is centered on retinoic acid and contains 13 focus genes, among which Cacnb4 and Carhsp1. Figure 1 shows the two most significant networks (the others are available on request to AH). Supplemental Table 2 lists the top related biological functions and diseases. Top biological functions include organismal injury and abnormalities (9 genes, including Fxn, Sod2, Igf1, Ahr and Ptgs2), cell cycle (12 genes including Rb1, Igf1 and Runx2), nervous system development and function (10 genes including Igf1, Ptgs2, Sod2, Gria2, Vldlr, Vtn and Rb1), organismal development and free radical scavenging (9 and 4 genes, respectively). Interestingly, five genes, including Gria2, Igf1, Ptgs2, Sod2 and Vldlr have been implicated in behavior, with the first four genes specifically in spatial memory formation. These five genes are all up-regulated in the hippocampus of *Rsk2*-KO mice. The p values in the range of 2.52×10^{-5} to 1.10×10^{-2} indicate statistical significance.

Confirmation of altered expression at the protein level

We confirmed increased expression at the protein level of GLUR2 (*Gria2* gene), CACNG8, VAMP4, EIF3A and DIABLO using quantitative western blot analysis (Fig. 2). These findings are in line with the changes detected by microarray-based analysis. We also confirmed differential expression of GLUR2, VAMP4 and IGF1 using immunohistochemical analysis. As shown in Fig. 3, we found increased expression of GLUR2 in the CA1 and CA3 regions and in the dentate gyrus of *Rsk2*-KO hippocampus. VAMP4 and IGF1 were increased in the whole hippocampus. Although the lack of specific antibodies precluded similar experimental validations for many other genes listed in Table 1, these observations suggested that the transcriptional changes observed in mutant mice may be generally reflected by matching changes in the levels of expression of their corresponding protein products.

Confirmation by in situ hybridization of Gria2 up-regulation

The expression of a number of genes involved in neurotransmission, including vesicle and receptor trafficking proteins, neurotransmitter receptors and ion channels, were altered in *Rsk2*-KO mice (Table 1). Of particular interest was the twofold increased level of expression of *Gria2*, encoding the subunit GLUR2 of the AMPAR. Since AMPARs mediate fast synaptic transmission at excitatory synapses in the brain and are thought to play key roles in synaptic plasticity, learning and memory (Seidenman et al. 2003) we wanted to further confirm up-regulation of the *Gria2* gene by in situ hybridization of WT and KO mice hippocampi. The expression of *Gria2* was significantly increased in all areas of the anterior hippocampus of mutant mice (Fig. 4a), whereas in the posterior hippocampus, the level of *Gria2*-mRNA was mainly increased in the dentate gyrus, in CA1 and in the CA3 region (Fig. 4b). We did not find any change in the level of expression of GLUR1 (not shown).

Increase in GLUR2 surface expression

We next wondered whether expression of GLUR2 at surface of synapses was also up-regulated since AMPARs in hippocampal neurons are mainly expressed as heteromers of GLUR1/2 as well as GLUR2/3. We compared the amount of GLUR2 at synapses in KO and WT cultured primary hippocampal neurons by staining surface GLUR2 (or GLUR1) clusters and counterstaining for PSD95. GLUR2 surface staining was punctate (Fig. 5a, b), and the number of GLUR2 puncta that were synaptic did significantly differ among WT and *Rsk2*-KO cultures (WT 8.1 ± 1.4, $n = 12$ embryos, KO 14.9 ± 2.2, $n = 12$, $p = 0.015$). This provided evidence that increase in total GLUR2 was correlated with increased surface-expressed GLUR2. No significant difference of surface GLUR1 was detected (WT 12.4 ± 7.4, $n = 4$, KO 10.7 ± 5, $n = 6$, $p = 0.8$) (Supplemental Fig. 1).

Determination of relative *Gria2* R/G editing and flip/flop splice levels

The great majority of native AMPA receptors are impermeable to calcium ions, due to the presence of the GLUR2 subunit. This subunit confers calcium impermeability on the channel due to RNA editing of a glutamine (Q) to an arginine (R) at codon 607. In addition to the Q/R site in GLUR2, the GLUR2, 3 and 4 subunits undergo RNA editing (arginine to glycine) at codon 764 (in GLUR2) (Lomeli et al. 1994). The pre-messenger RNA transcripts of all the four GLUR subunits can finally be alternatively spliced to produce either the flip or flop isoforms. Because the level of expression of GLUR2 was increased in *Rsk2*-KO mice, we wondered whether *Gria2* RNA editing and splicing were altered.

To determine whether there are changes in the Q/R, R/G site editing and flip/flop splice levels of the *Gria2*

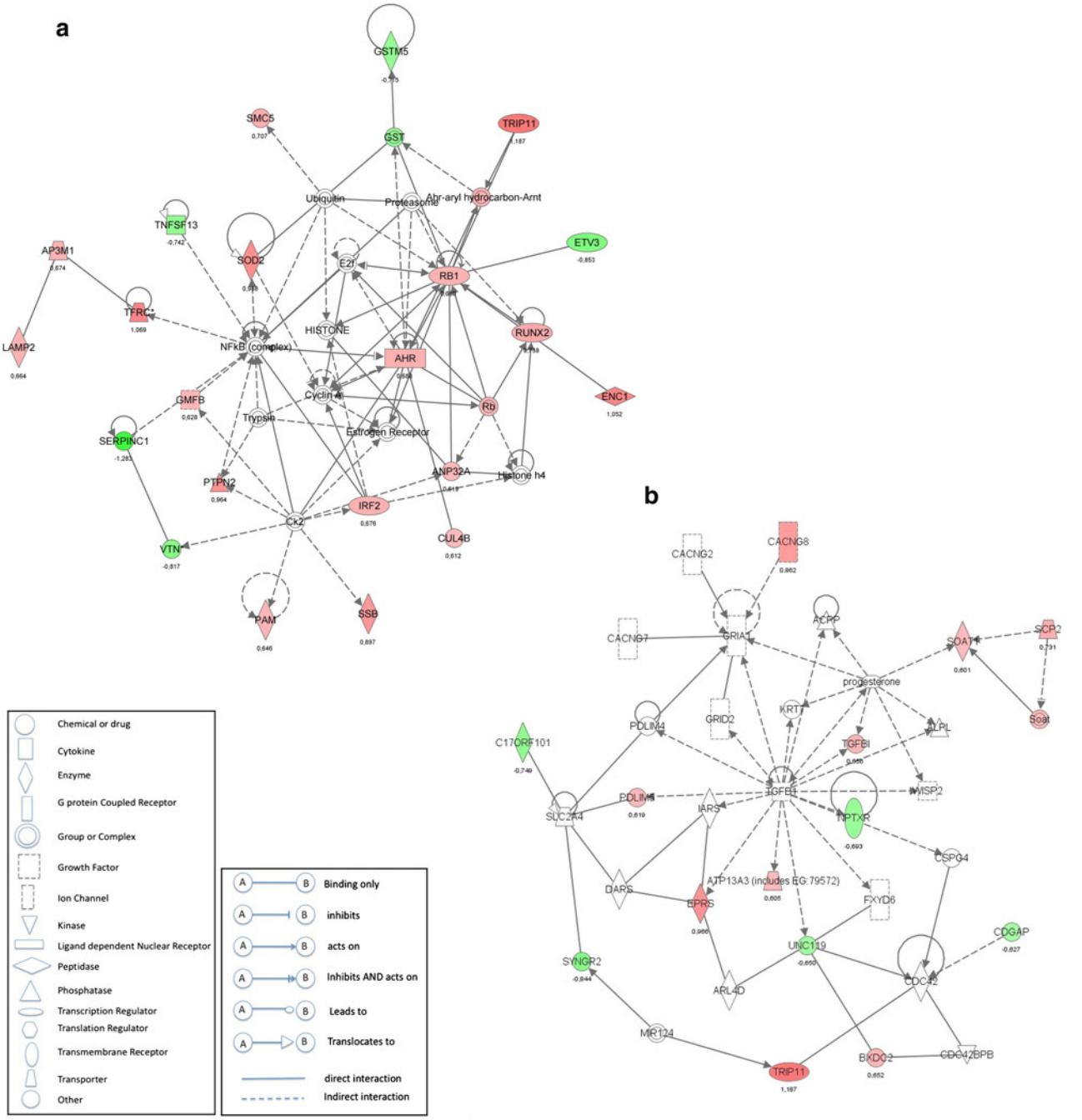

Fig. 1 Top integrated networks dysregulated in the hippocampus of *Rsk2*-KO. Networks were created by the Ingenuity Pathway Analysis Software. Up-regulated genes are listed in *red* and down-regulated in *green*. **a** This network is centered on NFκB. Twenty-five differentially expressed focus genes were brought into this network with a score of 45. **b** This network is centered on TGFβ1. Fifteen differentially expressed focus genes were brought into this network with a score of 26. Nodes and edges are described below the networks

messenger in 5-month-old *Rsk2*-KO hippocampi, the *Gria2* mRNA from five *Rsk2*-KO and five WT hippocampi was amplified by RT-PCR and the products sequenced to determine the relative levels of editing and splicing (Lee et al. 1998). No unedited form of the *Gria2* transcript at the Q/R site (codon 607) was detectable (not shown) neither in KO nor in WT mice, suggesting that the amount is very low. These data are in accordance with the previously reported results indicating that editing of Q/R site is $\sim$99% complete in postnatal brain (Carlson et al. 2000). At the R/G editing site (codon 764) edited (codon GGA) and unedited (codon AGA) forms were detectable in both WT and mutant mice.

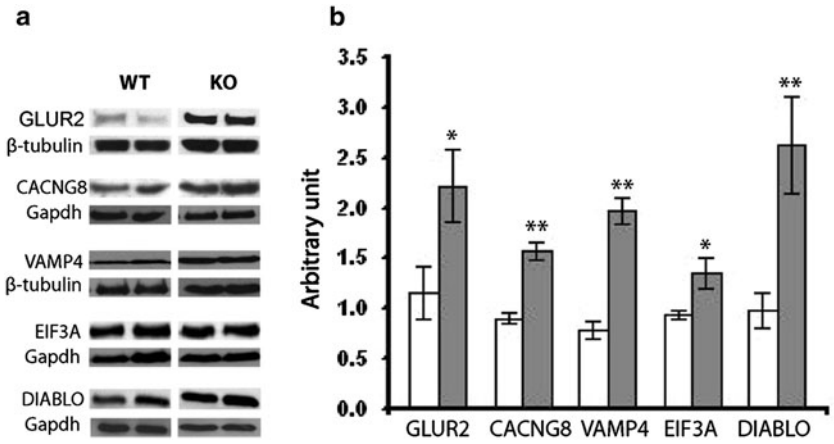

Fig. 2 Quantitative Western blot analyses. Levels of proteins expressed by the five up-regulated genes assayed are significantly increased in the hippocampus of *Rsk2*-KO mice. **a** Proteins detected in two *Rsk2*-KO and two WT mice are shown. **b** Data normalized either to GAPDH or to β-TUBULIN are represented as the mean ± SEM for six mice of each genotype for GLUR2 and CACNG8 and four mice for EIF3A, VAMP4 and DIABLO. *WT* white bar, *KO* gray bar $*p < 0.05$ and $**p < 0.01$

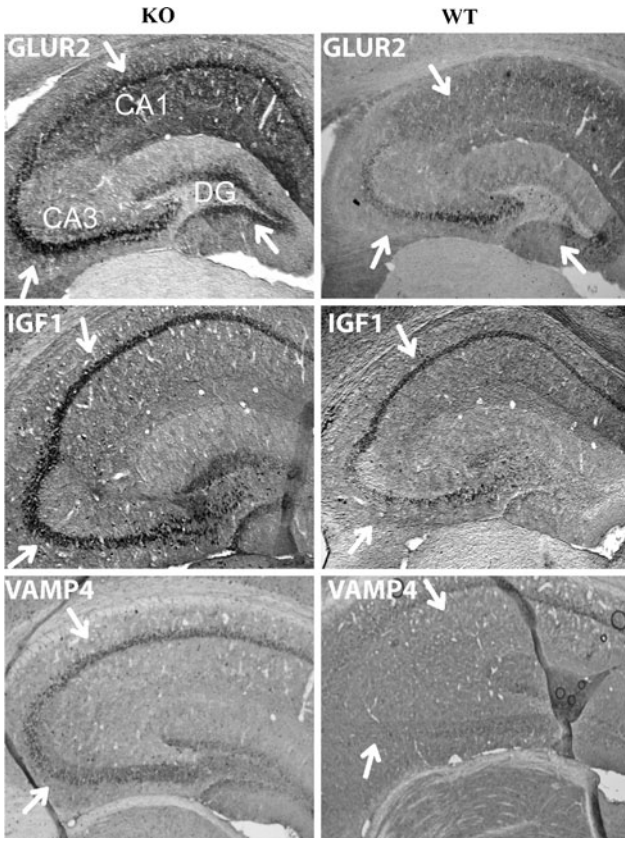

Fig. 3 Immunohistochemical analysis. Proteins expressed by three up-regulated genes show significantly higher expression in *Rsk2*-KO mice hippocampus. Three mice for each genotype were analyzed. Each *picture* represents one of the triplicates. *Arrows* point to hippocampus sub-regions showing increased expression in KO versus WT mice. CA1, CA3 and DG (dentate gyrus): hippocampus sub-regions

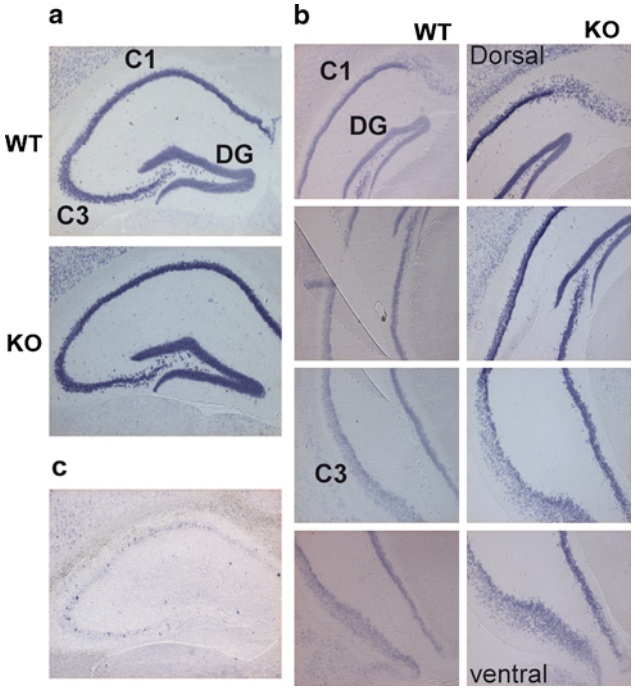

Fig. 4 In situ hybridization. Dig-labeled *Gria2* sense and anti-sense RNAs were hybridized to 25-µm coronal sections of three *Rsk2*-KO and three WT adult mouse brains. One *picture* from each genotype is shown. **a** Significantly increased expression in all areas of the anterior hippocampus of *Rsk2*-KO mice. **b** In the posterior hippocampus the level of *Gria2*-mRNA was mainly increased in the dentate gyrus, in CA1 and in the ventral CA3 region. **c** No staining was observed with sense RNA

The peak intensity of the G nucleotide signal at the edited position was measured and reported as a percentage of the total signal (A and G). Representative chromatograms from one KO and one WT littermate are shown in Supplementary Fig. 2. In WT hippocampi, the *Gria2* mRNA was approximately 61 ± 4% edited, whereas there was less editing in *Rsk2*-KO hippocampi (43 ± 3%, $p = 0.005$). Our data for WT mice are in accordance with the previous reports

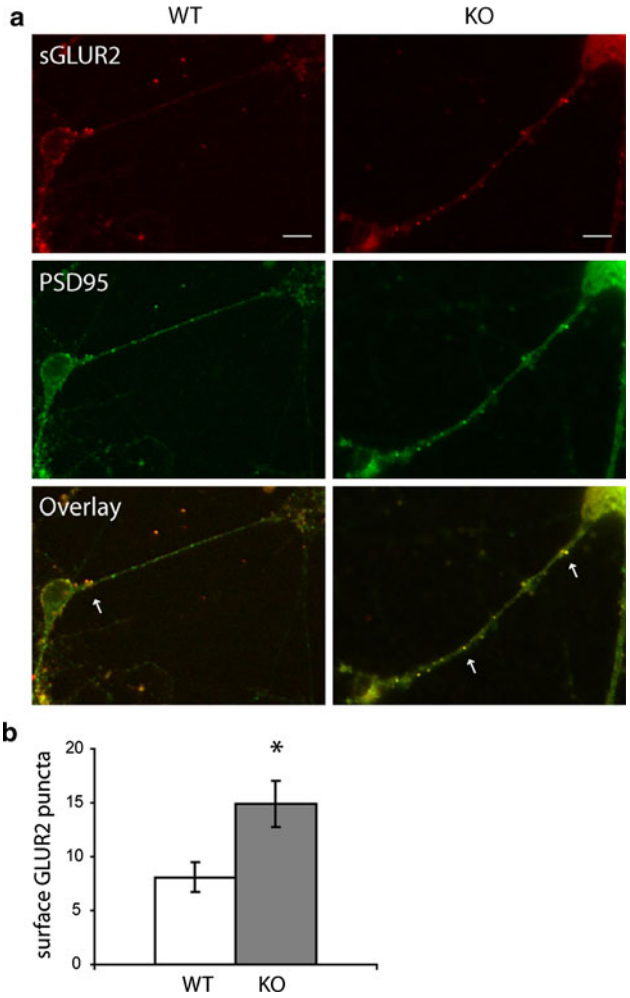

Fig. 5 Surface expression of AMPAR. **a** WT and *Rsk2*-KO hippocampal neurons were labeled with N-terminal GLUR2 antibody under non-permeabilized condition to stain surface GLUR2 (sGLUR2), followed by PSD95 staining (a post-synaptic marker). *Arrows* point to post-synaptic surface-expressed GLUR2. *Scale bar* 10 μm. **b** Quantification of sGLUR2 puncta. Data represent mean ± SEM of detected sGLUR2 clusters per unit dendrite length, from $n = 12$ (WT) and 12 (KO) embryos. $^*p < 0.05$

demonstrating an editing status at the R/G site of approximately 64% (Lai et al. 1997). Thus, in *Rsk2*-KO mice the extent of R/G editing was significantly decreased (rel. decrease: 18%, $p = 0.003$) in the hippocampal tissue.

To determine the ratio of transcripts in the flip/flop alternative splice form, the peak intensity of the first nucleotide difference (C vs. A) between the two variants was measured (Lee et al. 1998). In the WT hippocampi, there were approximately 55 ± 5% of Glur2 transcript in the flip form, whereas the percentage was significantly lower (43 ± 3%, $p = 0.002$) in the *Rsk2*-KO hippocampi (Supplemental Fig. 2c, d). No significant difference for the *Gria1* mRNA (encoding GLUR1) for the R/G editing site or flip/flop splice levels was found between WT and mutant mice (Supplemental Fig. 2a, b).

Reduced AMPA synaptic transmission

We then investigated whether changes in *Gria2* expression, editing and splicing affect basal AMPAR-mediated synaptic transmission in hippocampal slices from 4-week-old *Rsk2*-KO mice. To assess the strength of synaptic transmission, we compared the size of the presynaptic fiber volley (input) to the slope of the EPSP (output) in striatum radiatum and found a ~25% significant reduction in *Rsk2*-KO ($n = 9$) mice when compared with WT littermates ($n = 7$) (Fig. 6a). We evaluated also paired-pulse facilitation (PPF), a measure of release probability from presynaptic terminals. The PPF curves were essentially identical in slices from control and *Rsk2*-KO mice (Fig. 6b) indicating that RSK2 most likely modulates AMPA neurotransmission postsynaptically with no effect on presynaptic function.

Discussion

The absence of gross structural alterations in the brain of *Rsk2*-KO mice strongly indicates that their defective cognitive phenotype should be linked to subtler molecular or cellular alterations. In an effort to identify such alterations, we carried out a detailed characterization of the differences existing between the transcriptional profiles of the hippocampus of WT and KO animals. Our analysis by oligonucleotide microarrays yielded a list of 100 differentially expressed genes with high degree of statistical significance. These results were further confirmed for 24 genes by quantitative RT-PCR demonstrating their robustness.

Our study revealed a great variety of RSK2-influenced genes acting in various biological pathways. Indeed, the network with the highest score (as determined by ingenuity pathway analysis) centers on the NFκB complex, which plays a prominent role in cell differentiation and proliferation and apoptosis (Brand et al. 1997). Twelve genes are implicated at various stages of the cell cycle in *Rsk2*-KO neurons (including *Igf1, Rb1, Max, Sod2, and, Ptgs2*). This result suggests strongly that abnormal cell proliferation contributes to the CLS phenotype. In addition, 34 of the altered genes have been implicated in cell death or survival, out of which 7 (*Cacng8, Diablo, Gria2, Igf1, Ptgs2, Rb1, Sod2*) have been specifically associated with neuronal cell death. Interestingly, previous studies suggested that apoptotic and antiapoptotic cascades are tightly associated with cognitive dysfunctions and neurological disorders (Lutz 2007). Further studies are, therefore, necessary to investigate cell proliferation and death in *Rsk2*-KO mice. Four genes, including *Sod2, Fxn, Gmfb* and *Cp* are implicated in free radical scavenging, suggesting also a possible involvement of free radicals in the

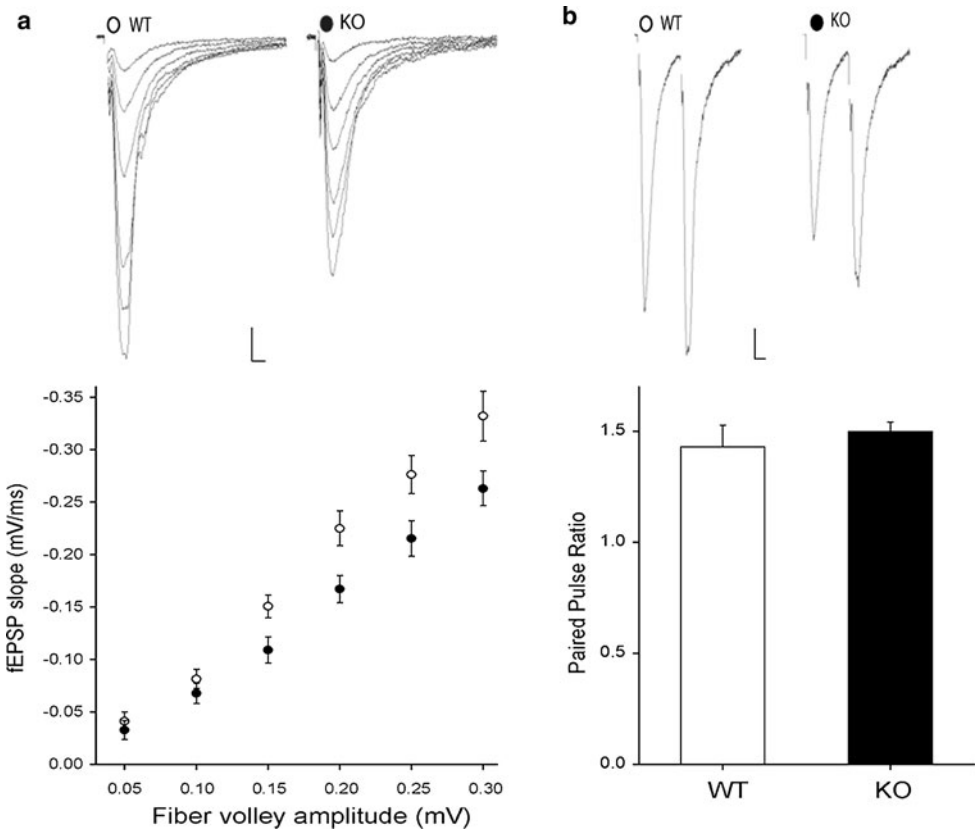

Fig. 6 Patch-clamp analysis**. a** Input–output curves for basal synaptic transmission in hippocampal slices. As illustrated in the sample traces and the *graph*, for each input (fiber volley $\geq$ 0.15 mV), the output (fEPSP) is reduced by 25% in *Rsk2*-KO slices ($p \leq 0.05$, WT $n = 10$; KO $n = 9$). *Scale bar* 0.1 mV, 5 ms. **b** Paired-pulse facilitation (PPF) does not differ between *Rsk2*-KO ($n = 9$) and WT ($n = 6$) cells. Sample traces are illustrated above the *bar graph*. *Scale bar* 0.05 mV, 10 ms

CLS phenotype. Ten genes (among which *Gria2, Igf1, Ptgs2, Sod2*, *Nptxr, Ahr* and *Vtn*) play a role in nervous system development and function. IGF1 for instance is essential for normal dendritic growth (Cheng et al. 2003). NPTXR is thought to be involved in activity-dependent synaptic plasticity (Xu et al. 2003). The AHR homologs in *Drosophila*, *Spineless* (*Ss*), and in *Caenorhabditis elegans*, *ahr-1*, regulate dendrite morphology (Kim et al. 2006) and neuronal differentiation (Qin and Powell-Coffman 2004). The expression of a number of genes involved in neurotransmission was also found affected. Up-regulation of the *Vamp4* and *Stxbp3* genes in mutant mice points to a role of RSK2 in pre-synaptic vesicle trafficking (Wang and Tang 2006). Up-regulation of *Cacnb4*, encoding a β-subunit of Voltage-gated Ca^{++} channels, suggests that influx of Ca^{++} into the cell upon membrane polarization is regulated via RSK2 (Birnbaumer et al. 1998). Moreover, alteration of *Gnb1* and *Gria2* expression suggests that RSK2 is involved in glutamate receptor signaling. GNB1 mediates the fast voltage-dependent inhibition of N-type Ca^{++} channels (Fu and Cheung 1999). The function of Gria2 will be discussed below. Finally, the expression of several genes encoding proteins implicated in gene expression was altered in *Rsk2*-KO hippocampi (including the transcription regulators *Etv3, Hip2, Rb1, Irf2, Max, Runx2* and *Trip11*) suggesting that some of the RSK2 effects may be direct and others indirect. Strikingly, two of the genes with altered expression in *Rsk2*-KO hippocampi, *Lamp2* and *Cul4b*, have previously been associated with syndromic forms of X-linked mental retardation (Nishino et al. 2000; Zou et al. 2007).

Among the genes associated with a specific neuronal function, *Gria2* was of particular interest because GLUR2 controls the key biophysical properties of AMPA receptors, which are implicated in learning and memory (Kessels and Malinow 2009). Most excitatory synaptic transmission in the brain being mediated through AMPAR, changes in the properties of these receptors are likely to have a major impact on brain function. Furthermore, GLUR2 was shown to bind directly to RSK2 in murine neurons suggesting a direct influence of RSK2 on GLUR2 function (Thomas et al. 2005). The GLUR family contains four closely related members (GLUR1-4). AMPARs are tetrameric, composed of various combinations of GLUR1-4 subunits, and the conductance properties of the receptors are highly dependent on their subunit composition (Kuner et al. 2001). GLUR2-lacking receptors have a higher Ca^{++} permeability, channel conductance, open probability and rectification than GLUR2-containing receptors (Isaac et al. 2007). Therefore, the presence or absence of the GLUR2 subunit can dramatically alter AMPAR properties and thereby synaptic transmission.

Our results provide evidence that in the hippocampus of *Rsk2*-KO mice total expression of GLUR2 is increased, and that the expression is also increased at the surface of synapses in cultured primary hippocampal cells. It was reported previously that surface insertion of GLUR2 occurs constitutively under basal conditions (Passafaro et al. 2001). Our results are compatible with these data. It may be speculated that over-representation of the GLUR2 subunit in synaptic AMPARs results in decreasing of Ca^{++} permeability and channel conductance. We show that there is, indeed, a 25% reduction in basal AMPAR-mediated transmission in the hippocampus of *Rsk2*-KO mice.

The Q/R site was completely edited in both WT and *Rsk2*-KO hippocampi, whereas the extent of R/G editing was significantly decreased. The GLUR2 subunit confers Ca^{++} impermeability on the channel due to a single arginine (R) residue located at amino acid position 607, which is a glutamine (Q) in the other AMPA receptor subunits. RNA editing of the Q/R site is specific to GLUR2 and is complete in postnatal brain. Our results in WT mice confirm further these latter data and show that Q/R editing is unaltered in *Rsk2*-KO mice. GLUR2-4 undergo also RNA editing [arginine (R) to glycine (G)] at amino acid position 743 (Lomeli et al. 1994). The presence of edited GLUR subunits at position 743 yields channels with faster kinetics. The R/G editing state of GLUR2–4 influences also the assembly and surface expression of AMPAR complexes. We show that R/G editing is significantly altered in *Rsk2*-KO mice. Finally, alternative splicing in the extracellular ligand binding domain of the AMPARs generates two variants, i.e., flip and flop. Native AMPAR are heteromeric assemblies of different subunits that may have different flip and flop isoforms. We show that in *Rsk2*-KO mice the proportion of GLUR2 molecules with a flop exon is significantly higher than in WT littermates. The flop variants desensitize at least 3 times faster but recover more slowly from desensitization than the flip counterparts (Pei et al. 2009). It was also previously shown that the flip/flop splicing has an effect on the maturation and cellular trafficking of AMPARs (Brorson et al. 2004). Alteration of R/G editing and splicing of GLUR2 in *Rsk2*-KO mice are therefore expected to alter AMPARs channel kinetic, desensitization and trafficking. Further functional studies are required to address the precise functional consequences of these editing and splicing changes in *Rsk2*-KO neurons. Furthermore, it was shown that proteins binding to GLUR2 are necessary for constitutive replacement of newly inserted GLUR1-containing receptors to maintain synaptic strength during LTP (Malinow and Malenka 2002). It has also been proposed that the expression of hippocampal LTD is critically dependent on GLUR2 (Malinow and Malenka 2002). Further studies will address the consequences of up-regulation of GLUR2 in *Rsk2*-KO mice for LTP and LTD. The signaling mechanisms involved in the increased levels of transcription of *Gria2* in *Rsk2*-KO hippocampal neurons are not yet known. The regulation of *Gria2* splicing and editing events is poorly understood as well. *Gria2* expression is influenced strongly at the transcriptional level by at least three regulatory elements in the 5′ proximal region of the promoter (Borges and Dingledine 2001). RNA editing is mediated by adenosine deaminase acting on RNA (ADAR) enzymes. Three structurally related ADARs (ADAR1 to ADAR3) have been identified in mammals. ADAR2 predominantly catalyzes RNA editing at the Q/R site of GLUR2 (Peng et al. 2006), whereas it is not yet clear how the R/G site is edited. The underlying mechanism of the R/G editing dysregulation may be caused by altered function or expression of one or several ADAR enzymes. However, the fact that editing of the Q/R site in GLUR2 is not affected in *Rsk2*-KO mice suggesting that ADAR2 is excluded. Further investigations are necessary to determine precisely the molecular events leading to up-regulation of the *Gria2* gene and alteration of RNA editing, and the contribution of each of these dysregulations to the cognitive dysfunction. The contribution of other pathways remains also to be investigated. Among the deregulated genes at least one other participates in regulation of AMPAR function: *Cacng8*. This gene encodes a synaptic protein, TARPγ-8 that participates in consolidation phase of memory and is involved in modulating neurotransmitter release. Evidence was provided that TARPγ-8 is critical for basal AMPAR expression and localization at extrasynaptic sites in the hippocampus (Rouach et al. 2005). Up-regulation of *Cacng8* may thus contribute to AMPAR dysfunction.

The data in this study provide a first glimpse of the gene expression profile of adult hippocampi in the absence of RSK2 expression. However, the *Rsk2*-KO animals represent a value model to study human Coffin–Lowry syndrome, it has significant limitations due to potential compensatory adaptation mechanisms in the developing nervous system (in particular through other RSK family members). Thus, it would be interesting to perform expression profiling following *Rsk2* gene silencing by RNA interference technology. Indeed, cellular or animal models based on this technology could offer further clues about the function of RSK2.

In conclusion, functional impairment of neurotransmission and plasticity due to AMPAR dysfunction may, indeed, contribute to the cognitive deficit of *Rsk2*-KO mice. However, further investigations are necessary to determine precisely the molecular events leading to alteration of GLUR2 expression and the contribution of this dysregulation to the cognitive dysfunction. The involvement of other pathways, including in particular cellular proliferation and apoptosis, remains also to be investigated.

Finally, the genes identified by our microarray analysis will help in further unravel the various functions of RSK2 in the hippocampus can be speculated to play a role in the pathogenesis of mental retardation in Coffin–Lowry syndrome and may provide targets for pharmaceutical intervention.

Accession number

The data of the expression arrays produced for this report have been submitted to NCBIs Gene Expression Omnibus (GEO: http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE22137.

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Conflict of interest The authors declare that they have no conflict of interest.

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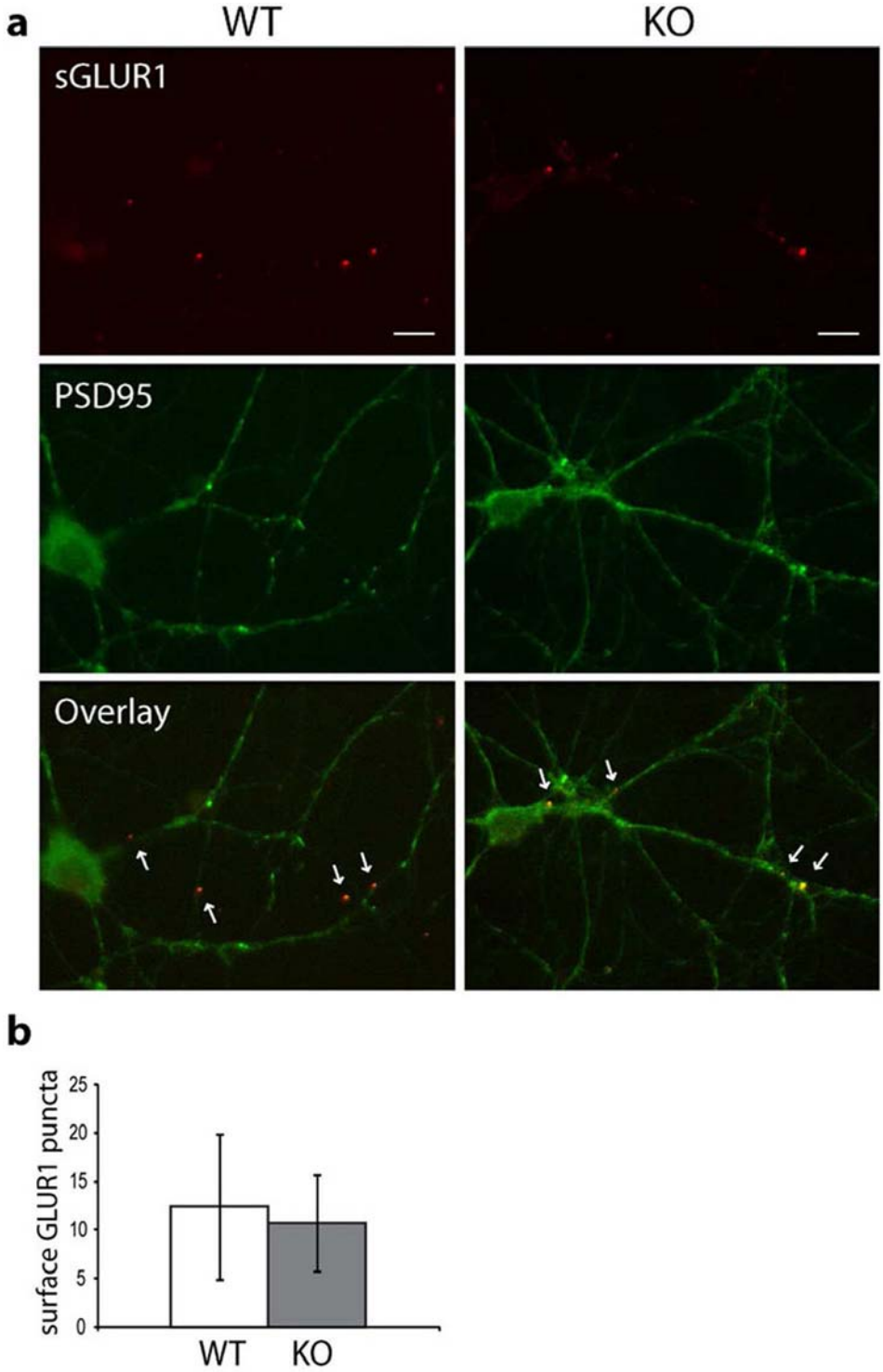
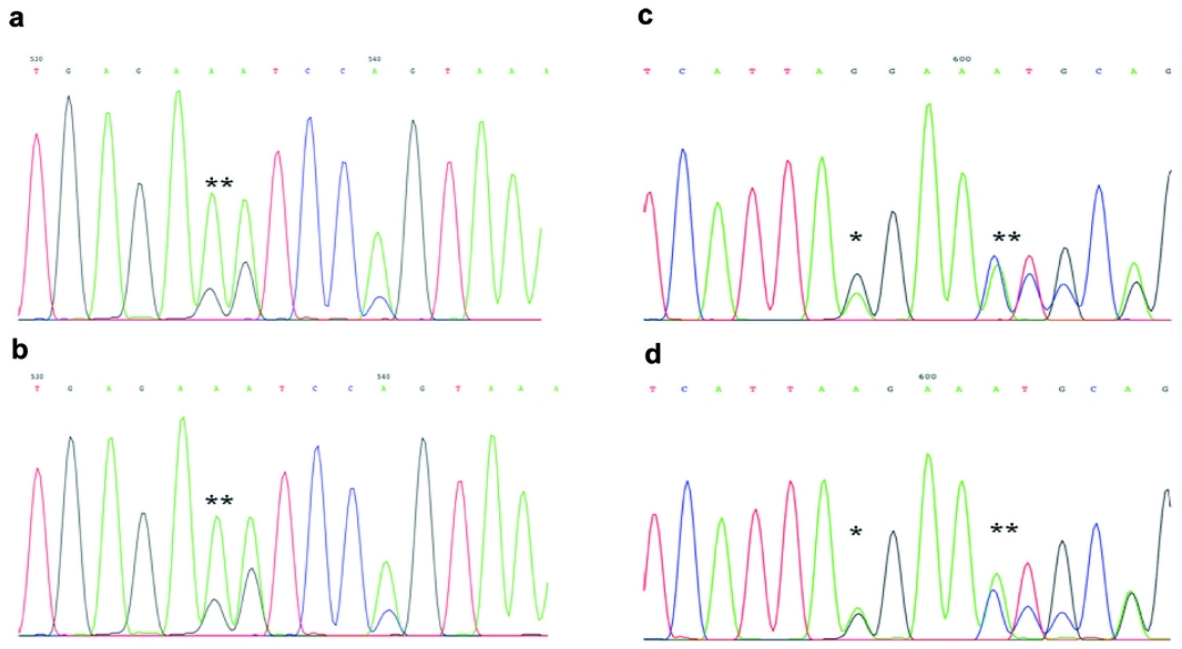
Supplemental data:



Supplemental Fig. 1 **(a)** WT and *Rsk2*-KO hippocampal neurons were labeled with N-terminal GLUR1 antibody under non-permeabilized condition to stain surface GLUR1 (sGLUR1), followed by PSD95 staining (a post-synaptic marker). Arrows point to post-synaptic surface-expressed GLUR1. **(b)** No significant difference for GLUR1 surface expression was found between WT and *Rsk2*-KO mice (n = 4 WT and 6 KO embryos).



Supplemental Fig. 2. Determination of relative *Gria2* R/G editing and Flip/Flop splice levels. The *Gria1*(encoding GLUR1) and *Gria2* (encoding GLUR2) mRNAs from five *Rsk2*-KO and five WT hippocampi were amplified by RT-PCR in triplicates and the products sequenced. Representative chromatograms from one KO and one WT littermate is shown. **(a)** *Gria1* for WT, **(b)** *Gria1* for KO, **(c)** *Gria2* for WT, **(d)** *Gria2* for KO. * points the G/R editing site (A: unedited, G: edited). The peak intensity of the G nucleotide signal was measured and reported as the mean percentage for the five mice of the total signal (A + G). ** Points the first nucleotide of the flip/flop exon (flop starts with A and flip with C). The peak intensity of the first nucleotide between the two variants was measured and reported as the mean percentage for the five mice of the total signal (A + C).

Supplemental Table 1. Sequences of primer used in qRT-PCR validation of 24 genes

Gene	Primer sequences
Arhgap12	Forward 5, –GGATCCAGCATGGATTCTTC
	Reverse 5' -ACTTTGGCACTGTCCATTC
Cacnb4	Forward 5' –ATGGCAGCACCACTTCTACC
	Reverse 5' -CAGGTTTGGACTTCGCTCTC
Cacng8	Forward 5' –TCGCCATCAGCACTGACTAC
	Reverse 5' -CACGCAGACACCTCTTTTA
Carhsp1	Forward 5' –GCCTGCAGCTTCTCATTCTT
	Reverse 5' -AAGGGCCATGGCTTCATTAC
Cul4b	Forward 5' –CTTCAGTGCACCATGCAAAC
	Reverse 5' -CTGAATGGCTTCCACTGCTT
Diablo	Forward 5' –ATTGATGAGGAGGGCTGTGT
	Reverse 5' -TTCCTCCTGGGAATTCATCTT
eIF3A	Forward 5' –AGATGAGGACAGAGGACCTAGAC
	Reverse 5′ -TCAGCATTCCGCCAGGATGA
Enc1	Forward 5' –TGGCCTCTCCGAAGTAGAAA
	Reverse 5' -GACTGTGGAACAGCATCACC
Gnb1	Forward 5' –TCTCTGGTGCTTGTGATGCT
	Reverse 5' -GGTCATGAGCTCCTGGTCTG
Gria2	Forward 5' –TTTCCTTGGGTGCCTTTATG
	Reverse 5' -GACAGATCCTCAGCACTTTCG
Igf1	Forward 5' –TGCAAAGGAGAAGGAAAGGA
	Reverse 5' -TGTTTTGCAGGTTGCTCAAG
Lamp2	Forward 5' –TGTGCAACAAAGAGCAGGTG
	Reverse 5' -CCAGCATAGGTCTTTCTTCTGC
Nptxr	Forward 5' –GCCAATGAGATCGTGCTTCT
	Reverse 5' -CACAGCCCATCCCTTGTAGT
Pdlim5	Forward 5' -AGGTGGCAAGGATTTCAACA
	Reverse 5' -CATATTCAAGGAGCCCGTACA
Phip	Forward 5' –TTCAGGCATGGAAGAAACAA
	Reverse 5' -TCTCTTGCTTGGTGTGTATGCT
Phkg1	Forward 5' –GGAAGCAAATGCTGATGTTG
	Reverse 5' -TCCTGAAAGAAAGGGTGTGC
Plek	Forward 5' –TCTGTGAGGAGAACTCCAGTGA
	Reverse 5' -GGTAGGCCGGGTCTTCTCT
Ptpn2	Forward 5' -CCTGACCATGGACCTGCAGT
	Reverse 5' -AGAGAAGGTGCCAGAGCGC
Rasl10a	Forward 5' –TGAGGCAGCGTATAGCAGA
	Reverse 5' -TGTATTTGGCTGAGCACTCG
Stk3	Forward 5' –AGCTGAGTGAAGACAGTTTG
	Reverse 5' -CCTGAAGATCTGACTCAACA
Stxbp3	Forward 5' –AGGCTCTTTTCATCAATCTTGT
	Reverse 5' -CCTGGTAATGCAAAGGAAA
Tmsb10	Forward 5' –TTGGCAGTCCGATTAGTGGAGG
	Reverse 5' -CGGAAGGAGAATCCACGAGTTG
Vamp4	Forward 5'-GCAGCGATCCTTTTGCTAAT
	Reverse 5' -ACCTGATTTCACTGGGCATC
Vldlr	Forward 5' –TCTCAATGATGCCCAAGACA
	Reverse 5' -GTACCCATTGGGACAGGAAC

Supplemental Table 2. Ontology analysis of the genes affected in the *Rsk2*-KO hippocampus.

Related functions and diseases	**Significance**	**Associated genes**
Organismal injury and abnormalities	$2.52\ e^{-5} – 1.52\ e^{-2}$	9
Cell cycle	$3.76\ e^{-5} – 1.52\ e^{-2}$	12
Nervous system development and function	$5.75\ e^{-5} – 1.52\ e^{-2}$	10
Organismal development	$5.75\ e^{-5} – 1.52\ e^{-2}$	9
Free radical scavenging	$5.75\ e^{-5} – 1.52\ e^{-2}$	4
Behavior	$9.71\ e^{-5} – 1.10\ e^{-2}$	5
Gene expression	$9.88\ e^{-5} – 1.52\ e^{-2}$	10
Cell death	$1.50\ e^{-4} – 1.52\ e^{-2}$	34
Skeletal and muscular system development and function	$1.72\ e^{-4} – 1.52\ e^{-2}$	7

2.2. Cobalt staining of primary hippocampal neurons:

NMDA receptors are highly permeable to Ca^{2+} ions, whereas AMPA receptors are normally less permeable to Ca^{2+}, due to the presence of GluR2 subunit in AMPA receptor. We have observed a two-fold higher level of GluR2 protein expression in *Rsk2*-KO mice than in WT mice, and the electrophysiology studies showed a significant reduction of basal AMPA receptor-mediated transmission in the hippocampus of *Rsk2*-KO mice. This prompted us to further explore the AMPA receptor functions, by using the Co^{2+} staining protocol on primary hippocampal neuronal cultures. The cobalt staining technique involves the pharmacological stimulation of AMPAR in the presence of $CoCl_2$, which leads to the uptake of Co^{2+} through Ca^{2+}-permeable AMPARs. Subsequently, one can visualize activated cells by precipitating histochemically the intracellular Co^{2+}. This technique allows identification of cells expressing Ca^{2+}-permeable AMPARs. Thus, this protocol was expected to reveal whether the proportion of cells with Ca^{2+}-permeable AMPARs is different in *Rsk2*-KO cultures than in WT cultures. We took the images of stained neuronal culture by using the light compound microscope. Co^{2+} treated neurons, without AMPA stimulation, showed no or very rare stained cells as shown in **(Figure 27A)**, in both KO and WT mice. Treatment with Co^{2+} and stimulation with AMPA (100µM) resulted in less stained neurons in *Rsk2*-KO cultures than in WT cultures. In addition the staining was stronger in WT neurons as compared to the KO cells **(Figure 27B)**. In these experiments AMPA act as an agonist on the AMPA receptor, resulting in uptake of Co^{2+}. Accumulation of Co^{2+} inside the neurons depends on the non-desensitization of AMPA receptors. Cyclothiazide is a positive allosteric modulator of the AMPA receptor, capable of reducing or essentially eliminating rapid desensitization of the receptor. Therefore, we tested a third condition by treating cells with Co^{2+}, AMPA (100µM) and cyclothiazide (30 µM). Again there were much more stained cells in WT cultures than in *Rsk2*-KOcultures **(Figure 27C)**.

Although, it was difficult to quantify the results, they suggested strongly that Ca^{2+} uptake through AMPA receptors/channels is affected by the over expression of the GluR2 subunit.

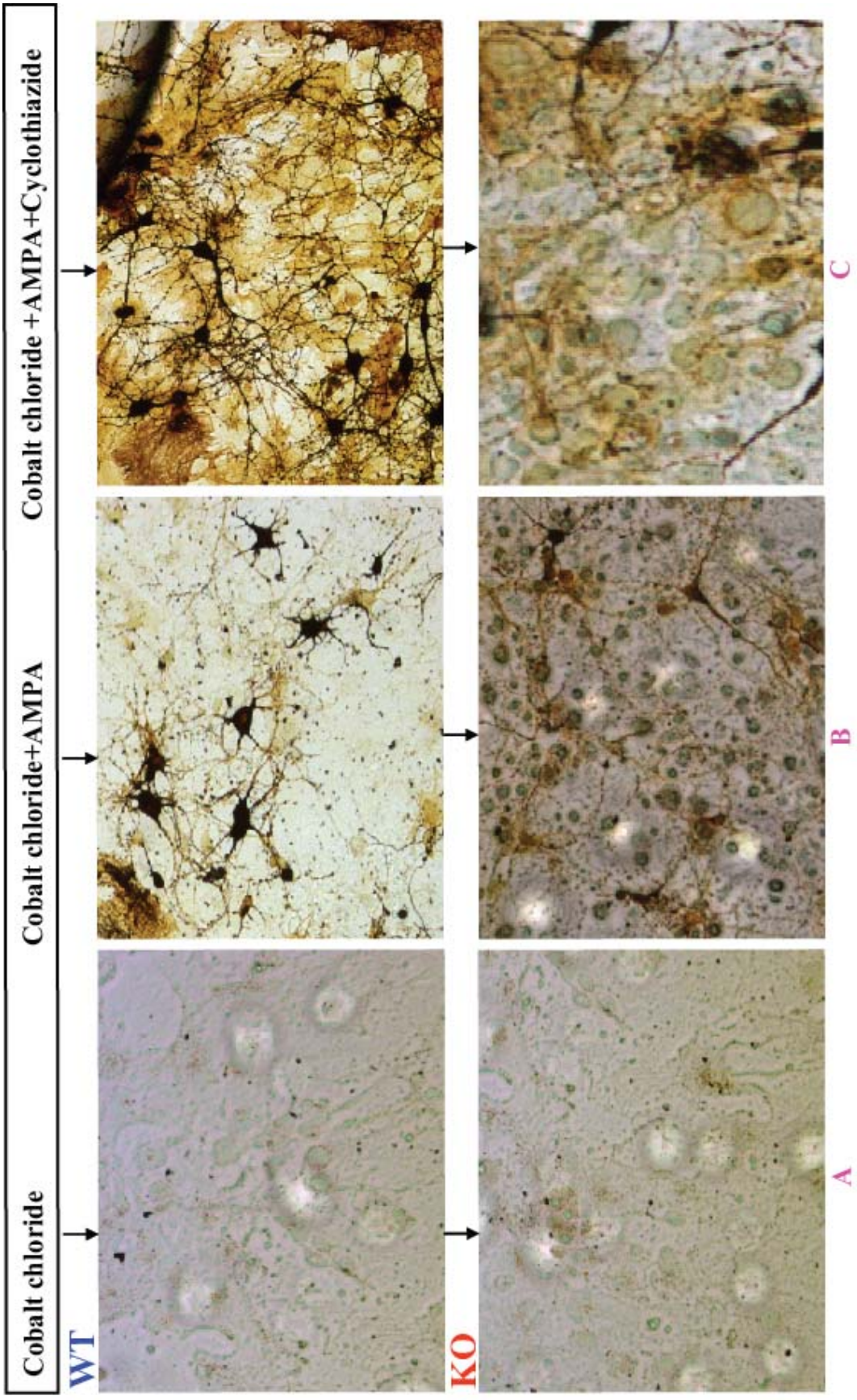


Figure 27: Cobalt staining of primary hippocampal neurons.
(A). Cobalt chloride treated neurons, without AMPA stimulation. **(B).** Neurons are treated with cobalt chloride and stimulated with AMPA (100µM). (C) Neurons have been treated with cobalt chloride, AMPA (100µM) and cyclothiazide (30 µM).

Publication-2

2.3. Alteration of ERK / MAPK signaling in hippocampal neurons of *Rsk2*-KO mice

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The objective of this work was to study the mechanism of regulation of ERK activity in the hippocampus of KO animals and to identify factors that may explain the deregulation of the transcriptional activity previously reported (Mehmood et al., 2011). To this end we analyzed in primary cultures of hippocampal neurons the activity of several upstream and downstream proteins of ERK1/2, at basal level and in response to glutamate stimulation.

RSK2 is a protein kinase acting at the distal end of the Ras-ERK/MAPK signaling pathway. ERK1/2 activates RSK2 by phosphorylating serine and threonine residues. After activation RSK2 in turn can phosphorylate numerous cytosolic and nuclear substrates. A few studies have identified RSK2 as a negative regulator of its own pathway. In particular, it was shown on cultured motor neurons that RSK2 negatively regulates axonal growth via inhibition of ERK (Fischer et al., 2009a), but also that the activity of ERK1/2 is increased in the cortex of KO mice, compared to the cortex of WT mice (Marques Pereira et al., 2008). A negative feedback of RSK2 on the ERK pathway, via phosphorylation of SOS, was proposed by Douville and Downward in 1997. We hypothesized that a deregulation of the ERK/ MAPK in the *Rsk2*-KO hippocampus, resulting from the absence of this feedback regulation, could cause alterations of many cellular processes and, thus, play a role in the molecular pathologic mechanism leading to cognitive impairment. We wanted to know if such a feedback exists in neurons of the hippocampus. We first analyzed the expression levels of ERK1/2 and P-ERK1/2 in the hippocampus of *Rsk2*-KO and WT littermate mice. A two- and threefold increase of the levels of Phospho-ERK1 and Phospho-ERK2, respectively, were observed when compared to WT littermates. We then analyzed phosphorylation of ERK1/2 in un-stimulated and glutamate stimulated hippocampal primary cultures derived from WT and *Rsk2*-KO littermate mice.

Glutamate is a major neurotransmitter that activates the ERK pathway in many brain regions (Kawasaki et al., 2004; Paul et al., 2010). Level of ERK1/2 phosphorylation was, indeed, significantly higher in *Rsk2*-KO neurons than in WT cells, at basal conditions and after glutamergic stimulation.

To identify the mechanism leading to this increase, we investigated the phosphorylation of kinases and phosphatases that may regulate the activity of ERK. The levels of phosphorylation of Raf-1 and MEK, two ERK upstream kinases, were also higher in WT and KO neurons, supporting the RSK2 retro control hypothesis reported by (Douville and Downward, 1997).

We then investigated the consequences of the increased ERK1/2 activity on the phosphorylation of some ERK1/2 substrates. Higher levels of phosphorylation of Elk1 and CREB (two transcription factors) were observed in *Rsk2*-KO hippocampal neurons than in WT cells. Inhibition of MEK with U0126 confirmed that the increased level of phosphorylation of CREB is ERK dependent. However, U0126 did not abolish glutamate-induced phosphorylation of ELK1, providing evidence that glutamate-induced ELK1 phosphorylation is not mediated by ERK1/2 in hippocampal neurons. Further data suggested that ELK1 activity is regulated via the p38 and JNK pathways Q-RT-PCR analyses showed an increased expression of target genes of these transcription factors (c-Fos, Zif268 and Arc) in *Rsk2*-KO neurons following glutamergic stimulation.

These results provided clear evidence that the transcriptional activity is impaired in RSK2 deficient neurons, which may account for the dysregulation of some genes evidenced by the transcriptional study. In this work we identified a dysregulation of the ERK/MAPK signaling pathway in RSK2-KO derived hippocampal neurons.

A comprehensive description and illustration of these results are found in publication-2.

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ORIGINAL ARTICLE

Altered ERK/MAPK signaling in the hippocampus of the *mrsk2_KO* mouse model of Coffin–Lowry syndrome

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Abstract

Coffin–Lowry syndrome is a syndromic form of mental retardation caused by mutations of the *Rps6ka3* gene encoding ribosomal s6 kinase (RSK)2. RSK2 belongs to a family containing four members in mammals: RSK1–4. RSKs are serine/threonine kinases and cytosolic substrates of extracellular signal-regulated kinase (ERK) in the Ras/MAPK signaling pathway. RSK2 is highly expressed in the hippocampus, and *mrsk2_KO* mice display spatial learning and memory impairment. In the present study, we provide evidence of abnormally increased phosphorylation of ERK1/2 in the hippocampus of *mrsk2*_KO mice. Further studies based on cultured hippocampal neurons revealed that glutamate activates ERK1/2 and RSKs, and confirmed a stronger activation of ERK1/2 in *mrsk2_KO* neurons than in WT cells. We, thus, provide further evidence that RSK2 exerts a feedback inhibitory effect on the ERK1/2 pathway. We also observed a transient sequestration of P-ERK1/2 in the cytoplasm upon glutamate stimulation. In addition, the transcription factors cAMP response element binding and Ets LiKe gene1 show over-activation in RSK2-deficient neurons. Finally, *c-Fos*, *Zif268* and *Arc* were significantly over-expressed in *mrsk2_KO* neurons upon glutamate stimulation. Importantly, the increased phosphorylation of other RSK family members observed in mutant neurons was unable to compensate for RSK2 deficiency. This aberrant ERK1/2 signaling can influence various neuronal functions, and thus play a significant role in cognitive dysfunction in *mrsk2_KO* mice and in the Coffin-Lowry syndrome.

Keywords: Coffin-Lowry syndrome, CREB, ERK, hippocampus, IEG, RSK2.

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Loss-of-function mutations in the gene encoding the ribosomal S6 protein kinase 2 (RSK2) are responsible for the Coffin-Lowry syndrome (CLS; OMIM 303600), a syndromic form of X-linked mental retardation. Cardinal features of CLS are growth and psychomotor retardation, typical facial and digital abnormalities, and progressive skeletal deformations (Hanauer and Young 2002). RSK2 is a serine/threonine kinase and belongs to the 90 kDa RSK family. Four highly related members are found in humans and mice, RSK1–4. These isoforms are derived from four independent genes. RSKs act at the distal end of the Ras/mitogen-activated protein kinase (MAPK) signaling pathway and are directly phosphorylated and activated by the extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Frödin and Gammeltoft 1999).

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Abbreviations used: Arc, activity-regulated cytoskeleton-associated protein (also known as Arg3.1); c-Fos, cellular FBJ murine osteosarcoma viral oncogene homolog; CLS, Coffin-Lowry syndrome; CREB, cAMP response element binding; DUSP, dual specificity phosphatase; ELK1, Ets LiKe gene1; ERK, extracellular signal-regulated kinase; IEG, immediate early gene; KO, knockout; MAPK, mitogen-activated protein kinase; MEK, MAPK/Extracellular signal-regulated Kinase; MSK, mitogen- and stress-activated protein kinase; Nur77, nuclear receptor subfamily; PDL1, phospholipase D; RAF-1, Rapidly Accelerated Fibrosarcoma-1; RSK, ribosomal s6 kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Shank1/3, SH3 and multiple ankyrin repeat domains protein 1/3; Zif268, zinc finger protein 268 (also known as Egr1).

ERK1/2 respond in neurons to a wide variety of stimuli including glutamate receptor activation (Bading and Greenberg 1991), synaptic activity that induces either long-term potentiation or long-term depression (Dudek and Fields 2001; Thiels *et al.* 2002) and growth factors (Segal and Greenberg 1996). Upon activation, both ERK1 and ERK2 can phosphorylate regulatory targets in the cytosol or translocate to the nucleus and phosphorylate common substrates, including extracellular proteins, effectors and transcription factors (Frödin and Gammeltoft 1999). Thus, ERK activation results in a variety of responses, such as cell proliferation, differentiation, gene expression, and cell cycle regulation in response to neural activity (Kolkova *et al.* 2000). Direct nuclear targets of ERK1/2 include the Ets LiKe gene1 (ELK1) transcription factor (Yang *et al.* 1998) and the immediate early gene (IEG) *c-Fos* (Murphy *et al.* 2002). The cAMP response element-binding (CREB) transcription factor is also a downstream nuclear target of the Ras-ERK pathway, and its phosphorylation can be carried out by several factors of the RSK and its homologous mitogen- and stress-activated protein kinase (MSK) families (De Cesare *et al.* 1998; Chwang *et al.* 2007). In addition to *c-Fos*, the expression of two IEGs, the Zinc finger protein 268 (*Zif268*) and the activity-regulated cytoskeleton protein (*Arc),* are dependent of the Ras-ERK/MAPK pathway, through mechanisms involving CREB, ELK1 or even *Zif268* (Li *et al.* 2005; Davis and Laroche 2006; Chotiner *et al.* 2010). Numerous studies implicate the ERK/MAPK signaling cascade and CREB and ELK1-mediated gene transcription in synaptic plasticity and memory (Thiels *et al.* 2002; Davis and Laroche 2006). The induction of *c-Fos*, *Zif268* and *Arc* expressions have also been linked to synaptic plasticity mechanism and memory processes (Davis and Laroche 2006; Messaoudi *et al.* 2007; Alberini 2009; Davis *et al.* 2010). Figure 1 represents schematically the simplified MAPK pathways.

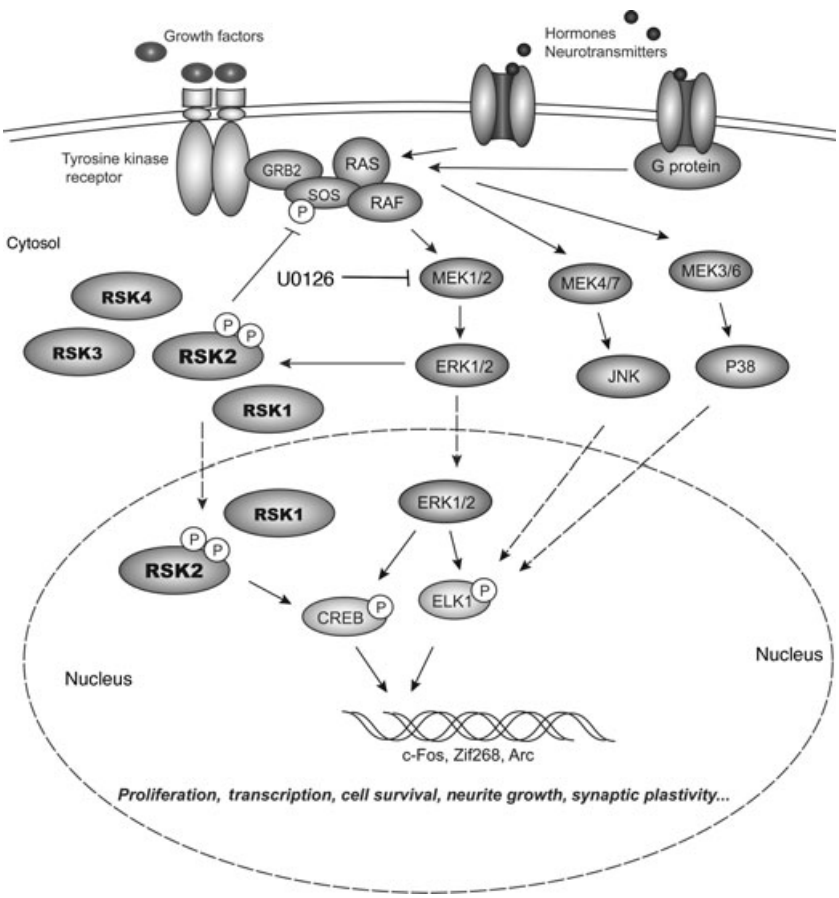

Fig. 1 Among the signaling cascades involved in the response to many extracellular signals in eukaryotic organisms are those that activate the MAPK family of serine-threonine kinases. Three major MAPK families have been identified in mammalian cells, such as the extracellular signal-regulated kinase (ERK) pathway and the stress-activated p38 and Jun kinase (JNK) pathways. The ERK pathway is strongly activated by growth factors, mitogens and neurotransmitters via the Ras protooncogene and is involved in cell proliferation, differentiation, death and neuronal function. RSK proteins are targeted by the MAPK/ERK pathway. Stress-activated MAPKs respond to stress factors or cytokines, but also growth factors and neurotransmitters, leading to cell death, survival, or defense and neuronal function. U0126 is a highly selective inhibitor of both MEK1 and MEK2.

Activation of ERK leads also to activation of RSKs in essentially all the cases where ERK and RSK kinase activities have been measured. The coordinate manner of activation is partially explained by the fact that ERK and RSK are physically associated in the cell (Scimeca *et al.* 1992). Once activated RSKs can target a wide range of cytosolic and nuclear proteins. Some of the known RSK2 substrates are, in the cytosol glycogen synthase kinase 3 (Angenstein *et al.* 1998), phospholipase D (PLD1) (Zeniou-Meyer *et al.* 2008) and the SH3 and multiple ankyrin repeat domains protein 1/3 (Shank1 and Shank3) (Thomas *et al.* 2005), and nuclear proteins including the activating transcription factor 4, c-FOS, the nuclear receptor subfamily 77 (NUR77), CREB and histone H3 (De Cesare *et al.* 1998; Sassone-Corsi *et al.* 1999; Yang *et al.* 2004). Together, the data suggest that RSK2 is involved in cellular and molecular events such as proliferation, apoptosis, and neuronal development as well as in learning and memory processes. Involvement of RSK2 in cognitive functions was further confirmed by the study of RSK2-deficient mice. Indeed, they show delayed acquisition of a spatial memory reference task and long-term spatial memory deficits (Poirier *et al.* 2007). Moreover, RSK2 shows a high level of expression in the hippocampus, which plays an important role in cognitive function and learning (Zeniou *et al.* 2002).

Recent studies showed that RSK has also an inhibitory role in the Ras-ERK pathway in *Drosophila* by anchoring ERK in the cytoplasm (Kim *et al.* 2006), and during mouse embryonic development by decreasing ERK activation and target gene expression (Myers *et al.* 2004). Previously, another study, based on PC12 cells, also pointed out that RSK2 could negatively regulate the Ras-ERK pathway, by phosphorylating son of sevenless (Douville and Downward 1997).

Glutamate is among the neurotransmitters that readily activate ERK. A large number of reports have well documented this point in neurons from various brain regions, such as the cortex, hippocampus, striatum and cerebellum (Wang *et al.* 2007). All three subtypes of ionotropic glutamate receptors and three subgroups of metabotropic glutamate receptors seem to modulate consistently the MAPK pathway in a stimulatory fashion. Active MAPKs translocate to the nucleus to activate a specific set of transcription factors for the facilitation of target gene expression. This glutamate receptor-dependent MAPK mediated transcription controls the development of multiple forms of synaptic plasticity related to various normal and abnormal neural activities, including memory. Very little is known about the specific role of RSK2 in glutamate signaling in neuronal cells.

We show in the present study that ERK1/2 is over-activated in the hippocampus of *mrsk2_KO* mice. To further investigate the cause and consequences of this ERK1/2 increased activation, we examined the phosphorylation of upstream actors [ERK, MAPK/Extracellular signal-regulated Kinase (MEK), Rapidly Accelerated Fibrosarcoma-1 (RAF-1)] of RSK2 in the ERK/MAPK pathway as well as the phosphorylation and/or expression of ERK1/2 and/or RSK2 targets (CREB, ELK1, *c-Fos*, *Zif268*) in *mrsk2*-defective cultured hippocampal neurons stimulated or not with glutamate. In addition, since there is evidence that *Arc* is regulated by *Zif268* (Li *et al.* 2005), we also tested the expression of this later gene.

Experimental procedures

Ethics statement

All experimental procedures for care and use of mice were performed according to agreements with the Departmental Direction of Veterinarian Services (Prefecture du Bas-Rhin, France No. 67-225) and IGBMC Animal Welfare Insurance (NIH, PHS No. A5100-01). *mrsk2*-KO mice were previously generated in our laboratory (Yang *et al.* 2004). WT and KO animals used in this study were male from the same littermates and housed four per cage in a 12 h dark/light cycle (light on from 7 AM to 7 PM), under controlled conditions of temperature and humidity. Killing of the mice used to derive primary cell cultures was carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC), and the study was specifically approved by the Ethics Committee in Animal Experiments (permit number 17) of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

Whole hippocampal protein extractions

Six male *mrsk2_KO* and 6 WT littermates with a C57Bl/6× genetic background were killed by cervical dislocation. Brains were rapidly dissected and the hippocampus was isolated using a standard dissection procedure. Whole protein extractions were performed as previously described (Marques Pereira *et al.* 2008).

Primary hippocampal cultures

Primary cultures of hippocampal neurons were obtained from WT and *mrsk2_KO* male mice, with a C57Bl/6× genetic background, at embryonic day E17. Dissected hippocampi were triturated and plated into wells of 24-well plates coated with poly-D-lysine (Sigma, St Louis, MO, USA), at a density of ~300 000 neurons/well. Growth media consisted in NeuroBasal (Gibco, Rockville, MD, USA; Invitrogen, Carlsbad, CA, USA) supplemented with 1× B27 (Gibco; Invitrogen), 0.5 mM L-glutamine and 1× penicillin/streptomycin. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and cultivated for 7 days *in vitro* prior to experimentation.

Cell culture treatments

At 7 days *in vitro*, hippocampal neurons were activated by glutamate. Glutamate was added to the primary culture media at a concentration of 100 μM for the duration indicated. The ERK pathway was inhibited with the MEK specific inhibitor U0126 (9903; Cell Signaling Technology, Beverly, MA, USA). Hippocampal cells were pre-treated for 30 min with 5 μM U0126 and then stimulated with glutamate. All drugs were prepared as concentrated stock solution in distilled water and diluted to final concentration in culture medium just before application. After treatment, cells were

washed twice with PBS1× and extracted in RIPA buffer (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (40 µL/well). The Bradford assay was used to determine protein concentration.

Phosphoprotein purification

After quantification, purification of Ser/Thr phosphorylated protein was done using the PhosphoProtein Purification Kit (37101; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After separation, eluted fractions were concentrated using Amicon Ultra-0,5 mL Centrifugal Filters (UFC5030BK; Millipore Corporation, Bedford, MA, USA), and denatured at 95°C for 5 min. Equal volume of each sample was loaded on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel as described below for western blot analysis, and resulting bands were normalized to the total input levels.

Cellular fractionation

Cytosolic and nuclear protein fractions were obtained using the ProteoExtract Subcellular Proteome Extraction Kit (539791; Calbiochem, San Diego, CA, USA) following the manufacturer's protocol. Briefly, after glutamate stimulation, hippocampal cells were washed twice with ice cold PBS1×. Several extraction buffers were subsequently added to the well to obtain four distinct fractions containing cytosolic, membranous, nuclear and cytoskeletal proteins respectively. The Bradford assay was used to determine protein concentration and 5 µg proteins were loaded on the SDS–PAGE gel as described below under western blot analysis. Resulting bands were normalized to a nuclear marker (TATA binding protein) and a cytoplasmic marker (CALPAIN).

Western blot analysis

Western blot analyses were performed as previously described (Marques Pereira *et al.* 2008), with minor modifications. For total extraction protocol, 5 µg hippocampal proteins were loaded on the SDS–PAGE gel, and proteins were transferred to an Immobilon-polyvinylidene difluoride membrane (IPVH00010; Millipore). Quantifications were carried out with the GeneTool software of the Chemigenius apparatus (Syngene, Cambridge, UK), and results were normalized to the level of the housekeeping protein βTUBULIN.

The following antibodies were used: anti-ERK (9102), anti-P-ERK (9106), anti-P-RSK (9344), anti-CREB (9197), anti-MEK (8727), anti-P-MEK (9121), anti-c-RAF (9422), anti-P-RAF (9427), anti-P-PP1 (2581), anti-CALPAIN (2539), anti-P-p38 (4631), anti-P-JNK (9255), anti-Elk-1 (9182) all from Cell Signaling Technology (Beverly, MA, USA); anti-RSK1 (sc-231-G), anti-RSK3 (sc-1431), anti-P-ELK1 (sc-8406) all from Santa Cruz Biotechnology; anti-P-CREB (Ser133) (NB300-273; Novus Biologicals, Littleton, CO, USA); anti-βTUBULIN (MAB3408, Millipore); anti-TATA Binding Protein (home made; IGBMC, Illkirch, France).

Real-time QRT-PCR analysis

Total RNA from hippocampal primary cultures was obtained using Tri-Reagent (TR-118; Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed on 1 µg RNA using the Transcriptor kit (03 531 317 001; Roche Molecular Biochemicals, Indianapolis, IN, USA) to generate cDNA with oligo dT following the manufacturer's protocol. Real-time QRT-PCR was performed with LightCycler^{480} Sybr Green I Master (04 887 352 001; Roche) and achieved using a LightCycler instrument (Roche). The sequence of PCR primers used for detection of *Zif268*, *c-Fos* and *Arc* expressions are the following: *Arc*; forward 5′-AGCAGCAGACCTGACATCCT, reverse 5′-TCTCAGCAGCCTTGAGACCT; *c-Fos*; forward 5′-AGCGAACAACCCTATGAGCA, reverse 5′-AGTCGTTTGGCTGGGTAAC, *Zif268*; forward 5′-AGCGAACAACCCTATGAGCA, reverse 5′-AGTCGTTTGGCTGGGTAAC. All results were normalized using quantification of the housekeeping gene *βTubulin;* forward primer 5′-CGATGAGCACGGCATAGAC, reverse primer 5′-TAAAGTTGTCGGGCCTGAAT).

Statistical analysis

All data are expressed as mean ± SEM except if stated otherwise. Comparisons between groups were made by a Student's *t*-test or a two-way ANOVA test. Differences with $p < 0.05$ between WT and *mrsk2_KO* groups were considered significant.

Results

Expression and activation of ERK1/2 in the hippocampus of *mrsk2_KO* mice

We first analyzed the expression levels of ERK1/2 and P-ERK1/2 in the hippocampus of 6 *mrsk2_KO* and 6 WT littermate mice. Detection of total ERK levels in whole hippocampal protein extracts, revealed no significant difference neither for ERK1 nor for ERK2 in KO mice when compared to WT mice. However, detection of phosphorylated ERK levels showed that the active forms of both ERK1 and 2 (P-ERK1/2) were significantly increased in mutated mice (P-ERK2/ERK2: WT: 1.26 ± 0.2; KO: 2.59 ± 0.1; P-ERK1/ERK1: WT: 1.04 ± 0.05; KO: 3.10 ± 0.4; $*p < 0.05$ by Student's *t*-test) (Fig. 2).

Expression and activation of ERK1/2 and RSK upstream kinases in primary hippocampal cell cultures

All the subsequent studies were then based on primary hippocampal neuronal cultures derived from *mrsk2_KO* mice and WT littermates. Total ERK levels, revealed no difference of ERK protein expression between WT and KO mice (Fig. 3). Analysis of phosphorylated forms of ERK showed that the active form of ERK 2 (P-ERK2) was significantly higher in *mrsk2_KO* neurons than in WT cells at the basal state (P-ERK2/ERK2: WT: 1.2 ± 0.1; KO: 2.9 ± 0.3; $*p < 0.05$), whereas, the level of P-ERK1, although not reaching significance, was also much higher in mutant cells as in WT cells: P-ERK1/ERK1; WT: 1 ± 0.1; KO: 2.9 ± 0.4 (Fig. 3). A strong increase of the level of both P-ERK1 and P-ERK2 was observed 5 min after glutamate stimulation in both WT and *mrsk2_KO* neurons, but which was significantly stronger in mutant-derived neurons (P-ERK2/ERK2: WT: 5.9 ± 0.7; KO: 8.7 ± 1.3; P-ERK1/ERK1: WT: 9.2 ± 1.3;

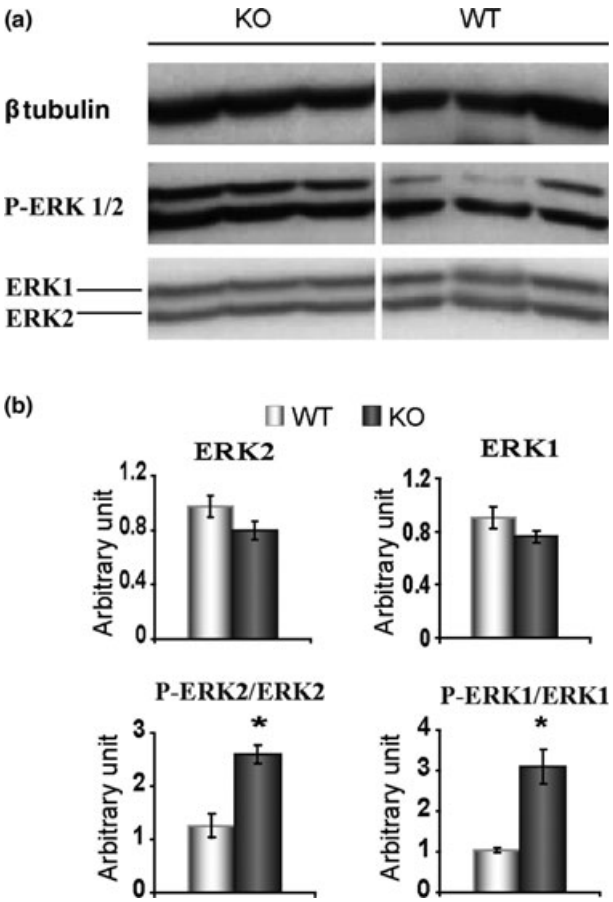

Fig. 2 Increased ERK phosphorylation in the *mrsk2_KO* hippocampus. (a) Proteins detected (ERK1/2, P-ERK1/2 and β-TUBULIN) in three *mrsk2_KO* and three WT mice are shown. (b) Data normalized to ERK1/2 and β-TUBULIN are represented as the mean values ± SEM for six mice of each genotype. Levels of P-ERK1/2 are significantly increased in the hippocampus of *mrsk2_KO* mice. *$p < 0.05$ by Student's *t*-test.

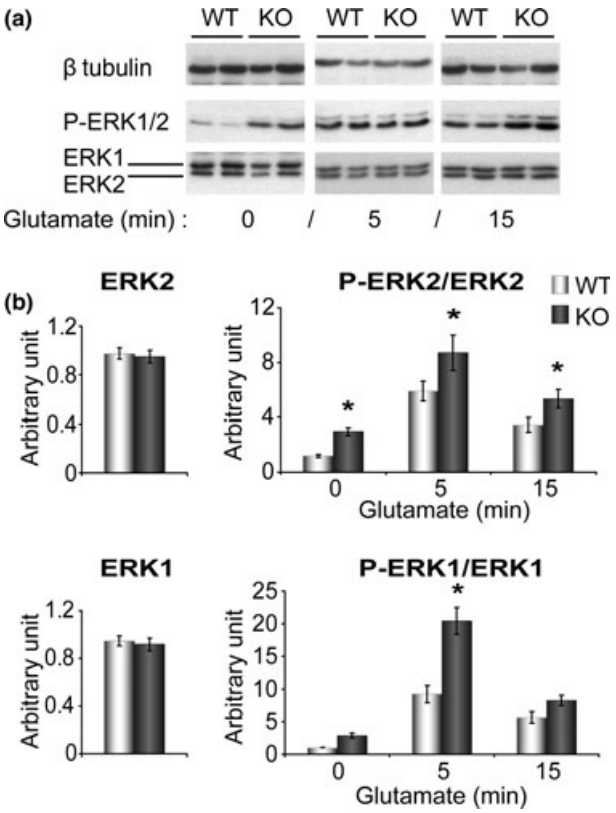

Fig. 3 Increased ERK activity after glutamate stimulation in *mrsk2_KO* hippocampal neurons. (a) Detection of the ERK1/2 and P-ERK1/2 protein levels in WT and *mrsk2_KO* hippocampal cell extracts, without treatment and after glutamate stimulation (100 μM). (b) Western blot data normalized to βTUBULIN and ERK are represented as the mean ± SEM (WT: $n > 10$ embryos; KO: $n > 13$ embryos). Glutamate stimulation induced an increase of ERK phosphorylation in both cell types, but this increase was higher and sustained in RSK2-deficient cells. *$p < 0.05$ by two-way ANOVA.

KO: 20.5 ± 2.1; *$p < 0.05$). Fifteen minutes after glutamate application, levels of both P-ERK1 and P-ERK2 decreased, but much more in WT neurons than in *mrsk2_KO* ones (P-ERK2/ERK2: WT: 3.5 ± 0.5; KO: 5.3 ± 0.7; P-ERK1/ERK1: WT: 5.7 ± 0.9; KO: 8.2 ± 0.8; *$p < 0.05$) (Fig. 3).

We then wondered whether the activation of ERK1/2 upstream kinases MEK and RAF-1 are also up-regulated in *mrsk2_KO* neurons. Analysis of total MEK and RAF-1 protein levels, revealed no difference between WT and KO mice (Fig. 4) However, levels of phosphorylated forms of MEK and RAF-1, were significantly higher in *mrsk2_KO* and WT neurons at the basal state (P-MEK: WT: 0.3 ± 0.04; KO: 0.5 ± 0.03; P-RAF-1: WT: 1.2 ± 0.06; KO: 1.5 ± 0.05; *$p < 0.05$), as well as after glutamate application (P-MEK: WT: 3.6 ± 0.6; KO: 7.2 ± 1.1; P-RAF-1: WT: 1.4 ± 0.08; KO: 2.2 ± 0.16; *$p < 0.05$) (Fig. 4).

RSK phosphorylation level

Analysis of total RSK1 and RSK3 protein levels revealed no significant difference of expression between WT and KO mice (Fig. 5). We assayed P-RSK levels with a rabbit anti-phospho-RSK antibody that does not discriminate between the RSK1, -2 and -3. phosphorylated forms. An increase of the level of P-RSK (for P-RSK1 + P-RSK2 + P-RSK3) was observed in WT and *mrsk2_KO* neurons 5 min after glutamate application, showing that RSKs are activated in response to glutamate (Fig. 5a and b). Interestingly, in spite of RSK2 loss, the level of P-RSK was higher in RSK2-deficient neurons than in WT neurons (WT: 3 ± 0.4; KO: 5.1 ± 0.6; *$p < 0.05$), suggesting that loss of RSK2 activity is over-compensated by an increase of the activity of RSK1 and/or RSK3 (Fig. 5a and b). To determine whether one or both of these latter RSKs are over-activated, a PhosphoProtein Purification protocol was used to isolate phosphorylated proteins from RSK2-deficient and WT cell lysates. The

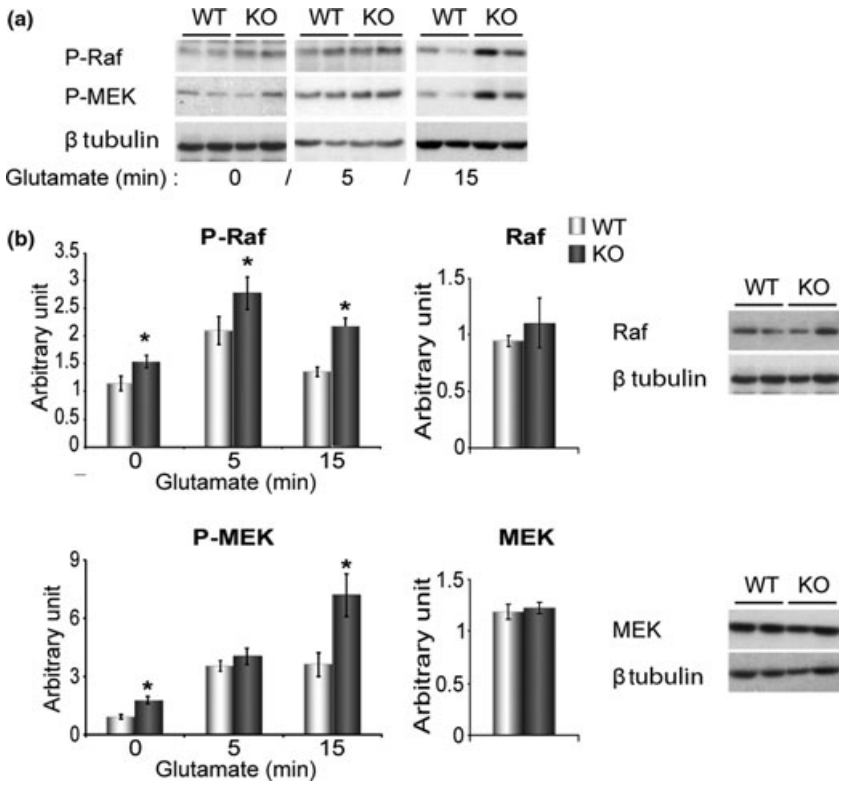

Fig. 4 Stronger activation of ERK upstream kinases in WT and KO neurons after glutamate induction. (a) Protein levels of ERK upstream kinases detected in WT and KO hippocampal culture lysates treated or not with 100 μM glutamate. (b) Western blot data normalized to βTUBULIN are represented as the mean ± SEM (WT: $n > 5$ embryos; KO: $n > 5$ embryos). Glutamate stimulation induced Raf-1 and MEK phosphorylation at a significantly higher level in *mrsk2_KO* cells than in WT ones. $^*p < 0.05$ by two-way ANOVA.

eluate fractions were then analyzed by western blot, with anti-P-ERK and ERK antibodies to confirm the effectiveness of the protocol (not shown) and subsequently with anti-RSK1 and anti-RSK3 specific antibodies. Although the results revealed only a tendency of over-activation of RSK1 in *mrsk2_KO* neurons after 5 min glutamate stimulation, the level of phosphorylated RSK3 was much higher and significant in mutant cells than in WT neurons (WT: 0.5 ± 0.01; KO: 1.2 ± 0.03; $^*p < 0.05$) (Fig. 5c and d).

Subcellular localization of activated ERK1/2

Since subcellular localization of the components of the ERK cascade was shown to play an important role in specificity determination, we also examined the subcellular localization of ERK and P-ERK in *mrsk2_KO* and WT neurons at basal state and after glutamate stimulation (Fig. 6). At the basal state, cytosolic and nuclear levels of both total ERK1 and ERK2 proteins were very similar in KO and WT cells (Fig. 6a–c). However, the level of P-ERK2 was much higher in the nuclear fraction of RSK2-deficient neurons than of WT counterparts, whereas the levels of nuclear P-ERK1 were similar in KO and WT cells (Fig. 6a and c). After 5 min of glutamate stimulation, we observed a similar small increase of both total ERK1 and -2 proteins in the nucleus and in parallel a decrease of both proteins in the cytosol of KO and WT neurons (Fig. 6a–d). There was also a translocation of the phosphorylated form of ERK2 into the nucleus in both WT- and RSK2-deficient neurons (Fig. 6a and c). However, at the same time a much higher level of P-ERK2 was still detectable in the cytosolic fraction of mutant neurons than in the cytosol of WT neurons (Fig. 6b and d). After 15 min of glutamate stimulation, levels of nuclear P-ERK2 decreased and cytosolic P-ERK2 increased similarly in neurons of both genotypes. P-ERK1 was also rapidly translocated into the nucleus after 5 min of glutamate stimulation, but at higher levels in WT neurons than in RSK2-deficient cells (Fig. 6a and c). At the same time and similarly to P-ERK2, a much higher level of P-ERK1 was detectable in the cytosolic fraction of *mrsk2_KO* cells (Fig. 6b and d). After 15 min of glutamate stimulation, levels of nuclear P-ERK1 decreased and cytosolic P-ERK1 increased similarly in both WT- and RSK2-deficient neurons (Fig. 6a–d).

Our results suggest that at the basal state there are more phosphorylated ERK2 (at least) molecules in the nucleus of *mrsk2_KO* neurons than in WT nuclei and that after short glutamate stimulation, higher fractions of both P-ERK1 and 2 are retained in the cytosol of RSK2-deficient neurons. After longer glutamate stimulation, the nuclear and cytosolic fractions became quite similar in WT and KO neurons.

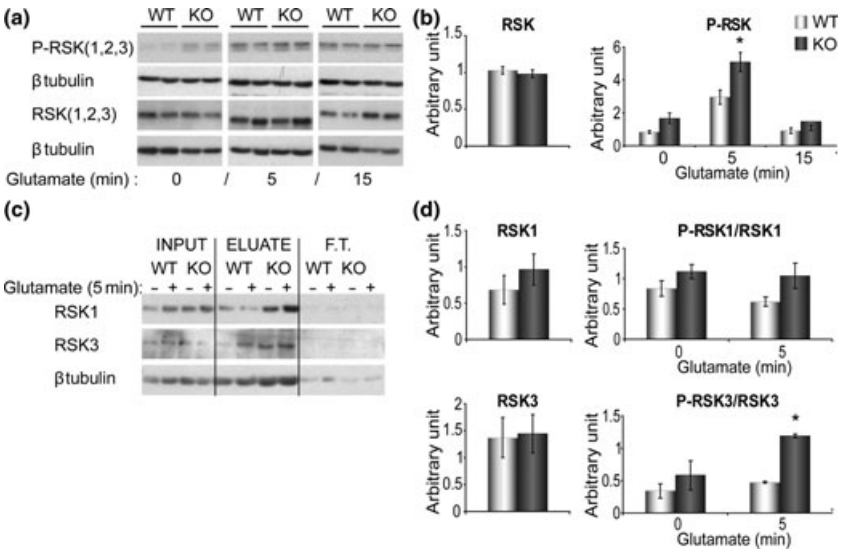

Fig. 5 Increased RSK phosphorylation in response to glutamate induction in *mrsk2_KO* hippocampal neurons. (a) Detection of RSK and P-RSK1/2/3 protein level in WT and KO hippocampal lysates, without stimulation, and after 5 and 15 min glutamate stimulation (100 μM). (b) Western blot data normalized to βTUBULIN are represented as the mean ± SEM (WT: $n > 8$ embryos; KO: $n > 9$ embryos). Glutamate stimulation induced an increased RSK phosphorylation in both cell types, but this increase was higher in RSK2-deficient cells after 5 min stimulation. $^*p < 0.05$ by two-way ANOVA. (c) Protein level detection of RSK1 and 3 after phosphoprotein purification of WT and KO neuronal extracts activated or not (Eluate fraction). (d) Western blot results normalized to both βTUBULIN and total input levels are represented as the mean ± SEM (for three embryos of each genotype). RSK3 phosphorylation was induced after glutamate stimulation, but at a significantly higher level in *mrsk2_KO* neurons than in WT cells. $^*p < 0.05$ by two-way ANOVA. (F.T., flow through)

To determine the consequences of the increased P-RSK level on subcellular distribution in *mrsk2_KO* cells, we also assessed RSK (for RSK1, 2 and 3) and P-RSK subcellular localization. Glutamate stimulation induced an increase of nuclear total RSK and P-RSK in both WT- and RSK2-deficient neurons, but the increase was higher and more sustained, for P-RSK, in mutant than in WT neurons (Fig. 6a–d).

Expression or activation of ERK1/2 and/or RSK2 targets

To investigate whether ERK over-activation has an influence on activity of ERK nuclear substrates, we analyzed phosphorylation of ELK1 and CREB. ELK1 is a member of the Ets family of transcription factors, which regulates serum response element-dependent gene expression. We tried to determine the level of total ELK-1 protein in WT and KO neurons, but unfortunately the antibody gave a strong background and no accurate quantification could be done. However, by visual examination the intensity of the 47 kDa band corresponding to the ELK-1 size was quite similar in KO and WT neuronal extracts. At the basal state, the level of P-ELK1 was significantly higher in RSK2-deficient neurons than in WT neurons (WT: 1 ± 0.1; KO: 2.1 ± 0.2; $^*p < 0.05$) (Fig. 7a and b). Five minutes of glutamate stimulation resulted in an increase of the phosphorylated form of ELK1 in WT and *mrsk2_KO* cells. After 15 min of glutamate treatment, the level of P-ELK1 decreased below the initial basal level in WT- and RSK2-deficient hippocampal neurons. However, the level of P-ELK1 always remained significantly higher in KO than in WT neurons (5 min: WT: 1.8 ± 0.12; KO: 2.51 ± 0.14; 15 min: WT: 0.87 ± 0.06; KO: 1.43 ± 0.11; $^*p < 0.05$) (Fig. 7). CREB is a transcription factor regulated via the ERK/MAPK pathway by RSK2 and MSK1. Total CREB protein was very similar in WT and KO neurons (Fig. 7). At basal state, the level of P(Ser133)-CREB was also significantly higher in RSK2_deficient neurons than in WT cells (WT: 1.2 ± 0.15; KO: 2 ± 0.27; $^*p < 0.05$). Glutamate treatment induced an increase of CREB phosphorylation in WT and KO neurons, but which was significantly higher in RSK2-deficient than in WT neurons (WT: 3.2 ± 0.23; KO: 3.9 ± 0.3; $^*p < 0.05$) (Fig. 7a and b).

U0126, a MEK specific inhibitor, was then used to confirm the ERK-dependent activation of RSK, ELK1 and CREB. Neurons were treated with U0126 for 30 min, before glutamate stimulation. As expected, glutamate-induced ERK phosphorylation was completely inhibited in WT and *mrsk2_KO* neuronal cultures (Fig. 8a). A dramatic decrease of RSK and CREB phosphorylation was also observed (P-CREB: decrease of 2.3 and 18.9* times in WT at basal versus basal + U0126 conditions, and activated versus activated + U0126 conditions respectively, and a decrease of 4.9* and 6.7* times respectively in KO cells; P-RSK: 1.5 and 6.1* respectively in WT neurons, and 4.3* and 6.4* respectively in KO cells, $^*p < 0.05$ by Student's *t*-test) (Fig. 8b). However, glutamate-induced ELK1 phosphorylation was

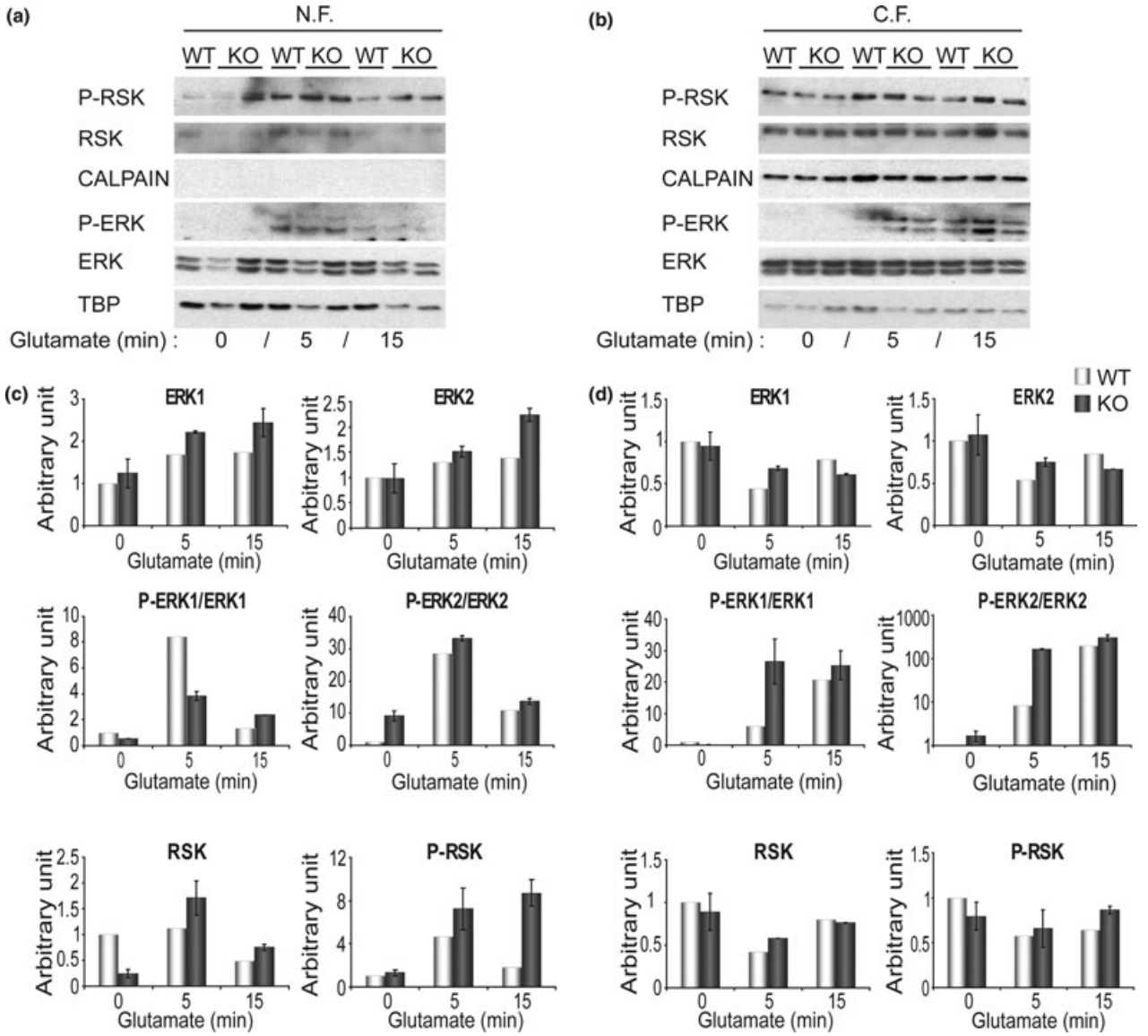

Fig. 6 Increased P-ERK and P-RSK localize in distinct compartments in *mrsk2_KO* neurons. (a, b) Level of ERK, P-ERK, RSK and P-RSK detected in WT and *mrsk2_KO* in (a) nuclear and (b) cytoplasmic fractions (N.F. and C.F. respectively). Subcellular fractionation was performed on basal and activated hippocampal cultures (100 μM glutamate) and analyzed by western blot. (c, d) Quantification of protein level, normalized to TBP (nuclear control), CALPAIN (cytoplasmic marker) or ERK are represented as the mean ± SEM (WT: $n = 1$ embryo; KO: $n = 2$ embryos). (c) Five minutes glutamate stimulation induced a similar increase of P-ERK2 in the nucleus of WT and KO neurons, whereas a higher proportion of P-ERK1 molecules were translocated to the nucleus in WT neurons than in KO cells. A higher increase of P-RSK level was observed in the KO nucleus compared to WT ones after treatment. (d) The cytosolic fractions of P-ERK1/2 increased in both WT and KO cells after 5 min glutamate stimulation, but at a much higher level in KO cells, suggesting retention in the cytosol of KO neurons. Cytosolic P-RSK was not modified by glutamate stimulation in both groups.

not inhibited by U0126 treatment neither in WT nor in KO neurons (Fig. 9a and b). As ELK1 activity is also regulated via the p38 and JNK pathways, we analyzed the phosphorylation state of P-p38 and P-JNK. P-JNK and -p38 revealed increased levels of phosphorylation in both cases (P-p38: increase of 1.5 and 3.6* times at activated versus activated + U0126 conditions in WT and KO cells respectively; P-JNK: increase of 2.5* and 0.9 times in WT and KO neurons respectively, $*p < 0.05$ by Student's *t*-test, but not by two-way ANOVA) (Fig. 9a and c).

Expression of immediate-early genes

To check whether increase of CREB and ELK1 activities in RSK2-deficient neurons leads to changes in transcriptional activity, we investigated the induction of the expression of three IEGs upon glutamate stimulation at the mRNA level

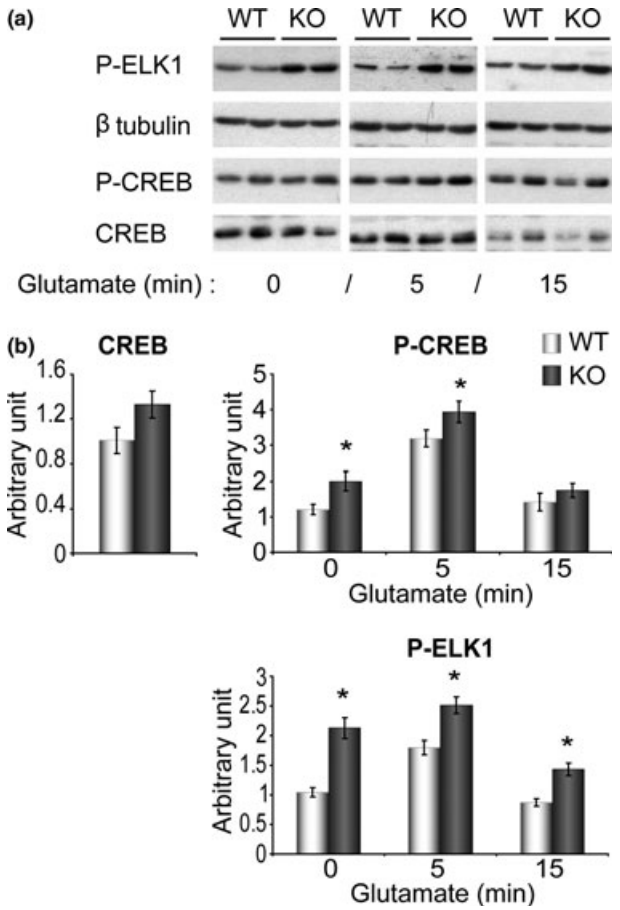

Fig. 7 Increased activation of transcription factors in *mrsk2_KO* cells after glutamate induction. (a) Western blot analysis of ERK targets, involved in regulation of gene expression, in WT and *mrsk2_KO* neurons untreated or treated with 100 μM glutamate. (b) Protein levels were normalized to βTUBULIN and are represented as the mean ± SEM (WT: $n > 12$ embryos; KO: $n > 14$ embryos). The level of P-ELK1 increased in both WT and KO neurons in response to glutamate stimulation. However, levels were significantly higher in *mrsk2_KO* neurons than in WT ones. Increase of CREB phosphorylation induced by glutamate was significantly stronger in KO neurons. *$p < 0.05$ by two-way ANOVA.

by QRT-PCR. Increase of transcription of all three IEGs, *c-Fos*, *Zif268* and *Arc*, was observed in WT and *mrsk2_KO* neurons after cellular stimulation, but, for the three genes, at a significantly higher level in RSK2-deficient neurons than in WT neurons (*c-Fos*: WT: 0.059 ± 0.009; KO: 0.08 ± 0.012; *Zif268*: WT: 0.1 ± 0.019; KO: 0.16 ± 0.015; *Arc*: WT: 0.013 ± 0.002; KO: 0.02 ± 0.004; *$p < 0.05$) (Fig. 10).

Discussion

We show in the present study that the levels of Phospho-ERK1 and Phospho-ERK2 are significantly higher in the hippocampus of *mrsk2*_KO mice as in WT littermates. To further investigate the cause and consequences of this ERK1/2 increased activation, we analyzed ERK1/2 signaling in RSK2-deficient cultured hippocampal neurons stimulated or not with glutamate.

Phosphorylation of ERK1 and ERK2 at the basal state and subsequent to glutamate stimulation was also stronger in RSK2-deficient neurons than in WT neurons. We first wondered whether this increased phosphorylation level might be ascribed to the loss of the RSK2 feedback inhibitory effect on the ERK pathway previously described (Douville and Downward 1997). Our data show that both MEK and RAF-1 are activated more strongly in RSK2-deficient neurons as in WT cells, confirming further this mechanism in hippocampal neurons. Strikingly, even in absence of RSK2, the level of phospho-RSK is much higher in mutant neurons than in WT counterparts, suggesting an over-compensation by other RSK members. We show that RSK3 is predominantly responsible for this over-compensation, the level of glutamate-induced RSK3 phosphorylation in KO hippocampal neurons being twice that in WT cells. The mechanism of this over-compensation is not yet known, but is likely to be the direct consequence of the over-activation of the upstream kinases ERK1/2.

The subcellular localization of ERK, an important factor controlling its activity, was also investigated in RSK2-deficient neurons. At the basal state, there are clearly higher levels of P-ERK2 in the nucleus of *mrsk2_KO* neurons than in WT nuclei. After glutamate stimulation, similar levels of ERK1/2 are detected in the nuclei of both WT and RSK2-deficient neurons, whereas high fractions of both P-ERK1 and -2 are transiently retained in the cytosol of RSK2-deficient neurons, but not in WT cells. Nuclear localization of ERK1/2 is essential for some of the phenotypic programs to which they contribute, including differentiation, transformation and altered transcription. Therefore, we wondered whether the transcription factors ELK1 and CREB are similarly activated in RSK2-deficient and WT neurons in response to glutamate stimulation. Increases of phosphorylation levels of both CREB and ELK1 were observed in WT- and RSK2-deficient neurons, which were, however, significantly stronger in RSK2-deficient neurons. As ELK-1 has been described as a direct nuclear target of ERK1/2 (Yang *et al.* 1998), this result suggested that the increased ELK-1 phosphorylation is directly the consequence of increased ERK1/2 activity in RSK2-deficient neurons. However, inhibition of ERK activity by U0126 did not abolish glutamate-induced phosphorylation of ELK1, providing evidence that glutamate-induced ELK1 phosphorylation is not mediated by ERK1/2 in hippocampal neurons. As glutamate also activates JNKs and p38 (Wang *et al.* 2007) we suspected involvement of these pathways in ELK1 activation. Both p38 and JNKs showed an increase of phosphorylation levels in WT and RSK2-deficient neurons after inhibition, with U0126, of the ERK/MAPK

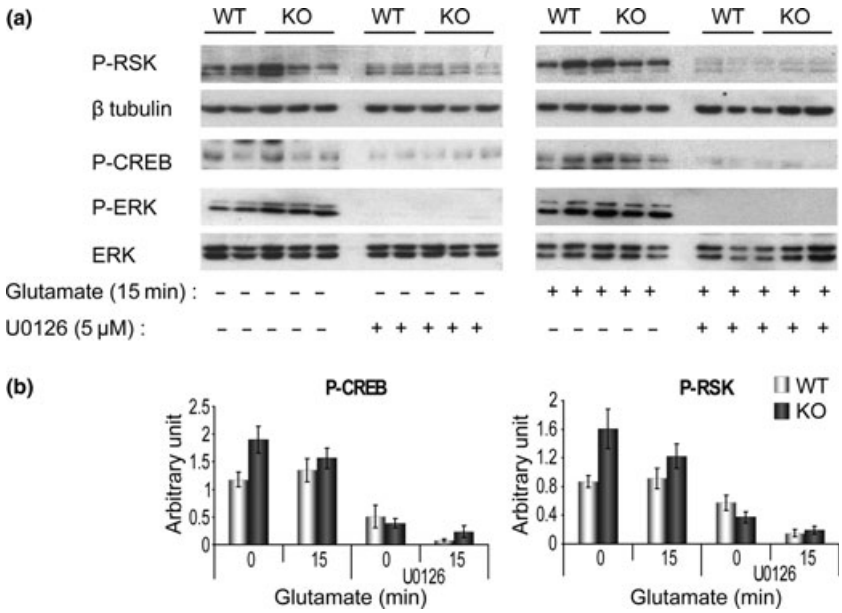

Fig. 8 RSK and CREB activation are dependent on ERK activity in hippocampal neurons. (a) Protein levels detection of P-RSK and P-CREB from WT and *mrsk2_KO* neurons treated with U0126 (5 μM), a MEK specific inhibitor and glutamate (100 μM). (b) Protein levels quantified by western blot analysis and normalized to βTUBULIN are represented as the mean ± SEM (WT: $n > 2$ embryos; KO: $n > 3$ embryos). U0126 treatment led to a strong decrease of ERK phosphorylation in both WT and KO cells. RSK and CREB phosphorylations were also significantly blocked by U0126 treatment in KO cells at the basal state and after glutamate stimulation in both WT and KO neurons. This suggests that ERK is the main upstream kinase of RSK and CREB in hippocampal cells.

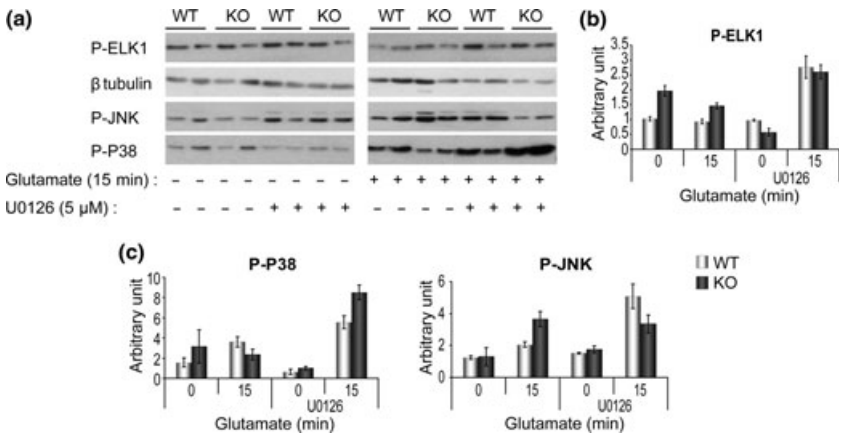

Fig. 9 ELK1 activation is ERK, p38 and JNK dependent in hippocampal neurons. (a) Protein levels of P-ELK1, P-p38 and P-JNK in WT and *mrsk2_KO* neurons treated with U0126 (5 μM) and glutamate (100 μM). (b, c) Western blot data normalized to βTUBULIN are represented as the mean ± SEM (WT: $n > 2$ embryos; KO: $n > 3$ embryos). (b) U0126 treatment leads to a decrease of ELK1 phosphorylation in KO cells at the basal state. However, an increase of P-ELK1 is observed after glutamate stimulation in WT and *mrsk2_KO* neurons. (c) Levels of phosphorylation of p38 and JNK were also increased in both cell types after U0126 treatment and glutamate stimulation. This suggests that ELK1 phosphorylation is in part due to p38 and JNK activation in these hippocampal cells.

pathway. A cross-talk between different MAPK pathways was already reported (Monick *et al.* 2006). The authors showed that ERK inhibition leads to JNK activation in macrophages. Our results indicate that it is also the case in neurons.

The increased CREB phophorylation observed in RSK2-deficient neurons indicated that RSK2 is not the major kinase of CREB in hippocampal neurons upon glutamate stimulation. However, it could not be excluded that the higher level of P-RSK, and mainly of P-RSK3, observed in mutant

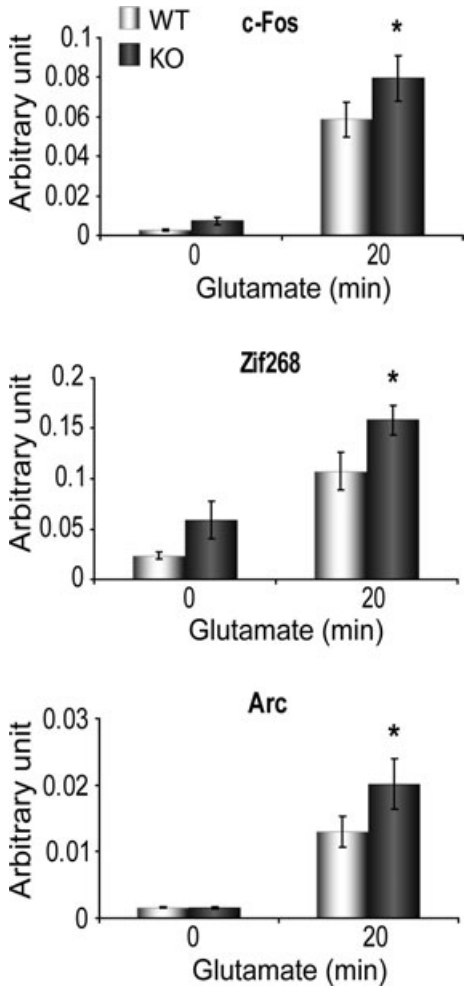

Fig. 10 Alteration of transcriptional activity in glutamate stimulated *mrsk2_KO* neurons. mRNA level of *c-Fos*, *Zif268* and *Arc* quantified in hippocampal cells derived from WT and KO embryos. mRNA levels were determined by real-time QRT-PCR using neuronal extracts stimulated by glutamate (100 μM) for 20 min. Each bar represents the mean ± SEM (*c-Fos*: WT: $n = 6$ embryos; KO: $n = 6$ embryos; *Zif268*: WT: $n = 7$ embryos; KO: $n = 7$ embryos; *Arc*: WT: $n = 4$ embryos; KO: $n = 4$ embryos). Glutamate stimulation induces an increase of these IEGs transcription in WT and *mrsk2_KO* cells. A higher level of expression is detected for each of them in RSK2-deficient neurons. *$p < 0.05$ by two-way ANOVA.

neurons over-compensates for absence of RSK2 in this cellular compartment and acts on some RSK2 substrates, including CREB. A strong reduction of CREB phosphorylation, below the basal level, was observed after U0126 treatment, demonstrating further that glutamate-induced CREB phosphorylation is ERK1/2-dependent in hippocampal neurons. This finding basically resembles those observed in striatal neurons for instance (Perkinton *et al.* 2002), indicating a significant role of MEK/ERK in Ca^{2+} signaling pathways to CREB phosphorylation in response to glutamate stimulation. The level of phosphorylated RSK1/3 decreased also dramatically after U0126 treatment, as expected for these direct downstream targets of ERK1/2. However, this result cannot exclude that (in absence of MEK inhibitor) over-activation of RSK3 is able to over-compensate for RSK2 knockdown in neuronal nuclei and act on CREB. Indeed, further investigations are necessary to determine whether over-activation of RSK1/3 or directly of ERK1/2 is responsible for increased CREB phosphorylation in RSK2-deficient neurons.

IEGs are induced soon after neuronal activity and they participate in diverse functions. Some IEGs are regulatory transcription factors (e.g. *c-Fos*, *Zif268/Egr1*) responsible for inducing transcription of late-response genes, whereas others are effector IEGs (e.g. *Arc*) that are directly involved in cellular changes at locations such as the cytoskeleton or receptors formation. The prototypic IEG, *c-Fos*, has been reported to be both rapidly and transiently transcribed in response to a variety of neurotransmitters that trigger Ca^{2+} influx (Bading *et al.* 1993; Curran and Morgan 1995). Although it was previously shown that transcription of the *c-Fos* gene was altered in fibroblasts derived from *mrsk2_KO* mice (Bruning *et al.* 2000), no data are available regarding the expression of *Zif268* and *Arc* in RSK2-deficient cells. In the present study, we show that transcription of *c-Fos*, *Zif268* and *Arc* is induced upon glutamate stimulation, but at significantly higher levels in RSK2-deficient than in WT neurons. This observation suggests a direct relationship between ERK1/2 over-activation and stronger induction of IEGs. Finally, our results show that the transient sequestration of P-ERK1/2 in the cytoplasm upon glutamate stimulation has no obvious consequence on CREB activation and induction of transcription of IEGs. However, additional studies are necessary to determine the consequences of ERK sequestration in *mrsk2_KO* cytoplasm and the roles played by other RSK family members, as well as MSK1/2.

The mechanism of feedback down-regulation of Ras activation by RSK2 described by Douville and Downward (1997) in epidermal growth factor stimulated PC12 cells has subsequently been suggested as an explanation for increased and prolonged ERK activation in skeletal muscle of *mrsk2*_KO mice (Dufresne *et al.* 2001). However, these latter authors also hypothesized that expression of ERK phosphatases may be reduced in RSK2-deficient cells and studies are ongoing in our laboratory to explore this hypothesis. Sheffler *et al.* (2006) more recently reported that basal and 5HT2A receptor-mediated ERK1/2 phosphorylation is increased in *mrsk2*_KO fibroblasts. Our data, showing that activation of RAF-1, MEK and ERK1/2 is up-regulated in RSK2-deficient neurons, further support and extend this mechanism to neuronal cells. Importantly, our data provide evidence for the first time that this deregulation has an impact on gene expression. Indeed, ERK1/2 over-activation may account, directly or indirectly, for some of the deregulated genes identified in our transcription profiling study

(Mehmood *et al.* 2011), through over-activation of transcription factors or over-induction of IEGs. Further studies, too explore this hypothesis, are ongoing in our laboratory.

Finally, our data provide an important contribution to a fascinating issue not yet clarified: do the various RSK proteins, which are highly homologous and all widely expressed, regulate distinct or unique (plus overlapping?) cellular functions? Our results show that increased phosphorylation of RSK1/3 in RSK2-deficient neurons neither is able to restore a normal ERK1/2 activity, nor to correct the expression of CREB, ELK1 and the IEG gene expression. The feedback inhibition exerted by RSK2 on the ERK1/2 pathway is clearly a specific function of RSK2 that cannot be compensated by other members of the RSK family. The phosphorylation/activation of other substrates of ERK1/2 has now to be investigated in the context of absence of RSK2 activity.

The aberrant ERK1/2 signaling report here is susceptible to influence various neuronal functions, and can, indeed, play a significant role in cognitive dysfunction of *mrsk2_KO* mice and of CLS patients.

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Publication-3

2.4: *Rsk2* Knockdown in PC12 cells results in *Sp1* dependent increased expression of the *Gria2* gene, encoding the AMPA receptor subunit GluR2, via up regulation of ERK1/2 activity

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The main objective of this study was to understand the molecular mechanism of up regulation of GluR2 (subunit of AMPA receptors) in RSK2 deficient cells. Previously we showed that in the hippocampus of *Rsk2*-KO mice GluR2 expression is up regulated at the transcriptional and protein levels (Mehmood et al., 2011). We also showed that ERK1/2 activity is abnormally increased in the hippocampus of *Rsk2*-KO mice (Schneider et al., 2011). The mechanism through which RSK2 deficiency, affects the expression of GluR2 in neural cells was unknown. Thus, we were interested to investigate, whether the GluR2 expression is directly controlled by the RSK2 kinase or indirectly via a RSK2 or ERK1/2 substrate. To the best of our knowledge no one else has reported on regulation of GluR2 expression by RSK2.

Since primary neural cultures are difficult to manipulate and transfect, we used, as a first step to understand the mechanism leading to GluR2 up regulation of expression, the RNA interference (shRNA) technology to knockdown the *Rsk2* gene in PC12 cells. PC12 cells are immortalized cells derived from a pheochromocytoma tumor of the rat adrenal gland. NGF treated PC12 cell line serves as a model system for primary neuronal cells. (Greene and Tischler, 1976; Satoh et al., 1988; Xia et al., 1995). First we investigated the efficiency to block RSK2 expression in PC12 cells by using three plasmids generating different RSK2-specific shRNA, targeted to different coding regions of the mRNA of *Rsk2*. We observed a dramatic decrease of the *Rsk2* mRNA level for one of them, shRNA (clone ID7). At the protein level RSK2 expression was decreased by 90% in cells transfected with this shRNA. There was also a strong increase of GluR2 expression at the RNA and protein levels in the cells transfected with clone ID7, as well as an increased level of ERK1/2 phosphorylation. Treatment of the *Rsk2*-knockdown cells with U0126, a MEK inhibitior, resulted in inhibition of ERK activity and in a dramatic decrease of GluR2 expression.

Therefore, we assumed that the increased GluR2 expression in *Rsk2*-KO mice and *Rsk2*-knockdown PC12 cells might be indirectly regulated by over activation of ERK1/2 activity. Ten years ago it was reported that the *Gria2* gene is regulated at the transcriptional level by the *Sp1* transcription factor (Ekici et al., 2011; Sroka et al., 2007). In addition, Chu and Ferro, (2005) reported that the MEK-ERK signal pathway mediates the *Sp1* phosphorylation at two sites T 453 and T739.

Therefore, we investigated the levels of *Sp1* phosphorylation at both of these sites. We observed an abnormally increased level of phospho-*Sp1* at both sites (T453 and T739) in *Rsk2*-knockdown cells compared to WT PC12 cells, where as there was no difference in total *Sp1* expression. We further found that treatment of *Rsk2*-knockdown PC12 cells with the MEK inhibitor U0126, completely abolished phosphorylation of *Sp1* at T453 and T739, confirming the specific implication of ERK1/2 in the phosphorylation of these sites. Partial silencing of *Sp1* expression in *Rsk2*-knockdown PC12 cells with *Sp1*-siRNA resulted in a remarkable decrease of GluR2 expression, confirming that *Sp1* plays a prominent role in the mechanism of GluR2 up-regulation of transcription in RSK2 deficient cells. I showed subsequently that in the hippocampus of *Rsk2*-KO mice, the level of pospho-*Sp1* is significantly higher than in WT littermate mouse, supporting our results in PC12 cells. Thus, the conclusion of our study is that over expression of GluR2 in RSK2 deficient cells is caused by increased *Sp1* transcriptional activity on the *Gria2* gene which itself is the consequence of ERK1/2 increased activity resulting from loss of retro inhibition activity exerted by RSK2 on the ERK pathway.

A complete description and illustration of these results are documented in publication-3 and paper is submitted in journal.

Rsk2 knockdown in PC12 cells results in a Sp1 dependent strong increase of expression of the *Gria2* gene, encoding the AMPA receptor subunit GLUR2, via up regulation of ERK1/2 activity

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Abstract

Background: The RSK2 protein is a member of the RSK serine-threonine protein kinase family and is encoded by the X-linked *rps6ka3* gene in human. Highly heterogeneous loss-of-function mutations affecting this gene are responsible for a severe syndromic form of cognitive impairment, Coffin-Lowry syndrome. RSK2, which is highly conserved in mammals, acts at the distal end of the Ras-ERK signaling pathway and is activated in response to growth factors and neurotransmitters. RSK2 is highly expressed in the hippocampus, and *Rsk2*-KO mice display spatial learning and memory impairment. We recently showed that ERK1/2 activity is abnormally increased in the hippocampus of *Rsk2*-KO mice as well as the expression of the AMPA receptor subunit GluR2. The mechanism via which RSK2 deficiency affects the expression of GluR2 in neural cells was unknown.

Results: To address this issue, we constitutively suppressed in the present study the expression of *Rsk2* in PC12 cells via vector-based shRNA. We show that *Rs*k2 silencing leads also to up regulation of ERK1/2 activity and GluR2 expression and that the increase of GluR2 expression results from up regulated ERK1/2 activity on the transcription factor Sp1. Our results strongly suggest that RSK2 modulates ERK1/2 activity on Sp1, which regulates GluR2 expression through transcriptional activation. Preliminary results show that Sp1 activity is also up regulated in the hippocampus of *Rsk2*-KO mice suggesting that the mechanism of GluR2 transcriptional up regulation is similar in mouse and PC12 cells.

Conclusions: Our present findings suggest an indirect role of RSK2 in modulating the expression of AMPA receptor GluR2 subunit and, therefore, in regulating glutamatergic neurotransmission and probably synaptic plasticity. Future studies are warranted to elucidate

the molecular mechanisms that underline the interplay between the *Rsk2* gene and the glutamatergic system.

Background

The 90kDa ribosomal S6 kinases (RSKs) constitute a family of four homologous Ser/Thr kinases (RSK1-4) that are activated by Mitogen activated protein kinase / extracellular-regulated kinases 1 and 2 (MAPK/ERK1/2) in response to growth factors, hormones, chemokines and neurotransmitters. When activated by Extracellular signal-regulated kinase (ERK), a fraction of RSK2 in turn phosphorylates a number of cytoplasmic substrates such as BCL2-associated agonist of cell death (Bad), p53, L1 cell adhesion molecule (L1CAM) and Glycogen synthase kinase 3 (GSK3) [1]. Another fraction of RSK2 translocates to the nucleus where it is thought to regulate gene expression through phosphorylation of transcription factors such as cAMP-response element binding protein (CREB), the cellular FBJ murine osteosarcoma viral oncogene homolog (c-fos), the oestrogen nuclear receptor, the nuclear receptor subfamily 77 (Nurr 77) and the Activating transcription factor 4 (ATF4) [1,2]. The roles of RSKs in cellular signaling, cell survival, growth and differentiation has been well established, however their implication in biological processes in vivo is less well known. An important physiological role of one RSK member, RSK2, was uncovered by the discovery of *Rsk2* (following the nomenclature: *Rps6ka3 gene*, but we will name it *Rsk2* gene) gene defects in the Coffin-Lowry Syndrome (CLS) [3]. CLS is an X-linked disorder with progressive skeletal abnormalities, facial dimorphism, and strong psychomotor impairment [4]. The vast majority of male CLS patients show severe cognitive deficiency with intelligence quotient (IQ) ranging from 15 to 60. Delay in speech acquisition is particularly common with most affected males having a very limited vocabulary. The mouse knockout (KO) model (*Rsk2*-KO) for CLS exhibits a severe impairment in spatial learning and a deficit in long-term spatial memory [5]. Moreover, in human and mouse brain, RSK2 is highly expressed in the hippocampus, an essential brain structure in cognitive function and learning

[6, 7]. Together the data suggest that RSK2 plays an important role in cognitive function in human and in mice.

To gain greater insight into the molecular mechanisms leading to learning and memory impairments in the *Rsk2*-KO mice and to mental retardation in CLS, we examined recently global gene expression profiles in hippocampus from KO mice, It revealed altered expression of 100 genes encoding proteins acting in various biological pathways [8]. We investigated in detail the consequences of the two-fold up-regulation of one of these genes, *Gria2*, which encodes the subunit GluR2 of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. Abnormally increased expression of GluR2 at the protein level was confirmed in the hippocampus of *Rsk2*-KO mice and at synapses from *Rsk2*-KO hippocampus primary neuron cultures. Basal AMPA receptor-mediated activity was found significantly decreased [8]. RSK2 deficient mice have also an increased baseline level of phospho-ERK1/2 as well as enhanced activation of ERK1/2 after glutamate stimulation of hippocampal neurons [9, 10]. These latter findings demonstrated that RSK2 exerts a feedback inhibitory effect on the ERK1/2 pathway in physiological conditions.

To define the molecular events leading to GluR2 over expression in RSK2 deficient neurons we used in the present study the RNA interference technology to knockdown the *Rsk2* gene in PC12 cells. We show that *Rs*k2 silencing in PC12 cells leads also to an increase of ERK1/2 activity and of GluR2 expression at the mRNA and protein levels. Furthermore, our data reveal that the increased GluR2 expression results from the increased ERK1/2 activity on the transcription factor Specificity Protein 1 (Sp1).

Results

Rsk2 knockdown of PC12 cells

For this study we used the PC12 cell line, an immortalized cell line derived from a pheochromocytoma tumor of the rat adrenal gland. NGF-treated PC12 cells cease proliferation, grow long neurites, and show changes in cellular composition associated with neuronal differentiation [11], and can therefore serve as a model system for primary neuronal cells. PC12 cells express a high level of RSK2 as shown in Fig. 1. We tested the efficacy to block *Rsk2* expression in PC12 cells of three plasmids producing different *Rsk2*-specific small hairpin RNAs (*Rsk2*-shRNA) targeted to different coding regions of the mRNA of *Rsk2*. The recombinant plasmids were transfected into the PC12 cells by lipofecamine 2000, recombinant cells selected 72 hours on G418, and mRNA and protein expression levels of the *Rsk2* gene were assayed by QRT-PCR and Western blot. One shRNAs (shRNA clone ID7), decreased *Rsk2* gene expression dramatically (Fig. 1a). Quantification of the immunoblots revealed a 90% reduction in the expression of the RSK2 protein, compared to untransfected PC12 cells. No significant change was found in the level of RSK2 mRNA and protein expression in cells transfected with a shRNA containing a scrambled sequence (negative control) (Fig. 1a). (Expression of the RSK2 protein in *Rsk2*-shRNA transfected cells: 0.07 ± 0.03; untransfected cells: 1.0 ± 0.09; scrambled-shRNA transfected cells: 0.917 ± 0.1; *$p<0.05$ by Student's t-test) (Fig. 1a). QRT-PCR revealed also a dramatic decrease of *Rsk2* mRNA expression (Expression of *Rsk2* mRNA in *Rsk2*-shRNA transfected cells: 0.316 ± 0.05; untransfected cells: 1.0 ± 0.16; scrambled-shRNA transfected cells; 0.865± 0.15; *$p<0.05$ by Student's t-test) (Fig. 1c). Thus, the shRNA clone ID7 was used in all subsequent studies. We then asked whether the severe down regulation of RSK2 would also affect the

expression of other RSK proteins in PC12 cells. The result for RSK1 is shown in Fig. 1b. (Expression of RSK1 protein in *Rsk2*-shRNA transfected cells: 0.895 ± 0.1; untransfected cells: 1.0 ± 0.28; scrambled-shRNA transfected cells: 0.894 ± 0.09; *$p<0.05$ by Student's t-test). RSK3 was very weakly expressed in untransfected PC12 cells and was unchanged in transfected PC12 cells (result not shown). Thus, none of these two latter RSK family members apparently exhibited any significant change in its expression levels, documenting that the *Rsk2*-shRNA approach was highly specific for RSK2 and reducing the possibility of compensation for the RSK2 loss via over expression of other RSK family members.

Immunocytofluorescence analysis of RSK2 expression

We also examined the expression of RSK2 in PC12 cells by immunocytofluorescence analysis. Most of untransfected, or scrambled-shRNA transfected PC12 cells showed strong RSK2 staining, whereas *Rsk2*-shRNA transfected cells expressed RSK2 very weakly (Fig. 2). It was concluded that the *Rsk2*-shRNA recombinant plasmid can effectively suppress the expression of the *Rsk2* gene in PC12 cells.

Rsk2 silencing increased ERK1/2 MAPK phosphorylation

We then asked whether *Rsk2* knockdown affects the level of ERK1/2 phosphorylation in PC12 cells. Detection of total ERK levels revealed no significant difference of total ERK protein expression between untransfected PC12 cells and cells transfected with the *Rsk2*- or scrambled-shRNA (Expression of ERK1 protein in *Rsk2*-shRNA transfected cells: 1.0 ± 0.11; untransfected cells: 0.875 ± 0.04; scrambled-shRNA transfected cells: 0.748 ± 0.08, Expression of ERK2 protein in *Rsk2*-shRNA transfected cells: 0.830 ± 0.07; untransfected cells: 0.950 ± 0.05; scrambled-shRNA transfected cells: 1.0 ± 0.09; *$p<0.05$ by Student's t-test) (Fig. 3a and b). In contrast, the analysis of phosphorylated ERK1 and ERK2 showed

that the active forms of both ERK1 (P-ERK1) and ERK2 (P-ERK2) were significantly higher in *Rsk2*-shRNA transfected PC12 cells than in scrambled-shRNA transfected or untransfected cells (Expression of P-ERK1 in *Rsk2*-shRNA transfected cells: 1.0 ± 0.07; untransfected cells: 0.03 ± 0.003; scrambled-shRNA transfected cells: 0.42 ± 0.06, Expression of P-ERK2 in *Rsk2*-shRNA transfected cells: 1.0 ± 0.09; untransfected cells: 0.04 ± 0.003; scrambled-shRNA transfected cells: 0.32 ± 0.05; *$p<0.05$ by Student's t-test) (Fig. 3a and c) . These results were consistent with our previous data in the hippocampus of *Rsk2*-KO mice, which also showed increased levels of ERK1/2 phosphorylation [9]. An increase of P-ERK1/2 levels was also observed in scrambled-shRNA transfected PC12 cells, but much lower than in *Rsk2*-shRNA transfected cells. The cause is not yet known but it might be due to the G418 selection.

Rsk2 knockdown results in up regulation of Gria2 gene (encoding GluR2) expression

We next examined the expression of GluR2 in *Rsk2* knockdown PC12 cells by Western blot analysis and QRT-PCR. Detection of GluR2 protein levels, revealed a very strong increase of expression in *Rsk2* knockdown PC12 cells (Expression of GluR2 protein in *Rsk2*-shRNA transfected cells: 1.0 ± 0.02; untransfected cells: 0.03 ± 0.008; scrambled-shRNA transfected cells: 0.382 ± 0.04; *$p<0.05$ by Student's t-test) (Fig. 4a). QRT-PCR revealed also an approximately four-fold higher *Gria2* mRNA expression in *Rsk2*-silenced PC12 cells compared to untransfected cells (*Expression of Gria2* mRNA in *Rsk2*-shRNA transfected cells: 1.0 ± 0.04; untransfected cells: 0.225 ± 0.007; scrambled-shRNA transfected cells: 0.476 ± 0.06; *$p<0.05$ by Student's t-test) (Fig. 4b). Cells transfected with the scrambled-shRNA showed a small increase of both GluR2 protein and mRNA but much lower than *Rsk2*-shRNA transfected cells, and which may be related to the small increase of ERK1/2 phosphorylation observed in these cells (Fig. 4a and b). We assayed also GluR1 and GluR3

levels of expression. Detection of GluR1 protein levels revealed a small decrease of expression in *Rsk2*-shRNA transfected PC12 cells compared to untransfected PC12 cells or cells transfected with the scrambled-shRNA (Expression of GluR1 protein in *Rsk2*-shRNA transfected cells: 0.803 ± 0.11; untransfected cells: 1.0 ± 0.14; scrambled-shRNA transfected cells: 0.951 ± 0.09; *$p<0.05$ by Student's t-test) (Fig. 4c). GluR3 was hardly detectable in untransfected PC12 cells as well as in *Rsk2*- and scrambled-shRNA transfected cells (not shown), suggesting that expression of GluR3 was not altered in RSK2 deficient cells. These results showed that only the expression of GluR2 is up regulated in response to RSK2 deficiency and suggested also that the regulatory mechanisms of expression are different for the various GluR genes.

Inhibition of GluR2 over expression in Rsk2 knockdown PC12 cells by U0126

We then evaluated if the increased level of active ERK may be the cause of the strong increase of GluR2 expression in *Rsk2* knockdown PC12 cells. To this end, *Rsk2*- and scrambled-shRNA transfected PC12 cells as well as untransfected cells were treated for 24 h, before being harvested, with U0126 (20 uM), a specific MEK inhibitor (MEK being the ERK1/2 upstream kinase in the ERK/MAPK pathway). U0126 dramatically inhibited the GluR2 up-regulation of expression induced by *Rsk2* silencing, as detected by Western blot analysis (Expression of GluR2, protein without U0126 treatment, in *Rsk2*-shRNA transfected cells: 1.0 ± 0.043; untransfected cells: 0.05 ± 0.03; scrambled-shRNA transfected cells: 0.35 ± 0.08; Expression of GluR2 protein after U0126 treatment in *Rsk2*-shRNA transfected cells: 0.08 ± 0.004 and scrambled-shRNA transfected cells: 0.043 ± 0.005; *$p<0.05$ by Student's t-test) (Fig. 5). These results suggested that ERK1/2 activity plays a crucial role in the up regulation of GluR2 expression induced by RSK2 depletion.

Involvement of Sp1 in the up regulation of GluR2 expression in Rsk2 knockdown PC12 cells

It was previously shown that GluR2 expression is strongly influenced at the transcriptional level by a positive Sp1 regulatory element in the 5' proximal region of the *Gria2* gene promoter [12]. The transcription factor Sp1 recruits basal transcription factor TFIID to DNA and induces transcription. It was also reported that Sp1 activity increases when phosphorylated by ERK at two specific threonine residues (Thr453 and Thr739) [13]. Thus, we wondered whether the expression and/or the phosphorylation of Sp1 are also up regulated in *Rsk2* knockdown PC12 cells. Total protein was extracted for analysis of Sp1 and Phospho-Sp1 (P-Sp1) expression by Western blotting. Total Sp1 levels, revealed no difference of Sp1 mRNA and protein expression between untransfected and *Rsk2*-shRNA transfected PC12 cells (mRNA expression of Sp1 in *Rsk2*-shRNA transfected cells: 1.0 ± 0.009; untransfected cells: 1.0 ± 0.006; scrambled-shRNA transfected cells: 0.921 ± 0.01; expression of Sp1 protein in *Rsk2*-shRNA transfected cells: 1.0 ± 0.12; untransfected cells: 0.89 ± 0.1; scrambled-shRNA transfected cells: 0.961 ± 0.06 *$p<0.05$ by Student's t-test) (Fig. 6a, b and c). However, immunoblot analysis of phosphorylated forms of Sp1 showed that the levels of phosphorylation at both phosphorylation sites, T453 and T739, were significantly higher in *Rsk2-shRNA* transfected cells than in untransfected cells (Expression of P-Sp1 (T453) in *Rsk2*-shRNA transfected cells: 1.0 ± 0.06; untransfected cells: 0.07 ± 0.01; scrambled-shRNA transfected cells: 0.376 ± 0.1; Expression of P-Sp1 (T739) in *Rsk2*-shRNA transfected cells: 1.0 ± 0.1; untransfected cells: 0.29 ± 0.03; scrambled-shRNA transfected cells: 0.26 ± 0.03; *$p<0.05$ by Student's t-test (Fig. 6 d and e).

To further determine whether treatment with the MEK inhibitor U0126 impacts Sp1 phosphorylation, *Rsk2*-shRNA transfected and untransfected PC12 cells were treated with this inhibitor (20uM) for 24 h before being harvested. Total proteins were extracted for Western

blot analysis and Sp1 protein levels, revealed no difference between *Rsk2*- transfected and scrambled shRNA transfected cells (expression of Sp1 in *Rsk2*-shRNA transfected cells: 1.0 ± 0.05; scrambled shRNA transfected cells: 0.91 ± 0.04). (Fig. 6a and b) Inhibition of ERK1/2 activity resulted in a dramatic decrease of Sp1 phosphorylation at both T739 and T453 sites (Expression of P-Sp1 (T453) after U0126 treatment in *Rsk2*-shRNA transfected cells: 0.13 ± 0.02; scrambled-shRNA transfected cells: 0.001 ± 0.005). Expression of P-Sp1 (T739) after U0126 treatment was undetectable (Fig. 6a, d and e). These results demonstrated that ERK is the major kinase that phosphorylates Sp1 at both of these sites.

To further confirm the crucial role of Sp1 in the up-regulation of *Gria2* expression, we down-regulated partially the expression of Sp1 by RNAi. Forty-eight hours after transfection, cells were harvested, total protein extracted and Western blotting performed. Sp1-siRNA reduced by about two-thirds the levels of Sp1 protein expression in *Rsk2*-shRNA transfected PC12 cells (Expression of Sp1 protein after Sp1-siRNA treatment in *Rsk2*-shRNA transfected cells: 0.37 ± 0.01; scrambled-shRNA transfected cells: 0.36 ± 0.08) (Fig. 6a and b). Expression of P-Sp1 was also drastically decreased (Expression of P-Sp1 (T453) after Sp1-siRNA treatment in *Rsk2*-shRNA transfected cells: 0.15 ± 0.01; scrambled-shRNA transfected cells: 0.30 ± 0.04 (Fig. 6a and d) and expression of P-Sp1 (T739) after Sp1-siRNA treatment in *Rsk2*-shRNA transfected cells: 0.53 ± 0.05; scrambled-shRNA transfected cells: 0.18 ± 0.06 (Fig. 6a and e).

The level of GluR2 expression was also much lower in Sp1-siRNA transfected *Rsk2* knockdown PC12 cells as in *Rsk2* knowkdown cells not transfected with Sp1-siRNA, and similar to untransfected PC12 cells (Expression of GluR2 protein before Sp1-siRNA transfection in *Rsk2*-shRNA transfected: 1.0 ± 0.03; untransfected cells: 0.084 ± 0.04; scrambled-shRNA transfected cells: 0.341 ± 0.04; and after Sp1-siRNA transfection in *Rsk2*-shRNA transfected cells: 0.08 ± 0.008; scrambled-shRNA transfected cells: 0.126 ± 0.04;

*p<0.05 by Student's t-test). (Fig. 7 a). QRT-PCR revealed also a similar decrease of *Gria2* mRNA expression following Sp1-siRNA application (Expression of *Gria2* mRNA before Sp1-siRNA transfection in *Rsk2*-shRNA transfected cells: 1.0 ± 0.04; untransfected cells: 0.225 ± 0.07; scrambled-shRNA transfected cells: 0.475 ± 0.06; and after Sp1-siRNA transfection, *Rsk2*-shRNA transfected cells: 0.05 ± 0.008 ; scrambled-shRNA transfected cells: 0.03 ± 0.001; *p<0.05 by Student's t-test) (Fig. 7b). These results further demonstrate that Sp1 is involved in the increased GluR2 expression in RSK2 deficient PC12 cells.

Together, with previously reported data on regulation of the *Gria2* gene and Sp1 activity, our results show that an abnormally increased level of Sp1 phosphorylation in response to ERK1/2 over activation is responsible for up regulated transcription of the *Gria2* gene in RSK2 deficient cells.

Significant increase of Sp1 phosphorylation in the hippocampus of Rsk2-KO mice

Western blot analysis of total protein extracts from hippocampi of *Rsk2*-KO adult mice revealed also significantly increased levels of P-Sp1 (T453) as compared to WT littermate. No significant difference was observed for Sp1 protein levels (Sp1: WT: 1.0 ± 0.18; KO: 0.95 ± 0.08; and P-Sp1 (T453): WT: 0.34 ± 0.04; KO: 1.0 ± 0.07. *p<0.05 by Student's t-test.) (Fig. 8).

Discussion

The (AMPA)-type glutamate receptor channels are expressed ubiquitously in brain neurons and mediate fast excitatory neurotransmission in most excitatory synapses. AMPA receptors are assembled from combinations of four subunits, GluR1, GluR2, GluR3 and/or GluR4. AMPA receptors play also a crucial role in synaptogenesis and formation of neuronal

circuitry, as well as in synaptic plasticity [14]. There is a huge amount of evidence indicating that the GluR2 subunit plays a pivotal role in AMPA receptors function. The Ca^{++} permeability, rectification, and single-channel conductance of AMPA receptors are all dominantly influenced by inclusion of an edited GluR2 subunit in the receptor complex [15-21]. Receptors that contain a single edited GluR2 subunit have maximally reduced Ca^{++} permeability; whereas inward rectification is reduced in a graded manner as the number of GluR2 subunits in a receptor increases [21]. Thus, moderate changes in GluR2 expression may have functional consequences on receptor subunit rearrangement and lead to functional differences in the functioning of synaptic circuits. GluR2 expression is governed by neuronal activity [22], and its synaptic distribution and levels are modulated as part of the mechanism of synaptic plasticity [23,24]. GluR2 interacts also with various molecules implicated in receptor trafficking [25-27]. The crucial role played by GluR2 in neuronal function may therefore explain why it is the most tightly regulated of the AMPA receptor subunits [27].

The two-fold increased level of GluR2 expression in *Rsk2*-KO mice prompted us to elucidate the molecular mechanism leading to this alteration of expression by silencing the *Rsk2* gene in PC12 cells. The PC12 cell line, cloned from rat pheochromocytoma cells, can be induced to differentiate and to acquire a neuronal-like phenotype [11]. PC12 cells express endogenous RSK2 at a relatively high level, whereas the level of GluR2 is low (present study, Fig. 1a and Fig. 4a and b). Therefore, the PC12 cell line was a good model for the present study. As anticipated from our previous data in *Rsk2*-KO neurons [9], silencing of the *Rsk2* gene led to an up-regulation of ERK1/2 activity and to an increase of GluR2 expression, both being, however, stronger than those observed in *Rsk2*-KO neurons [8 ,9]. Increase of GluR2 expression was observed at both the protein and mRNA levels, indicating that it was predominantly the transcription of the *Gria2* gene that was affected by the absence of RSK2 activity. Since it was previously reported that the ERK pathway plays a role in the regulation

of GluR2 expression [28], we hypothesized that the increased GluR2 expression level in the *Rsk2*-KO mice and in *Rsk2* knockdown PC12 cells might be related to the elevated ERK1/2 activity. As expected, inhibition of ERK1/2 activity in the *Rsk2* knockdown PC12 cells with the U0126 inhibitor reversed the effect of RSK2 depletion on GluR2 expression, implicating clearly ERK1/2 in the elevated GluR2 expression.

Previous investigations demonstrated a positive regulatory role for Sp1 in the transcriptional regulation of *Gria2* gene via cis-acting elements in its proximal promoter regions [12]. Multiple signaling pathways converge on Sp1, including ERK, Akt, and c-Jun N-terminal protein kinase [29]. ERK-regulated phosphorylation sites on Sp1 were identified at T453 and T739 in response to growth factor regulation of the vascular endothelial growth factor gene [30]. Phosphorylation of Sp1 on T453 and T739 increases Sp1 DNA binding activity [30]. Our data show that levels of phosphorylation at both Sp1 sites is strongly increased in *Rsk2* knockdown PC12 cells, whereas treatment with the MEK inhibitor U0126 inhibits phosphorylation at both of these sites, and also abolishes the increased level of GluR2 expression in RSK2 deficient cells. Thus, our results confirm the implication of ERK1/2 in Sp1 phosphorylation at both T453 and T739 Sp1 sites, and indicate that the increased levels of Sp1 phosphorylation and GluR2 expression in RSK2 deficient cells are related to the elevated ERK1/2 activity. Finally, the evidence that a significant reduction of Sp1 expression, with a Sp1-specific siRNA, prevents the increased level of GluR2 expression in RSK2 deficient cells further supports the critical role of Sp1 in the up regulation of GluR2 expression. How phosphorylated Sp1 activates the *Gria2* promoter remains now to be investigated. It is possible that Sp1 phosphorylation may change its interaction with other transcription factors. Sp1 phosphorylation induced by the ERK pathway was also shown to cause the release of a histone deacetylase corepressor complex from a gene promoter [31].

Previously it was shown that changes in the expression levels of RSK2 can modify both the expression levels and the subcellular distribution of GluR2 subunits [8]. Our present data, showing that RSK2 can modulate indirectly GluR2 expression by increasing or decreasing ERK1/2 activity, provide now a potential mechanism for these modifications. RSK2 was also shown previously to directly interact with GluR2 [32]. Together the data provide increasing evidence that RSK2 plays an important role in the regulation of the function of AMPA receptors.

Gria1 (encoding GluR1) and *Gria2* promoters share common features, such as multiple transcription start sites, GC rich, lack of a TATA box, and presence of Sp1 binding site in vitro [33, 12]. However, RSK2 deficiency causes only an enhancement of GluR2 but not GluR1 or GluR3 protein expression levels both in hippocampal neurons [8] and PC12 cells (present study), which further underscores the specific role of the GluR2 AMPA receptor subunit. In the present study, the expression of GluR1 is even slightly down regulated in RSK2 depleted PC12 cells. Our data provide evidence that the regulation of *Gria1* and *Gria3* genes expression might be under the control of distinct signals that remain to be identified.

The vast majority of male patients affected with Coffin-Lowry syndrome have severe-to-profound intellectual disability, and currently there is no cure for this disorder. Our finding of an abnormally increased level of GluR2 expression in RSK2 deficient neurons could lead to therapeutic strategies for CLS. Indeed, in the Fragile X syndrome (FXS), observations suggesting increased metabotropic glutamate receptor (mGluR5) expression led to preclinical studies showing that the inhibition of mGluR5 can ameliorate multiple mutant phenotypes in mouse and drosophila models of FXS, and clinical trials based on this therapeutic strategy are underway [34]. Modulation of GluR2 levels may be a useful pharmaceutical approach for improving cognitive function. However, it is first necessary to validate the mechanism described in the present report in *Rsk2*-KO mice. We have already shown in the present report

that the basal level of phospho-Sp1 is significantly higher in the hippocampus of RSK2 deficient mice than of WT mice (Fig. 8). It is also essential to further dissect the molecular mechanism of *Gria2* transcriptional control, including the identification by which Sp1 activates the *Gria2* promoter. In addition to Sp1, *Gria2* expression is also influenced at the transcriptional level by another positive (Nrf-1) and a negative (Re1/Nrse-like silencer) regulatory element in the 5' proximal region of the promoter [12]. Myers *et al*. [33] showed that the *Gria2* Re1/Nrse-like silencer binds the RE1-silencing transcription factor (NRSF/REST), and that co-transfection of REST into neurons reduced *Gria2* promoter activity in a silencer-dependent manner. The nuclear respiratory factor 1 (NRF-1) is a transcription factor that was also shown to regulate *Gria2* gene transcription in neuroblastoma cells [35]. It is clearly necessary to understand the roles of the different regulatory elements and their interplay in neurons. In addition, although there is evidence that rapid (AMPAR) excitatory synaptic transmission is affected in *Rsk2* mutant mice [8], the extent of glutamate transmission alteration, and whether synaptic plasticity is modified, remain to be explored. These studies will provide more insights into the feasibility of targeting *Gria2* transcription in Coffin-Lowry syndrome treatment.

Conclusion

Our results provide evidence that loss of RSK2 activity results in over activation of the transcription factor Sp1, via an abnormally increased level of ERK1/2 activity, which in turn leads to over expression of GluR2. Thus, these findings suggest an indirect role of RSK2 in modulating the expression of AMPA receptor GluR2 subunit and, therefore, in regulating glutamatergic neurotransmission. It can also be anticipated that the increased level of ERK1/2 activity is responsible for several additional functional deregulations in RSK2 deficient cells. Further studies, too explore these hypotheses, are ongoing in our laboratory.

Methods

Animals and tissue dissection

All experimental procedures for care and use of mice were performed according to agreements with the Departmental Direction of Veterinarian Services (Prefecture du Bas-Rhin, France No. 67-225) and IGBMC Animal Welfare Insurance (NIH, PHS No. A5100-01). WT and KO animals used in this study were males from the same littermates and housed 4 per cage in a 12h dark/light cycle (light on from 7 am to 7 pm), under controlled conditions of temperature and humidity. Euthanasia of the mice was carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC), and the study was specifically approved by the Ethics Committee in Animal Experiments (permit number 17) of the Institut de Génétique et de Biologie Moléculaire et Cellulaire.

Male *Rsk2*-KO and WT mice with a C57Bl/6x genetic background were killed by cervical dislocation. Brains were rapidly dissected and the hippocampus was isolated using a standard dissection procedure. Tissue samples were immediately frozen in liquid nitrogen and kept at -80 °C until use, whole protein extractions were performed as previously described [10].

Cell Culture

PC12 cells were grown on Dulbecco's Modified Eagle Medium (DMEM) medium (glucose 4.5g/L) (Gibco by Invitrogen, Carlsbad, Calif) supplemented with 10% horse serum and 5% fetal bovine serum. To induce terminal differentiation, we added 50 ng/ml Nerve Growth Factor-β (NGF), (Chemicon Millipore). NGF was supplied for a minimum of 4-5 days before transfection.

Transfection of PC12 cells with shRNA vectors

We first tested two mouse shRNAs (from SureSilencing) matching completely with the rat *Rsk2* gene, which reduced only modestly *Rsk2* expression in PC12 cells. We then tested a shRNA directed against the human *Rsk2* gene (according to the nomenclature: *RPS6KA3* gene) (SureSilencing TM shRNA Plasmids for Human *RPS6KA3*, Clone ID7) and matching completely with the rat *Rsk2* gene, which decreased Rsk2 expression dramatically. Thus, the shRNA clone ID7 was used in all subsequent studies (and named *Rsk2*-shRNA). Approximately 10^3 to 10^4 undifferentiated cells were plated in each of six well plates. After differentiation the cells were used for transfection. Briefly, 5 µg of *Rsk2*- or scrambled-shRNA, diluted in Opti-MEM® I, were added to each well and transfection was performed with Lipofectamine 2000 according to the manufacturer's instructions. Cells were incubated for 6 hours at 37°C in a CO_2 incubator, then the medium was changed for fresh medium and the plates stored again in the incubator. After 24 hours of transfection, G418 (0.6mg/mL) (Gibco by Invitrogen) was added to the medium and the plates again stored in the incubator.

siRNA Transfection

Untransfected and shRNA transfected PC12 cells were used 24 hours after transfection for a second transfection to silence the Sp1 gene expression. The complex of siRNA (Sp1-siRNA: sc-61895, Santa Cruz Biotechnology) and Lipofectamine® RNAiMAX in Opti-MEM® I (both from Invitrogen, Carlsbad, CA, USA) was prepared according to the manufacturer's instructions. After this second transfection cells were incubated for 48 hour at 37° C in a CO_2 incubator, and then proteins were extracted using the RIPA buffer (Santa Cruz Biotechnology) (150-200µl/well).

Cell cultures treatment with the MEK inhibitor

U0126 (20mM) (9903; Cell Signaling Technology, Beverly, MA, USA). was added to the culture medium of shRNA transfected PC12 cells four days after transfection and stored at 37°C in the incubator. Twenty-four hours later proteins were extracted as above.

Whole hippocampal protein extractions

Five male *Rsk2*-KO and 5 WT littermates with a C57Bl/6 genetic background were killed by cervical dislocation. Brains were rapidly dissected and the hippocampus was isolated using a standard dissection procedure. Whole protein extractions were performed as previously described [10].

Western blot analysis

Western blot analyses were performed as previously described [10]. Thirty µg of protein extracts for each sample were loaded on the SDS-PAGE gel. After scanning of the autoradiography films, quantifications were carried out with the GeneTool software of the Chemigenius apparatus (Syngene), and results were normalized to the level of the housekeeping protein GAPDH.

The following antibodies were used: anti-ERK (9102), anti-P-ERK (9106), anti-RSK1 (9333) (all three from Cell Signaling Technology, Beverly, MA, USA), anti-RSK2 (sc-1430) and anti-RSK3 (sc-1431) (both from Santa Cruz Biotechnology), GluR2 (AB 1768-25UG), Sp1 (07-645), p-Sp1 (T453, ab37707), p-Sp1(T739, BS4755) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (MAB374) (all five from Millipore Corporation, Bedford, MA, USA).

Real-time QRT-PCR analysis

Total RNA from PC12 cultures was extracted using Tri-Reagent (TR-118, Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed on 1µg RNA using the Transcriptor kit (03 531 317 001; Roche Molecular Biochemicals, Indianapolis, IN, USA)) to generate cDNA with oligo dT following the manufacturer's protocol. Real time QRT-PCR was performed with LightCycler480 Sybr Green I Master (04 887 352 001, Roche) and achieved using a Light Cycler instrument (Roche) [10]. The sequence of PCR primers used for detection of *Rsk2* (Forward 5' ACAAGGGGTGGTTCACAGAG, Reverse 5' GCATCATAACCTTGCCGT TT), Gria2 (Forward 5' TTTCCTTGGGTGCCTTTATG, Reverse 5' GACAGATCCTCAG CACTTTCG) and Sp1 (Forward 5' CAGACTCAGTATGTGGACCAA, Reverse 5' GTTGAATAGCTGTTGGCAT). All results were normalized using quantification of the housekeeping gene *Gapdh* (Forward 5' CCAAAAGGGTCATCATCTCC, Reverse 5' GAGGGGCCATCCACAGTCTT).

Immunohistofluorescence

Untransfected and Transfected PC12 cells grown on slides were washed three times with 1X Phosphate Buffered Saline (PBS), fixed for 10min with 4% PFA and 2.5M glycine and washed again 3 times (1X PBS). Slides were then immersed in PBS-Triton 0.1% for 15min and rinsed again 3 times with PBS. The cells were then incubated with the primary monoclonal antibody (Goat-anti-RSK2, 1:500, Santa Cruz Biotechnology) overnight, followed by incubation with a fluorescence-conjugated secondary antibody for 1 h, and finally dehydrated and mounted with KAISER's glycerol gelatin (Merck).

Statistical analysis

All data are expressed as mean ± SEM and comparisons between groups were made by a Student's t-test. Differences with $p<0.05$ were considered significant.

Abbreviations

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATF4, Activating transcription factor 4; Bad, BCL2-associated agonist of cell death; c-Fos, cellular FBJ murine osteosarcoma viral oncogene homolog; CREB, cAMP response element-binding; ERK, Extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase GluR, Glutamate receptor; GSK3, Glycogen synthase kinase 3; gria, glutamate receptor, ionotropic; IQ, intelligence quotient; KO: knockout; L1CAM, L1 cell adhesion molecule; MAPK, Mitogen activated protein kinase; NRSF/REST, RE1-silencing transcription factor / RE1-silencing transcription factor; Nur77, nuclear receptor subfamily 77; RSK: Ribosomal s6 kinase; shRNA, Small hairpin RNA; siRNA, small interfering RNA; Sp1, Specificity Protein 1.

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Authors' contributions

AH designed and directed the study, and wrote the manuscript. TM prepared the figures. TM, AS and SP conducted the experiments and data analysis. AH, TM, AS and SP read and approved the final manuscript.

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LEGENDS OF FIGURES

Fig. 1 Silencing of the *Rsk2* gene in PC12 cells.

(A). Detection of the RSK2 protein level in Untransfected (UT), transfected with the *Rsk2*-shRNA (T) or the scrambled-shRNA (negative control, NC) PC12 cells. Data normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are represented as the mean values ± SEM (UT: n>8, T: n>8, NC: n>8). RSK2 expression is drastically decreased in *Rsk2*-shRNA transfected cells (*p <0.05 by Student's t-test). (B) RSK1 protein expression in PC12 cells. Western blots results showed no significant difference between UT, T and NC cells (UT: n>3, T: n>3, NC: n>3). (C) Quantification of the expression level of the *Rsk2* mRNA by real time QRT-PCR (T: n>3, UT: n>3, NC: n>3). Data are normalized to the *Gapdh* gene expression. Bar represents the mean ± SEM. *p <0.05 by Student's t-test.

Fig. 2 RSK2 expression by immunocytofluorescence analysis.

RSK2 expression in untransfected (UT), transfected with the *Rsk2*-shRNA (T) or the scrambled-shRNA (negative control, NC) PC12 cells. The anti-RSK2 antibody reveals a strong RSK2 staining in UT and NC cells, whereas the signal is very faint in *Rsk2*-shRNA transfected (T) cells, confirming the dramatically decreased expression of RSK2 in *Rsk2*-shRNA transfected cells (UT: n>3, T: n>3, NC: n>3). DAPI: 4',6'-diamidino-2-phénylindole.

Fig. 3

Strong increase of Phospho-ERK1/2 (P-ERK1/2) activity in RSK2 depleted PC12 cells. (A) Western blot analysis of the ERK1/2 and P-ERK1/2 protein levels in untransfected (UT), transfected with the *Rsk2*-shRNA (T) and scrambled-shRNA (negative control, NC) PC12 cells. The results revealed a much higher level of P-ERK1/2 in RSK2 depleted PC12 cell than in the other conditions. (B) Quantification of ERK1 and 2 normalized to GAPDH revealed no significant difference between the various conditions. (C) Quantification of P-ERK1 and 2 expressions, normalized to ERK1 or 2, respectively. The levels were significantly higher in *Rsk2*-shRNA transfected (T) cells than in the other conditions. Data are represented as the mean ± SEM (UT: n > 6, T: n > 6, NC: n > 6, *p <0.05 by Student's t-test).

Fig. 4 Quantitative analysis of GluR2 protein and *Gria2* gene expression.

Untransfected (UT), transfected with the *Rsk2*-shRNA (T) or the scrambled-shRNA (negative control, NC) PC12 cells. (A) The GluR2 protein expression level is much higher in *Rsk2*-shRNA transfected cells as compared with (UT) and (NC). Data, normalized to GAPDH expression, are represented as the mean ± SEM (UT: n >6, T: n > 6, NC: n > 6, *p <0.05 by Student's t-test. (B) Quantification of the expression level of the *Gria2* mRNA by real time QRT-PCR and normalized to the *Gapdh* gene expression. A significantly higher expression of

the *Gria2* mRNA was observed in *Rsk2*-shRNA (T) transfected cells, than in UT or NC PC12 cells (UT: n>3, T: n>3, NC: n>3). Bar represents the mean ± SEM *p <0.05 by Student's t-test. (C) Quantitative analysis of GluR1 protein expression. After normalization with GAPDH, no significant difference was observed between the three conditions (UT: n>3, T: n>3, NC: n>3).

Fig. 5 Treatment with the MEK inhibitor U0126 inhibits GluR2 increased expression

Untransfected (UT), transfected with *Rsk2*-shRNA (T) or with scrambled-shRNA (negative control, NC) PC12 cells. The level of expression of the GluR2 protein was dramatically decreased in the *Rsk2*-shRNA transfected PC12 cell line and treated with U0126 when compared to the same *Rsk2*-shRNA transfected cells but without U0126 treatment. Data are normalized to GAPDH and are represented as the mean ± SEM (UT: n > 4, T: n > 4, NC: n > 4, *p <0.05 by Student's t-test).

Fig. 6 Expression levels of Sp1 and phospho-Sp1 (P-Sp1)

Untransfected (UT), transfected with the *Rsk2*-shRNA (T) or the scrambled-shRNA (negative control, NC) PC12 cells. (A). Western blot results. (B) Quantification of Sp1 protein expression levels. Data, normalized with GAPDH, are represented as the mean ± SEM (UT: n >3, T: n > 3, NC: n > 3). Sp1 protein expression is strongly decreased in Sp1-siRNA transfected cells, whereas in all other conditions it is very similar. (C). Quantification of Sp1 mRNA expression by QRT-PCR and normalized to the *Gapdh* gene expression (UT: n>3, T: n>3, NC: n>3). Bar represents the mean ± SEM. *p <0.05 by Student's t-test. (D, E). Levels of P-Sp1 (T453 and T739) are much higher in *Rsk2*-shRNA transfected (T) cells as in all other conditions. Treatment with U0126 or Sp1-siRNA reduced drastically Sp1 phosphorylation at both T453 and T739 sites in RSK2 depleted cells (T) (UT: n >3, T: n > 3,

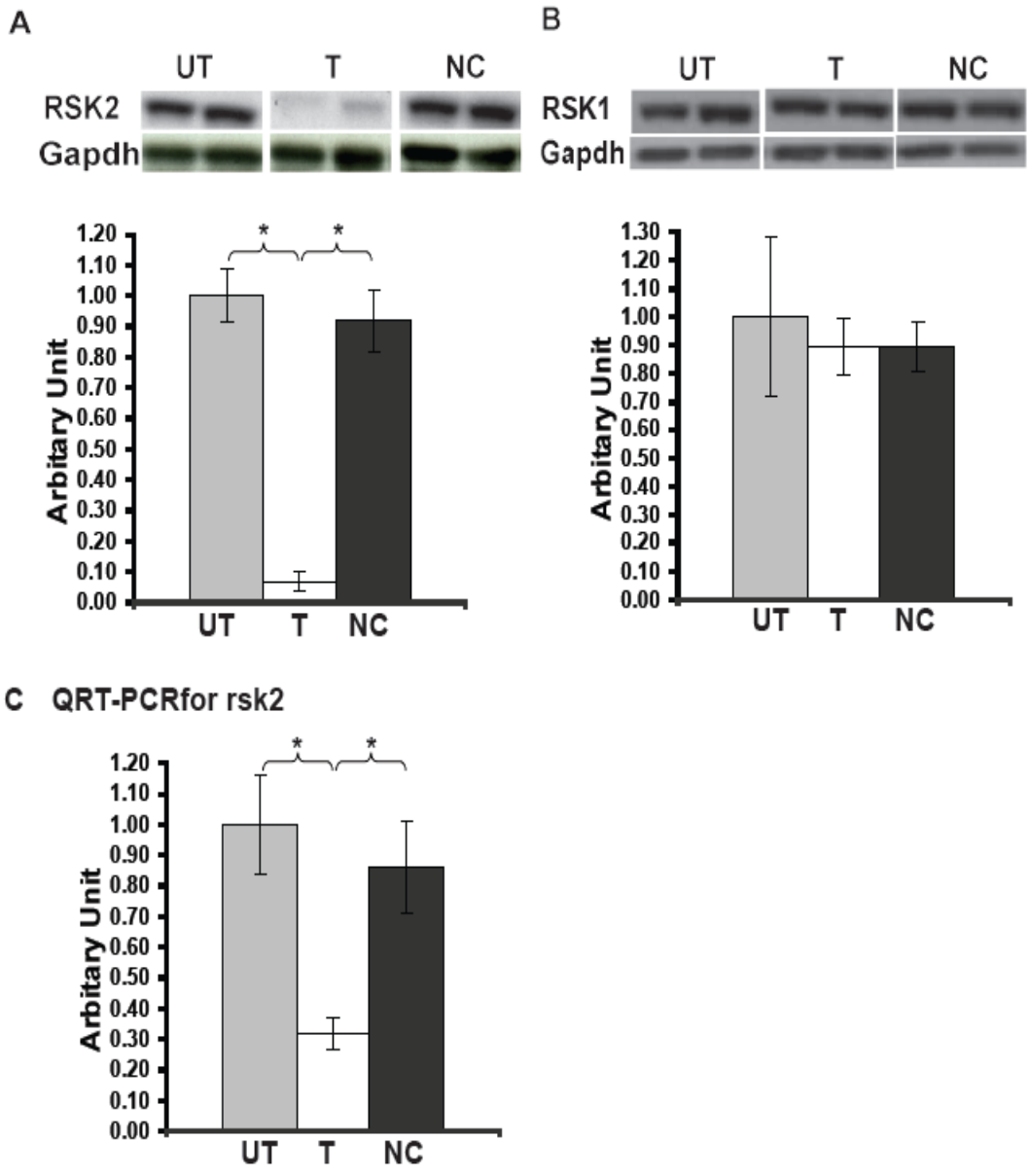
NC: n > 3). Differences with *p<0.05 between UT and T and NC groups were considered significant by Student's t-test.

Fig. 7 GluR2 expression is dramatically decreased in *Rsk2* knockdown cells treated with the Sp1-siRNA.

(a) Western blot results revealed a strong decrease of GluR2 expression in Sp1 silenced *Rsk2* knockdown PC12 cell, as compared to *Rsk2* knockdown cells without Sp1 silencing. (b) Quantification of *Gria2* mRNA expression by QRT-PCR. Data are normalized to *Gapdh* mRNA expression and are represented as the mean ± SEM (UT: n > 3, T: n > 3, NC: n > 3) (*p <0.05 by Student's t-test).

Fig. 8 Sp1 and Phospho-Sp1 (P-Sp1) expression levels in the hippocampus of *Rsk2*-KO and WT littermate male mice.

(A). Western Blot data and quantification of Sp1 expression levels, normalized to GAPDH expression and represented as the mean values ± SEM (WT: n>5; KO: n>5). (B) Western blot results and quantification of P-Sp1 (T453) expression levels. Data are normalized to Sp1 protein expression and are represented as the mean values ± SEM (WT: n>5; KO: n>5). The level of phospho-Sp1 (T453) expression is significantly higher in *Rsk2*-KO mice than in WT littermate. *p <0.05 by Student's t-test.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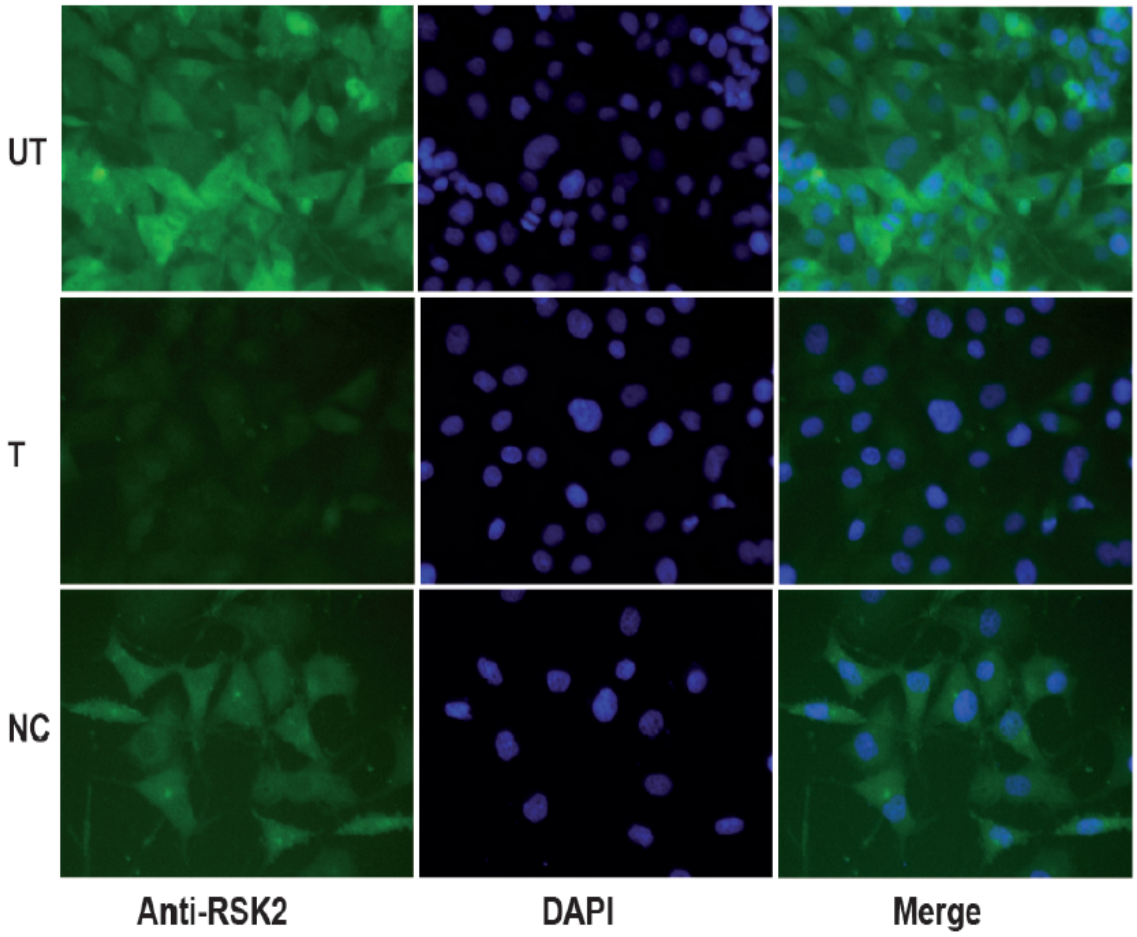
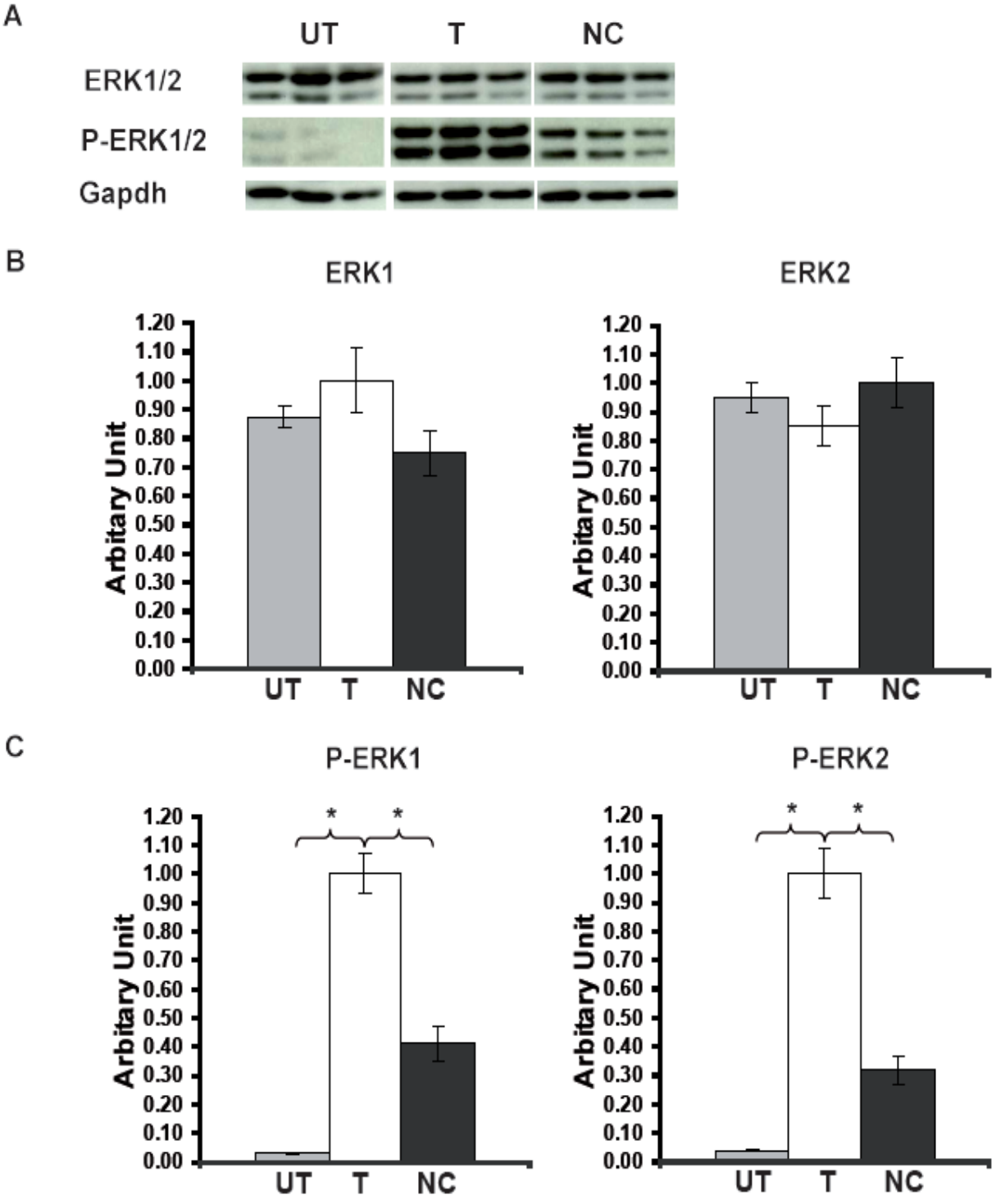


Fig. 2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UT
T
NC
ERK1/2
P-ERK1/2
Gapdh
B
ERK1
ERK2
Arbitary Unit
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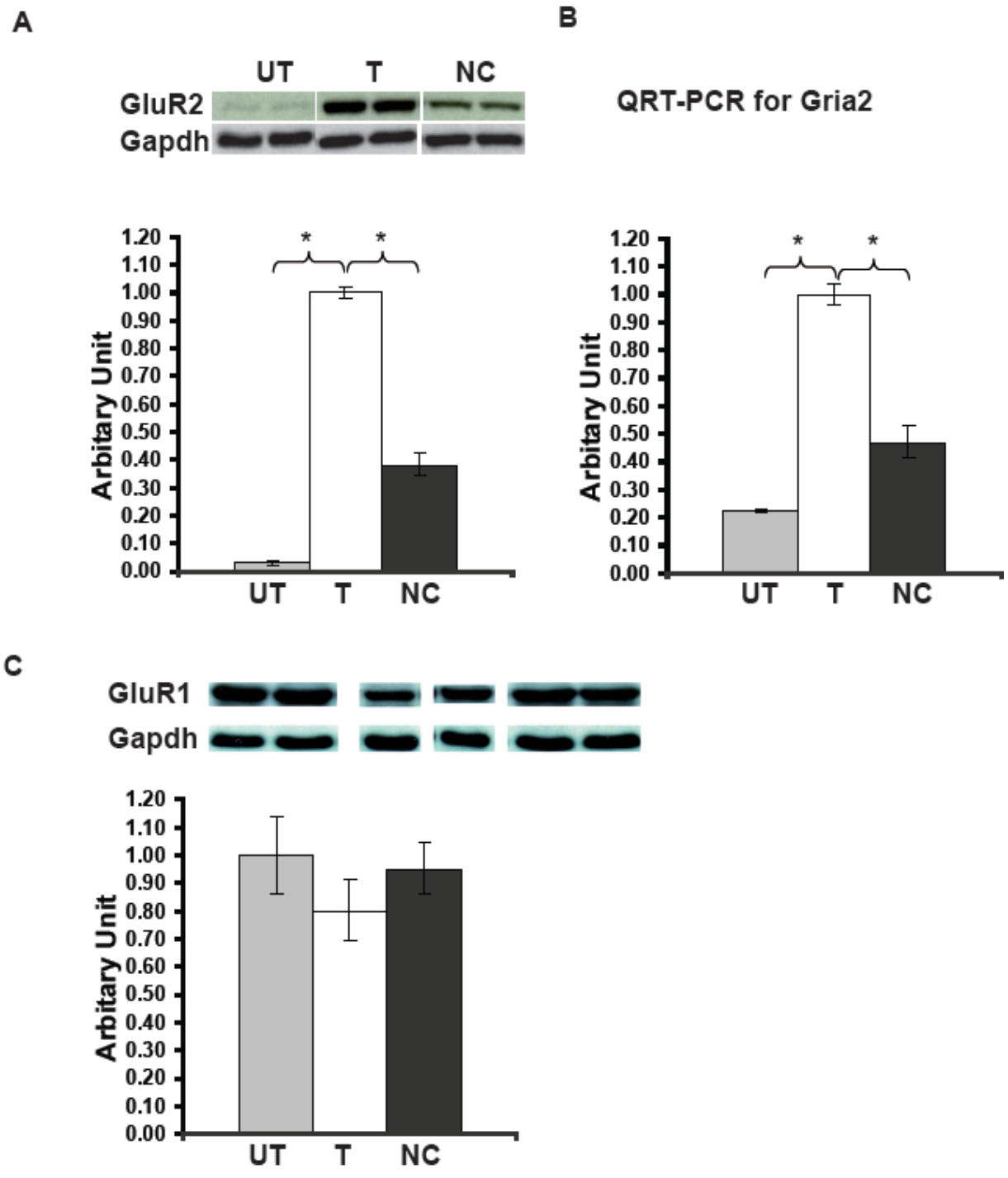
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
UT
T
NC
C
P-ERK1
P-ERK2
*
*

Fig. 3.


A
UT
T
NC
GluR2
Gapdh
B
QRT-PCR for Gria2
Arbitary Unit
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1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
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*
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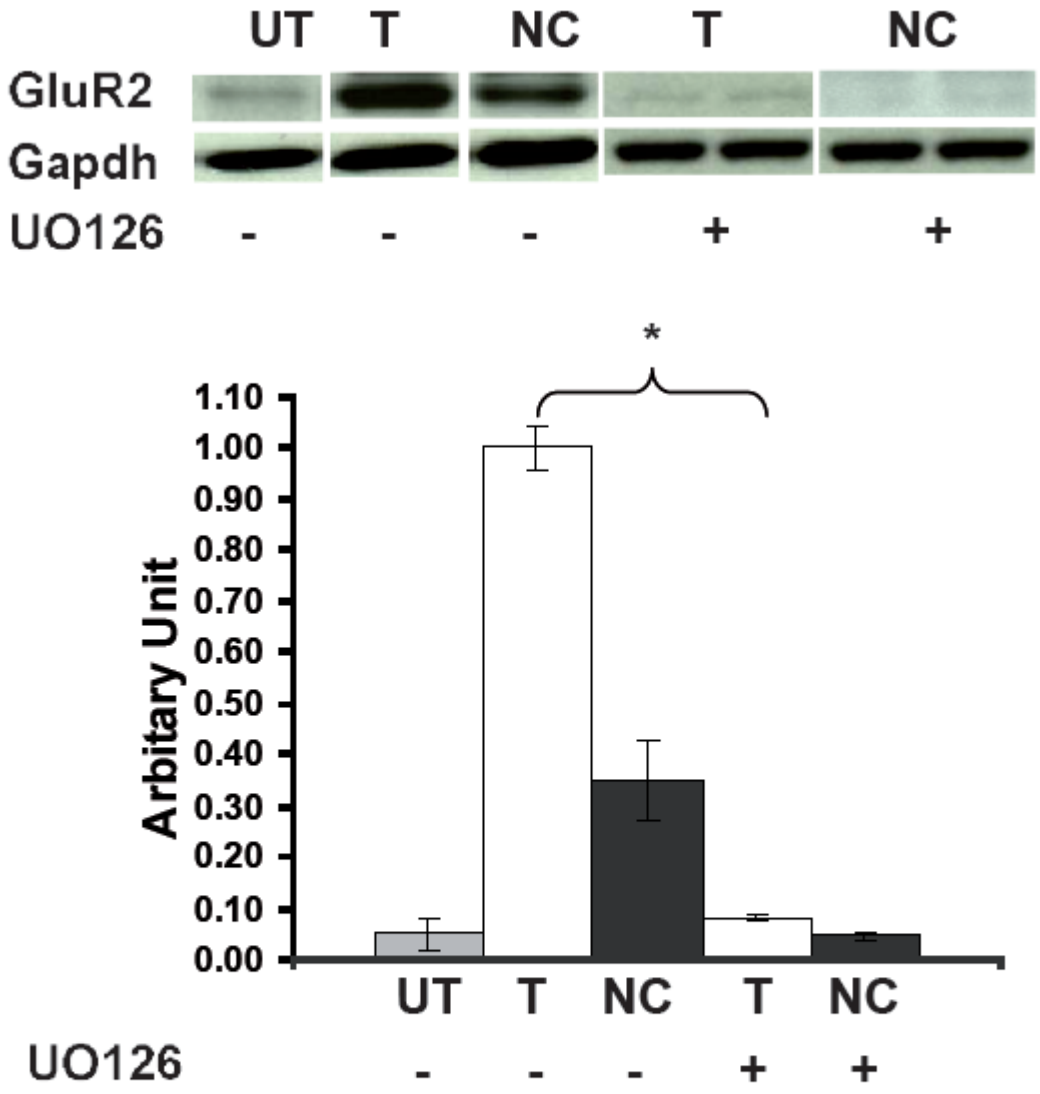
UT
T
NC
C
GluR1
Gapdh
Arbitary Unit
UT
T
NC

Fig. 4.


UT
T
NC
T
NC
GluR2
Gapdh
UO126
- - - + +
*
Arbitary Unit
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
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UT T NC T NC
UO126
- - - + +

Fig. 5.

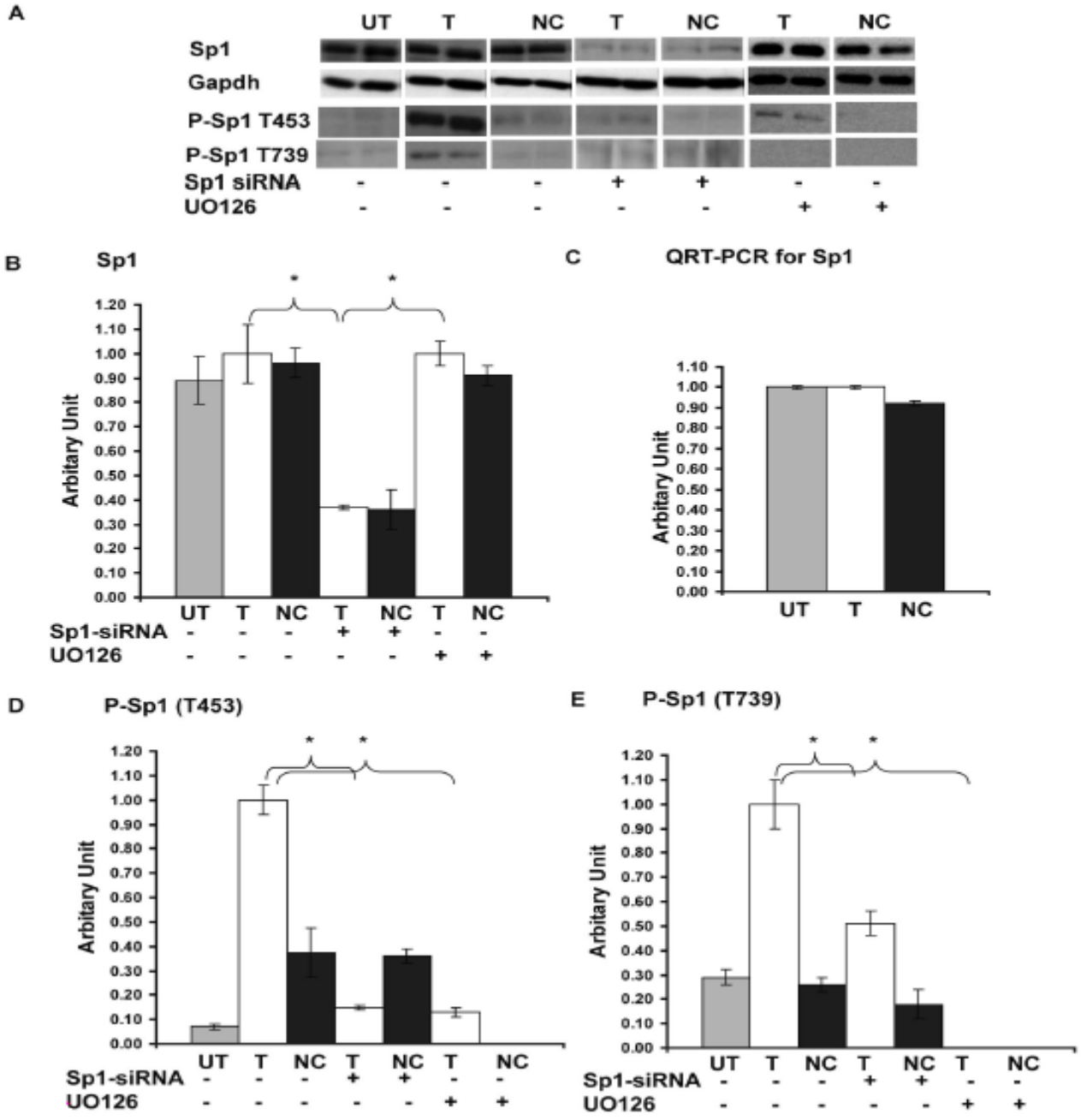
A
UT T NC T NC T NC
Sp1
Gapdh
P-Sp1 T453
P-Sp1 T739
Sp1 siRNA - - - + + - -
UO126 - - - - - + +
B
Sp1
Arbitary Unit
Sp1-siRNA
UO126
C
QRT-PCR for Sp1
D
P-Sp1 (T453)
E
P-Sp1 (T739)

Fig. 6

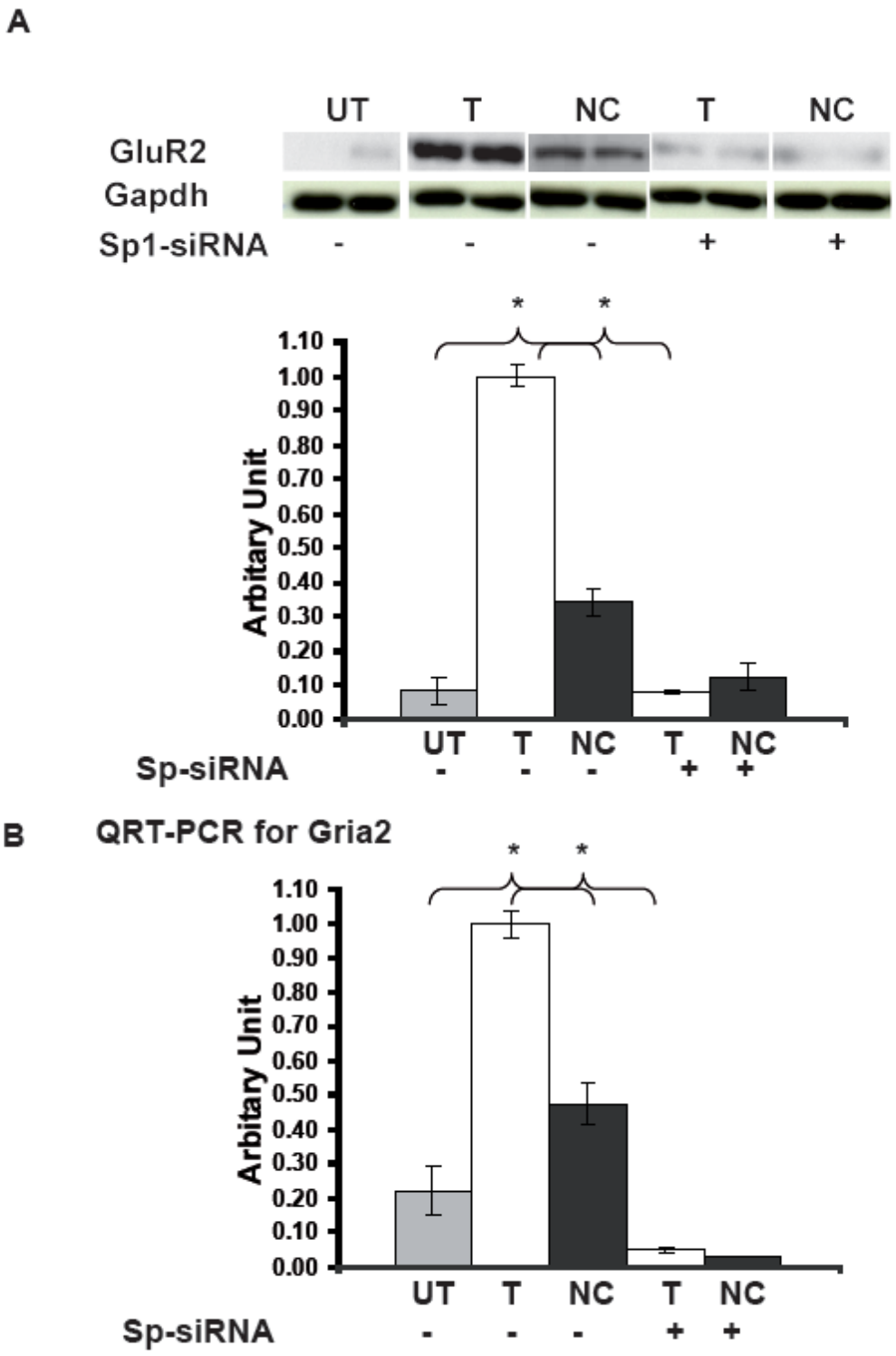
A
UT
T
NC
T
NC
GluR2
Gapdh
Sp1-siRNA
- - - + +
*
*
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Arbitary Unit
UT T NC T NC
Sp-siRNA
- - - + +
B
QRT-PCR for Gria2
*
*
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Arbitary Unit
UT T NC T NC
Sp-siRNA
- - - + +

Fig. 7.

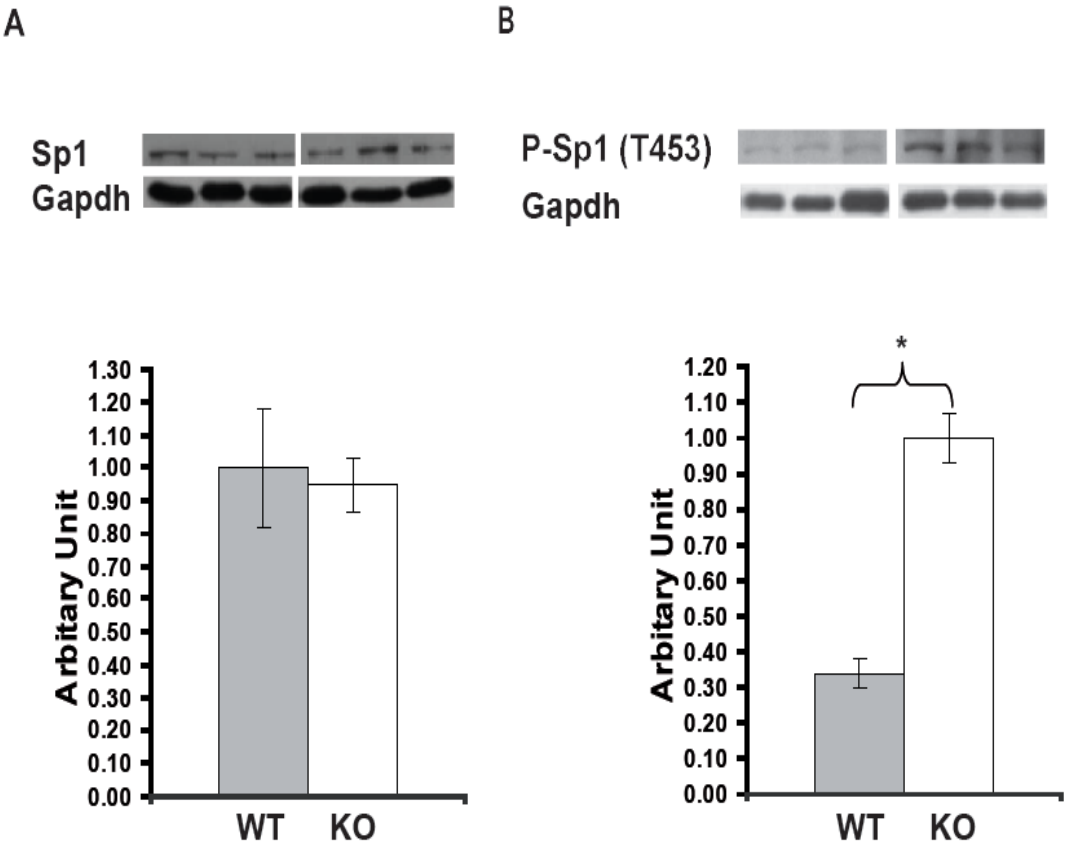
A
B
Sp1
Gapdh
P-Sp1 (T453)
Gapdh
Arbitary Unit
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
WT
KO
*
Arbitary Unit
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
WT
KO

Fig. 8.

2.5. NMDA receptor-mediated synaptic transmission:

The electrophysiology result obtained by our collaborator Nathalie Rouach shows that the AMPA based mediated transmission is significantly decreased in the hippocampus of *Rsk2*-KO mice. Her data showed also that the ratio of AMPA to NMDA currents was unaltered, suggesting that the NMDA basal transmission was also decreased. Therefore, Nathalie Rouach further investigated the level of basal NMDA receptor mediated synaptic transmission. To this end, 4 weeks old *Rsk2*-KO mice as well as WT littermate were used. NMDA receptor-mediated input-output curves in hippocampal slices show that for each input (fiber volley ≥ 0.2mV), the output (fEPSP) is reduced in *Rsk2*-/- slices ($p \leq 0.05$, +/+ n = 7; -/- n = 6). Scale bar 0.1 mV, 50 ms. These preliminary results show that NMDA synaptic transmission is also decreased by ~ 40% in *Rsk2*-KO mice as compared with WT littermate mice. However, at this stage we do not know the mechanism, which may account for this alteration of NMDA synaptic neurotransmission in RSK2 deficient mice.

The curves obtained for NMDA transmission are given in **(Figure 28A)**.

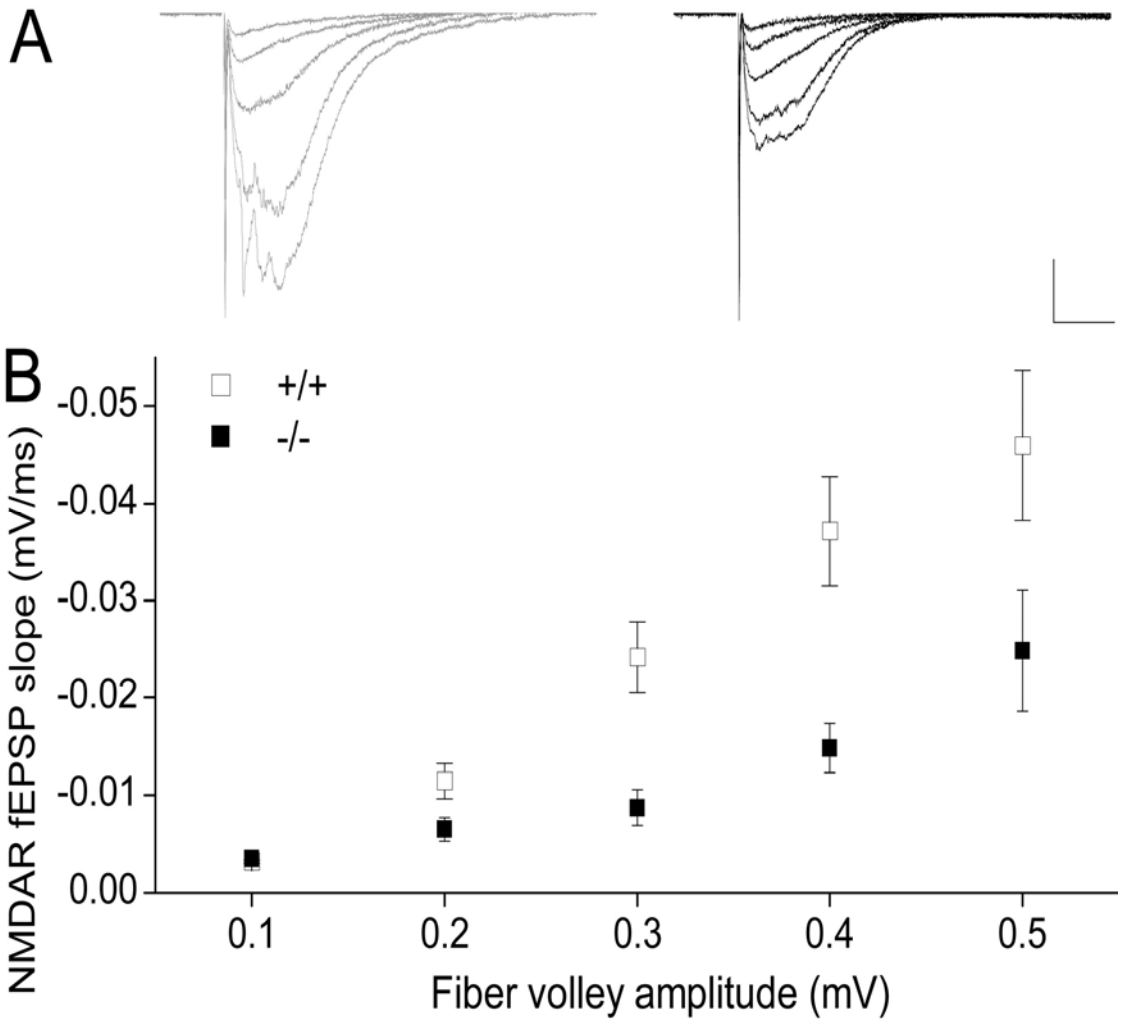

Figure 28: NMDA receptor-mediated synaptic transmission is decreased in *Rsk2*-/- mice.

(A). The curves obtained for NMDA based transmission and **(B)** NMDA receptor-mediated input-output curves in hippocampal slices show that for each input (fiber volley ≥ 0.2mV), the output (fEPSP) is reduced in Rsk2-/- slices ($p \leq 0.05$, +/+ n = 7; -/- n = 6). Scale bar 0.1 mV, 50 ms.

Discussion

Discussion:

Loss of function mutations in the *RPS6KA3* gene cause coffin-Lowry syndrome (CLS), a syndromic form of intellectual disability (ID). The *RPS6KA3* gene encodes the RSK2 protein, which acts at the distal end of the MAPK/ERK cell signaling pathway. RSK2 is a member of the RSK family, which are implicated in many fundamental cellular mechanisms.

To facilitate the study of the physiopathology of ID and other symptoms of CLS, a mouse model (*Rsk2*-KO) was previously generated in our team. This mouse exhibits delayed acquisition of a spatial memory reference task and long-term spatial memory deficits. To gain better insight in the molecular mechanisms leading to learning and memory impairment in the *Rsk2*-KO mice, and to intellectual disability in CLS, we performed a transcriptomic analysis of the *Rsk2*-KO hippocampus. This study aimed to identify genes with altered expression, and as a result altered biological pathways, in absence of RSK2 kinase activity. My particular interest was to understand the molecular events leading to over expression of one of the genes identified in this study, the *Gria2* gene encoding the AMPA receptor subunit GluR2 and the consequence on synaptic neurotransmission in RSK2 deficient neurons. I will now discuss the results, the mechanisms involved and their possible role in the molecular and cellular physiopathology of intellectual disability in CLS patients.

3.1. Alteration of transcriptional activity in the hippocampus of *Rsk2*-KO mice:

The activation of the RSK2 protein by ERK1/2 leads to the translocation of a fraction of this kinase into the nuclear compartment, where it participates in regulation of gene expression, (as previously mentioned). We therefore hypothesized that altered transcriptional activity in the hippocampus of *Rsk2*-KO animals could participate in the pathological mechanism underlying, cognitive impairment.

Our transcriptomic analysis revealed change in expression, with a 1.5-fold or more difference at a p-value <0.03, of 100 genes. Expression of one fourth of these genes was down regulated; whereas the remaining three fourth were up regulated in RSK2 deficient mouse.

3.2. Genes and biological pathways deregulated in the absence of RSK2:

Because information about the function was available for most of the genes they have been grouped by categories. Genes coding for enzymes (Rasl10A, Sod2), regulating the transcription factors (Etv3, Runx2), kinases (FXN, Stk3) and ion channels (*Gria2*, Cacng8 and Cacnb4) have been identified for example in this study.

As anticipated, the data suggest that several cellular mechanisms are affected in the absence of RSK2, including the regulation of cell cycle, gene expression and cell death, and the development and functioning of the skeletal muscles and the nervous system. In addition to determine whether there are potential interactions among the differentially expressed genes, we assembled biologically networks by using the computer soft wear "ingenuity pathway." Seventy-eight genes out of 100 differently expressed genes were mapped and assembled into five major biological networks with a score ≥20 (publication-1).

Strikingly, several genes with important functions in the central nervous system were altered in the *Rsk2*-KO mouse model, including *GRIA2, CACNG8, VAMP4*, and *EIF3A*. The *Gria2* gene was particularly interesting because it encodes the GluR2 protein, a subunit of the AMPA receptor. This receptor is an ionotropic glutamate receptors, is the predominant excitatory neurotransmitter receptors in the mammalian brain and is activated in a variety of normal neurophysiologic processes. This protein is sensitive to AMPA, and acts as a ligand-activated cation channel. The *Gria2* gene is subject to RNA editing (CAG->CGG; Q->R) within the second transmembrane domain, which is thought to render the channel impermeable to Ca^{2+}. The *CACNG8* gene encodes the type I transmembrane AMPAR regulatory protein (TARP).

TARP controls both trafficking and channel gating of AMPA receptors, supports their targeting to the cell membrane and synapses and modulates their gating properties by controlling their rate of activation, deactivation and desensitization and by mediating their re-sensitization. (Chu et al., 2001; Kato et al., 2010). The Vesicle-associated membrane protein 4 is a protein that in humans is encoded via the *VAMP4* gene (Advani et al., 1998). This protein is a member of the VAMP/synaptobrevin family and plays a role in trans-golgi network-to-endosome transport. The eukaryotic translation initiation factor 3 subunit A is a protein that is is encoded by the *EIF3A* gene. This protein is required for the initiation of the protein synthesis mechanism. The two fold increased expression of the *GRIA2* gene was particularly attractive, because it encodes the AMPA receptor subunit GluR2 which has been described as a critical actor in learning and memory (Kessels and Malinow, 2009).

In addition we also observed that many of the identified genes play a role in nervous system development including the *IGF1* and *AHR* genes. Previously it was reported that IGF1 is essential for normal dendritic growth (Cheng et al., 2003).

3.2.1. Alteration of the apoptotic process:

The transcriptomic results detailed in publication-1 revealed that one third of the deregulated genes have been implicated in apoptosis and cell survival. Seven of them are involved specifically in neuronal death (Cacng8, Diablo, *Gria2*, Igf1, Ptgs2, Rb1, sod2) and their expression is increased in the absence of RSK2. These results were not surprising because several previous studies have assigned such a role to RSK2. Subsequently, Anne Schneider In our laboratory obtained confirmatory results, based on cultures of primary hippocampal neurons and by using the tunel method. She showed that the number of apoptotic neurons was significantly higher in KO than in WT cultures after treatment with staurosporine. Staurosporine, an inhibitor of protein kinases, can induce apoptosis via the mitochondrial pathway (Kajta et al., 2006). Stimulation of this pathway allows the release of cytochrome C from the mitochondria, released cytochrome C binds with Apoptotic protease activating factor - 1 (Apaf-1) and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3. This cascade involves also the activation of caspase-3 and -7, and ultimately cleavage of Poly (ADP-

ribose) polymerase (PARP). Anne Schneider observed, by Western blot, an increase of the expressions of cleaved Caspase-3, -9 and PARP in KO neurons. Thus, her findings revealed a greater induction of apoptosis in RSK2 deficient cells.

One other gene identified by microarray encodes a protein that is a second mitochondria-derived activator of caspases, Smac/DIABLO. This mitochondrial protein induces apoptosis after release into the cytosol. Therefore it binds to the X-linked inhibitor of apoptosis protein (XIAP), which prevents it from inhibiting caspases. The expression of this gene is regulated by the transcription factor E2F1, and decreased expression of DIABLO by siRNA reduces apoptosis (Xie et al., 2006). However, RSKs can phosphorylate C/EBPβ, leading to its homodimerization and, hence, inhibition of factor E2F1 (Sebastian et al., 2005, Lee et al., 2010). Thus, absence of RSK2 could result in maintenance of E2F1 in active form and promote the expression of its target genes, including Diablo. However, although, increased DIABLO gene and protein expression was confirmed in the hippocampus of KO animals (Publication-1), no significant increase was found in neuronal cultures. To determine whether its release from the mitochondria can still be involved, a mitochondrial fractionation was performed. This experience indicated a greater release of DIABLO in the cytosol of KO neurons after treatment with staurosporine. This could contribute to greater sensitivity to staurosporine in KO neurons, but this hypothesis should be confirmed with a larger number of animals.

In addition, several other deregulated genes are attractive candidates to explain the susceptibility of *Rsk2*-KO neurons to apoptosis (as SOD2, NFκB, Bad…). Bad is particularly an attractive target because its activity can be regulated by RSKs (She et al., 2002, Chaturvedi et al., 2009). Bad in its non-phosphorylated form inhibits the anti-apoptotic factor Bcl-2, preventing the release of cytochrome C and DIABLO.

In addition, many studies showed that ERKs are involved in regulating cell survival, by assigning them either an anti-apoptotic or a pro-apoptotic function (Boston et al., 2011; Campbell et al., 2010; Nakata et al., 2011).

We may, through the use of the inhibitor UO126, investigate whether the inactivation of ERK promotes or reduces the susceptibility of neurons to apoptosis.

Lutz et al. (2007) suggested that apoptotic and anti-apoptotic cascades can be associated with cognitive dysfunctions and neurological disorders. Taken together, our results suggest that apoptotic processes are altered in the hippocampus of KO mice, which can contribute to the mechanism of cognitive deficits. Apoptosis and cell survival of *Rsk2*-KO neurons are functions that will be further investigated in the team. Following the description of a decrease in the total size of the brain of people with CLS, Kesler et al. suspected already in 2007 a deregulation of the cell death process.

3.2.2. Potential involvement of additional mechanisms in ID of CLS:

As mentioned above, various cellular mechanisms appear to be affected in the absence of RSK2 in the hippocampus. In addition to the deregulated ERK1/2 function and synaptic transmission, many other biological functions may be affected, as suggested by our transcripomic data.

3.2.2.1. Potential causes for alteration of transcription:

Before discussing the molecular mechanisms potentially altered in mice *Rsk2*-KO, we will try to understand some potential mechanisms leading to gene expression alteration. Our study suggests several possible molecular mechanisms that may explain the transcriptional deregulation observed in the *Rsk2*-KO mouse model.

3.2.2.2. Modification of the activity of NF-κB:

A link between RSK2 and NF-κB had already been described by Peng et al. in 2010. They observed that RSK2 can phosphorylate IκBα at Ser-32 directly, which enhances IκBα degradation and releases NF-κB, allowing its passage into the nucleus and activation of transcription.

The deregulation of the expression of some genes observed in *Rsk2*-KO mice might be the result of a decreased phosphorylation of IκBα, itself due to the absence of RSK2. Consequently, it would result in a higher retention of NF-κB in the cytoplasm, where its activity is blocked. This hypothesis could be interesting and should be confirmed, in

particular through analysis of cell fractionations to locate NF-κB in the cell. Inhibition of IκBα could also be undertaken to determine whether the expression of certain deregulated genes (Use of mutants or constitutional siRNA) can be restored.

NF-κB activity is also controlled by phosphorylation, which contributes to its freeing from the inhibitor, its transport into the nucleus and binding to DNA. It has been shown that phosphorylation of the p65 subunit of NF-κB at Ser276 by MSK1, allows induction of transcription of target genes through co-operation of the histone H3 and p300 (Vermeulen et al., 2003). This residue, highly homologous to CREB Ser133 is part of a consensus sequence recognized by RSKs, suggesting that NFkB activity might also be regulated via the RSKs. Wang et al. (2010a) confirmed RSK dependent phosphorylation of the p65 subunit following stimulation of protease-activated receptors (PAR) in epithelial cells HT-29. Inhibition of ERK either with UO126 or silencing of RSKs by siRNA led to decrease of Ser276 phosphorylation of p65 and reduction of NF-kB-dependent transcription. In addition, the authors noted that the action of RSKs is isoform specific. The absence of RSK2 could therefore also influence in this manner gene transcription. Preliminary data of Anne Schneider suggested, however, that phosphorylation of this factor is not altered in the absence of RSK2 in hippocampal cell cultures. These studies must be repeated for confirmation, but also after mitogenic or glutamate stimulation.

3.2.2.3. Modification of the ERK/MAPK signaling:

Our transcriptomic results revealed that most of the genes (75/100) with altered expression are over-expressed in hippocampus of *Rsk2*-KO mice. This was expected, because RSK2 is mainly known for its role in activation of gene expression.

In the second article of this thesis, we have shown that RSK2 exerts a negative feedback on its own pathway. The mechanism leading to over-activation of ERK is described by Douville and Downward in 1997 and reported that a loop of feedback-inhibition to regulate the way, ERK/MAPK dependent on SOS factor. Following stimulation of PC12 cells by EGF, they observed that RSK2 phosphorylates SOS between two proline-rich regions, the region for binding to the SH3 domain of Grb2. This phosphorylation leads to dissociation of the SOS-Grb2 complex, which affects the

activation of the signaling pathway. While the phosphorylation of SOS participates in the regulation of the channel Ras-ERK/MAPK in PC12 cells, previously it does not seem involved in hippocampal neurons. Indeed, we did not observe any difference in total MEK and RAF-1 proteins level, however the glutamate activated neurons shown the levels of phosporylated forms of the MEK and RAF-1 were significantly higher in *Rsk2*-KO neurons than WT. activation of MEK and Raf-1, activating kinases ERK and RSK in KO neurons. Dysregulation of the entire signaling pathway is therefore included (publication-2).

This regulation leads to an increased activity of ERK1/2 (ERK pathway / MAPK) in *Rsk2*-KO hippocampal neurons, but also to an increase in activity of two transcription factors and substrates of ERK: Elk1 and CREB. This activation stimulates their transcriptional activity because the induction of c-Fos, Zif268 and Arc (three IEGs) expression is stronger in RSK2 deficient neurons than in WT cells in response to glutamatergic stimulation. A change in the expression of these IEGs could lead to an alteration of transcription of late response genes. It can thus be anticipated that the increased level of ERK activation, in *Rsk2*-KO neurons, can participate to the deregulated transcriptional activity, either in response to over-activation of transcription factors or to over expression of IEGs. Moreover, some genes whose expression is increased in the *Rsk2*-KO hippocampus have been described as being regulated by ERK at the transcriptional level (Yoon and Seger, 2006). This is an interesting hypothesis to explain the expression alteration of at least some of the genes reported. An abnormal increase in ERK phosphorylation at the baseline level has been reported in many other tissues in the absence of RSK2 (Dufresne et al. 2001; Marques Pereira et al., 2008). Deregulation of the MAPK/ERK pathway and its influence on gene expression may, indeed, have deleterious consequences for many cell functions. In order to confirm this hypothesis, partial in vitro inhibition of ERK through the MEK inhibitor U0126, may be considered followed by transcriptome analysis. In addition this study would determine whether the altered expression of some of these genes can be rescued.

At least six other nuclear and cytoplasmic MAPKs can regulate the activation of ERK have been described (Bermudez et al., 2010) and are potential targets.

Indeed, we showed in the last part of my thesis work that phosphorylation of the transcription factor P-*Sp1* is increased at T453 and T739 in RSK2 depleted cells. Milanini-mongiat et al., in 2002 reported that ERK kinase phosphotylate transcription factor *Sp1* at

these two sites (Thr453 and Thr739). Phosphorylated form of *Sp1* influences positively its transcriptional activity (Tan and Khachigian, 2009). We observed higher levels of phosphorylation for both sites in RSK2 silenced PC12 cells, leading to up regulation of *Gria2* transcription. This result confirmed the assumption made above about the role of ERK1/2 up-regulated activity on gene expression.

3.3. Positive regulation of *Gria2* gene expression via phospho-*Sp1*:

(Boggio et al., 2007), by using immunolabelling, showed that in vivo stimulation increases p-ERK expression at presynaptic and postsynaptic sites of axospinous junctions, suggesting that ERK plays an important role in the local modulation of synaptic function.

Our results showed that the expression and function of the AMPA receptor subunit GluR2, a crucial actor of synaptic function, are positively regulated via ERK1/2 and that RSK2 can indirectly modulate this action. Expression of the *Gria2* gene is strongly controlled by a *Sp1* regulatory element located in the GC-rich promoter region (Myers et al., 1998). In 1999, Merchant et al. observed that the DNA binding activity of the transcription factor *Sp1* is stimulated by ERK phosphorylation. Interestingly, in 2009 it was reported that phosphorylation of *Sp1* influences positively its transcriptional activity (Tan and Khachigian, 2009). The *Sp1* transcription factor is phosphorylated at various sites by different kinases as mentioned in the introduction of this thesis. To elucidate the molecular mechanism leading to GluR2 over expression, we constitutively suppressed the expression of RSK2 in PC12 cells via a vector-based shRNA. The PC12 cell line, cloned from rat pheochromocytoma cells, can be induced to differentiate and to acquire a neuronal-like phenotype (Greene & Tischler, 1976). PC12 cells express endogenous RSK2 at a relatively high level, whereas the level of GluR2 is low (present study) and can be easily transfected. Therefore, the PC12 cell line was a good model for the present study. We showed that *Rsk2* silencing in PC12 cells also leads to an elevation of ERK1/2 phosphorylation as well as of GluR2 expression and that the increased level of GluR2 expression results from the increased ERK1/2 activity on the transcription factor *Sp1* (publication-3). Our results strongly suggest that RSK2 modulates ERK1/2 activity on *Sp1*, which regulates GluR2 expression through transcriptional activation. Together our data provide evidence that RSK2 plays an important role in the regulation of the function of AMPA receptors. How

phosphorylated *Sp1* activates the *Gria2* promoter remains now to be investigated. It is possible that *Sp1* phosphorylation may change its interaction with other transcription factors.

Sp1 phosphorylation induced by the ERK pathway was also shown to cause the release of a histone deacetylase co-repressor complex from a gene promoter (Liao et al., 2008). Furthermore, in addition to *Sp1*, *Gria2* expression is also influenced at the transcriptional level by another positive (Nrf-1) and a negative (Re1/Nrse-like silencer) regulatory element (Borges and Dingledine, 2001). Myers et al. (1998) showed that the *Gria2* Re1/Nrse-like silencer binds the RE1-silencing transcription factor (NRSF/REST), and that co-transfection of REST into neurons reduced *Gria2* promoter activity in a silencer-dependent manner. The nuclear respiratory factor 1 (NRF-1) is a transcription factor that was also shown to regulate *Gria2* gene transcription in neuroblastoma cells (Dhar et al., 2009). It is clearly necessary to understand the roles of the different regulatory elements and their interplay in neurons. Finally, it is now necessary to validate the mechanism described in *Rsk2*-Knocckdown PC12 cells in *Rsk2*-KO mice. Preliminary results show that *Sp1* activity is also up regulated in the hippocampus of *Rsk*2-KO mice suggesting that the mechanism of GluR2 transcriptional up regulation is similar in mouse and PC12 cells. *Sp1* phosphorylation and GluR2 expression can, for example, be assayed in cultured primary hippocampal neurons treated with the MEK inhibitor, and *Sp1* can be knocked down partially in cultured neurons using lentiviral-mediated shRNA expression vectors.

3.4. Consequences or potential consequences of the deregulation of GluR2 expression:

No systematic descriptions of anatomical abnormalities of the brain in CLS patients or animal models have been reported. Therefore, we hypothesized that more subtle cellular events were altered.

Transmission of neuronal signals being dependent on the type of receptors and their neurotransmitters, alteration of one of these factors can lead to a failure of synaptic transmission. In our study, we have revealed alteration of the expression and function of the

AMPA-type ionotropic receptor. Preliminary results very recently obtained reveal also decreased NMDA basal neurotransmission. The molecular basis of this later deficit is not yet known.

These alterations may, indeed, contribute to the cognitive impairment of CLS patients. In the following sections, we will analyze our results and propose a molecular hypothesis for the occurrence of these defects.

It is now well established that the nervous system response to a short lasting change in the efficiency of synaptic transmission requires the expression of new genes. Many genes have been implicated in this process, *Gria2* is one of them. AMPA receptors play a crucial role in synaptogenesis and formation of neuronal circuitry, as well as in synaptic plasticity (Santos et al., 2009). There is a huge amount of evidence indicating that the GluR2 subunit plays a pivotal role in AMPA receptors function. The Ca^{2+} permeability, rectification, and single-channel conductance of AMPA receptors are all dominantly influenced by inclusion of an edited GluR2 subunit in the receptor complex (Hollmann et al., 1991; Burnashev et al., 1992; Bowie and Mayer, 1995; Koh et al., 1995; Seeburg, 1996; Swanson et al., 1997; Washburn et al., 1997). Receptors that contain a single GluR2 subunit have maximally reduced Ca^{2+} permeability; whereas inward rectification is reduced in a graded manner as the number of GluR2 subunits in a receptor increases (Washburn et al., 1997). GluR2 expression is governed by neuronal activity (Bai and Wong-Riley, 2003), and its synaptic distribution and levels are modulated as part of the mechanism of synaptic plasticity (Keifer and Zheng, 2010; Sprengel, 2006). GluR2 interacts with various molecules implicated in receptor trafficking (Dingledine et al., 1999; Collingridge et al., 2004; Isaac et al., 2007). Therefore, it can be anticipated that moderate changes in GluR2 expression may have functional consequences on receptor subunit rearrangement, neurotransmission and lead to functional differences in the functioning of synaptic circuits.

Our data reported in publication 1 showed that basal AMPA neurotransmission is altered in the hippocampus of *Rsk2*-KO mice. Preliminary data, not yet published, reveal also that NMDA basal neurotransmission is significantly decreased in *Rsk2*-KO mouse.

3.4.1. Alteration of Ca^{2+} uptake:

The fact that AMPA receptor dependent neurotransmission was altered in RSK2 deficient mouse, prompted us also to investigate Ca^{2+} uptake of hippocampal neurons. For this purpose we used the cobalt chloride staining approach. As already mentioned AMPA receptors without GluR2 subunit are permeable to Ca^{2+} (or other divalent cations, like Co^{2+}) (Cull-Candy et al., 2006; Konig et al., 2001), whereas in the presence of GluR2 subunit(s) they are impermeable to Ca^{2+}. The results obtained with this approach suggested strongly a decrease of AMPA dependent divalent ions uptake in RSK2 deficient neurons. Thus, our results strongly suggest that up regulation of GluR2 expression in *Rsk2*-KO neurons, affects the AMPA receptor subunit composition, resulting in decreased Ca^{2+} entrance in neurons after AMPA receptors stimulation. Unfortunately, the cobalt approach does not allow quantification of the uptake. Therefore, our conclusion has to be further supported by additional electrophysiological studies, as well as more precise calcium imaging technologies that allows quantification.

3.4.2. Alteration of synaptic transmission and plasticity:

Many studies have reported an alteration of synaptic transmission following changes of expression and recycling of GluR2. The increased synaptic expression of this subunit, suggests an increased proportion of AMPA Ca^{2+} impermeable receptors in *Rsk2*-KO mice. This would well fit with the decreased synaptic transmission observed in hippocampus slices.

In addition to the increased synaptic GluR2 expression, other molecular defects can contribute to alter synaptic transmission. The editing and splicing also play a role in the receptors activity. With regard to the R/G site, Lomeli et al. (1994) reported that edition increases the kinetics of the channel. A reduction of edition is thus assumed to result in a decreased receptor activity, which could contribute to the decrease of transmission in RSK2 deficient neurons. In addition, in 2009, Pei et al. discovered that the flop variant leads to a faster closing of the channel than the flip variant; as a result inactivation of the receptor is quicker. Thus, the increased proportion of flop variants to the detriment of the flip variants

may also contribute to the reduction of basal AMPA receptor transmission in the hippocampus of *Rsk2*-KO mice.

The cause of the decreased AMPA receptor dependent basal transmission, might also be the presence of silent synapses in KO hippocampal neurons. This hypothesis seems interesting because in our lab Anne Schneider observed a decrease of the number of potential excitatory synapses in primary neuronal culture. To test this hypothesis, we have determined in collaboration with Nathalie Rouach the ration of AMPARs/NMDARs currents. Silent synapses are expected to express less AMPAR than NMDAR, or in our case, less functional AMPA receptors. However, the results indicated that the ratio is similar in WT and *Rsk2*-KO mice. More specific studies of the AMPA receptor properties (as the conductance, the rectification, the closing of the channal…) would give more information as to the origin of the alteration of AMPA receptor transmission. The consequences of AMPA receptor dysfunction for synaptic plasticity should also be studied, by assessing LTP and LTD. The alteration of these forms of synaptic plasticity would also explain part of the cognitive deficits associated with MR in CLS patients.

3.5. Consequences or potential consequences of GLUR2 editing and splicing alterations:

AMPA receptor subunits are synthesized and assembled in the Endoplasmic reticulum (ER), before insertion in the plasma membrane. Their assembly and exit from the ER are highly controlled. They can be influenced by post-transcriptional mechanisms, such as RNA splicing and editing.

Two editing sites were identified for *Gria2*, the Q / R site in the transmembrane domain (TMD) domain and the R / G site (residue 607) in the ligand binding domain (LBD). Both of these sites are involved in the membrane recycling of GluR2. Greger et al., in 2002 reported that the Q/R site controls the function and trafficking in AMPA receptors. Editing at the GluR2 Q/R site regulates AMPAR assembly at the step of tetramerization. Specifically, edited R subunits are largely unassembled and ER retained, whereas unedited Q subunits readily tetramerize and traffic to synapses (Greger et al. (2003). This assembly mechanism restricts the number of the functionally critical R subunits in AMPAR tetramers. The same mechanism was subsequently implicated in the edition of the R/G site

at position 743 (Greger et al., 2006). Edition of Q / R and R / G sites thus controls the maturation of the GluR2 subunit. The results reported in publication 1 of the present thesis showed that edition of the Q / R site is not altered whereas the R / G site is significantly less edited in KO mice than in WT animals. By taking into account the data from Greger et al. (2006), our results opens the possibility than the increased expression of unedited GluR2 subunits leads to an increased formation of unedited GluR2 homotetramers in *Rsk2*-KO cells. The over representation of GluR2 at the synapse in *Rsk2*- KO mice might thus be due, at least in part, to the decreased R/G edition.

On the other hand, AMPA receptors are subject to alternative splicing in the LBD domain, generating flip and flop variants. This alternative splicing influences certain parameters of the AMPA receptor, including their assembly and synaptic expression as outlined by Brorson et al. in 2004. Our results (reported in publication 1) revealed an increase of the flop isoform in the hippocampus of *Rsk2*-KO mice. In their study in 2006, Greger et al. showed that the flop isoform promotes ER export and synaptic expression of AMPA receptors. Therefore, an increase in the proportion of this variant could also contribute to the stronger expression of synaptic GluR2 in RSK2 deficient neurons.

It would be interesting now to analyze the cause of abnormal *Gria2* editing in RSK2 deficient neurons. Editing is regulated by adenosine deaminases (ADAR) generating the conversion of adenosine to inosine at the pre-mRNA level (Seeburg et al., 1998). Three ADAR have been identified in mammals (ADAR1, 2 and 3) and the Q/R site is predominantly ADAR2 dependent (Peng et al., in 2006). Through the use of siRNA stereotaxically injected in the hippocampus of rats, they showed that ADAR2 is required for the edition of the Q / R site and thus for the expression of synaptic Ca^{2+} impermeable AMPA receptors. We have shown that edition of the Q/R is not altered in *Rsk2*-KO animal, leading to the conclusion that the ADAR2 function is not affected. However, Sergeeva et al. (2007) implicated ADAR2 in the regulation of Q/R site editing, which is decreased in *Rsk2*-KO mice.

They showed that increased R/G editing in neurons of the tuberomammillary nucleus (TMN) is correlated with higher expression of ADAR2 and inhibition of ADAR3. But they also stated that editing of the Q/R site is not always associated with ADAR2. An analysis of the levels of expression and phosphorylation of the various ADAR isoforms

might give a first indication of the factor involved in the reduction of R/G editing in the hippocampus of *Rsk2*-KO mice. We may then try to influence the editing rate by inhibiting or over-expressing the defective ADAR isoform.

3.6. Other potential deregulation: Phosphorylation of AMPA receptors:

As mentioned in the introduction, AMPA receptor recycling can be regulated by phosphorylation, and many kinases appear to participate in this activity. It has to be reminded here that in addition to its impact on nuclear targets, deregulation of ERK has also an impact on the function of cytoplasmic substrates. For example, an increased level of phosphorylation of RSK1 and 3 was observed in *Rsk2*-KO neurons (publication-2). In addition to suggest a compensatory mechanism in the absence of RSK2 by other RSK family member, this result further demonstrates that the activity of ERK cytosolic substrates is affected. The wide variety of cytosolic ERK substrates suggests that many other cellular mechanisms, and not depending on transcriptional alterations, can be altered in *Rsk2*-KO mice. A role in regulating synaptic transmission has been attributed to ERK via phosphorylation of glutamate receptors (Thomas and Huganir, 2004). Alteration of these regulatory mechanisms could contribute to ID in CLS patients.

Insertion of GluR1 and GluR2 at the synapse is dependent on the activation of the Ras-ERK/MAPK. Qin et al. (2005) noted that stimulation of NMDA receptors induces phosphorylation of GluR2 at Ser841 by ERK/MAPK, thus promoting synaptic expression. Other studies also assigned a role to ERK1/2 in synaptic trafficking. Li and Keifer (2009) reported that synaptic localisation of GluR1 and 4, following stimulation with BDNF, correlates with ERK activation. These studies showed that over-activation of ERK results in an increased density of these subunits at the synapse.

The increased density of synaptic GluR2 subunits observed in *Rsk2*-KO neurones (publication-1) may thus be explained by an increased level of GluR2 phosphorylation. Thus, to determine the role of ERK in this increased synaptic GluR2 expression, it would be interesting to assay the level of GluR2 phosphorylation, and particularly at Ser841 (Qin

et al., 2005). The influence of ERK on synaptic GluR2 expression may also be investigated by immunofluorescent labeling of hippocampal neurons treated with the inhibitor U0126.

3.7. Potential causes for spine morphology/ maturation alteration:

It was reported that the size of the PSD, and thus the head of the spine, is correlated to the number of synaptic glutamate receptors on its surface (Cingolani and Goda, 2008). The proportion of synaptic AMPAR can influence the morphological development of dendritic spines. Passafaro et al. in 2003 described that GluR2 over-expression leads to an increase of the size of the spines head of hippocampal neurons at 22 DIV. This is specific to GluR2 over expression, whereas GluR1 or GluR3 over expression do not produce the same effect. The authors confirmed the role of GluR2 by showing that its over expression leads to the formation of spines in GABAergic interneurons. Their results were validated by the subsequent study of GluR2-KO mouse model in which a decrease in the volume of the "mushrooms" has been reported (Medvedev et al., 2008). The increased expression of GluR2 protein in the hippocampus of *Rsk2*-KO mice (publication-1) could therefore contribute to the increased proportion of mature dendritic spines observed in the derived primary neuronal cultiures (Anne Schneider, manuscript in preparation). To confirm this hypothesis, a new analysis of the morphology of spines could be carried out after partial silencing of GluR2 expression in cultured neurons or by injecting directly siRNA or shRNA stereotacticaly in the hippocampus of *Rsk2*-KO mice.

3.8. Potential causes of NMDA receptor-mediated synaptic transmission alteration:

Preliminary results show that NMDA synaptic transmission is also significantly decreased in *Rsk2*-KO mice. The NMDA receptor has a key role in the regulation of synaptic transmission in the central nervous system of mammalians. In particular, he has a long channel open time and high Ca^{2+} permeability and in consequence a prolonged synaptic conductance coupled with a large Ca^{2+} influx. Activation of the NMDA receptors triggers widespread signal transduction cascades that regulate synaptic plasticity as well as the formation and elimination of synapses (Malenka and Nicoll, 1999; Carroll and Zukin, 2002). Little is known about plasticity of the NMDAR-mediated transmission (Malenka and Bear, 2004). Extensive studies indicated that NMDA receptor activity in the

hippocampus is essential for the formation of both spatial and non-spatial memory (Li and Tsien, 2009; Nakazawa et al., 2004; Tsien, 2000).

There are several potential causes for NMDA receptor dysfunction, which may not be exclusive. In 2005, Morishita and his group reported that inhibition of endogenous cofilin by the Ser3-p-cofilin peptide blocks the actin-dependent NMDA-receptor-LTD (Morishita et al., 2005). They showed that actin anchors NMDA receptors by interacting with a cohort of scaffolding protein. Anne Schneider in our team showed that the level of phosphorylated Cofilin, a well known regulator of actin polymerization and spine morphology, was increased abnormally in *Rsk2*-KO cells. While LIMK activity, a Cofilin upstream kinase, was found unaltered, preliminary data suggest that the Cofilin phosphatase SSH1 activity may be affected. Anne Schneider also detected altered activation of SynGAP, a Ras GTPase-activating protein, which participates in actin dynamics regulation. The SynGAP also play an important role in NMDA glutamate receptors trafficking (Rumbaugh et al., 2006). Thus, altered trafficking of NMDA receptors may contribute to the decreased neurotransmission. Another hypothesis concerns a possible implication of CaMKII. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine kinase regulated by intracellular calcium and by the activity of the NMDAR in spine PSDs. In dendritic spines, CaMKII is associated with the NMDA receptor subunits NR1 and NR2B (Merrill et al., 2005). The results obtained by Anne Schneider showed that the activity of CaMKII is significantly decreased in the *Rsk2*-KO hippocampus. The molecular mechanism leading to NMDA receptor dysfunctions as well as the consequences have now to be further investigated.

3.9. Other potential cause of cognitive dysfunction: alteration the processes of neurogenesis:

As mentioned above, many cellular mechanisms appear to be affected by the absence of RSK2 in the hippocampus. In addition to the regulation of ERK / MAPK and mechanisms of plasticity and synaptic transmission that we have mentioned, preliminary results (a collaboration with S. Laroche, Orsay) also show that the rate of neurogenesis is decreased in the hippocampus of *Rsk2*-KO mice.

In a study conducted in 2010, Dugani et al. suggested that the decrease in the total size of the brain of patients CLS would be the consequence of a reduction in the neurogenesis. They indeed showed that the inhibition of RSK2 by shRNA does not affect cell survival but rather decreases the differentiation of cortical precursors into neuronal cells. This decrease in neuronal cells was attributed to the maintenance of cell proliferation at the expense of the differentiation process. According to these authors, RSK2 would regulate the process via phosphorylation of transcription factors such as C/ EBP or via the regulation of chromatin structure by CBP. RSKs, and RSK1 in particular, can phosphorylate C/EBP at Thr217 and, hence, control neurogenesis (Menard et al., 2002). Activation of C/EBP allows the transcription of genes such as neuron-specific Tα-1 tubulin, which promotes the differentiation of cortical progenitors into neurons. On the other hand, Wang et al. in 2010b indicated that the histone acetyltransferase (HAT) activity of CBP is essential for the expression of genes necessary for the differentiation of cortical precursors into neuronal cells, glia or oligodendrocytes. In 2001, Merienne et al. showed that RSK2 could interact with CBP at baseline and their dissociation following stimulation increases its HAT activity. Impairment of this activity can be anticipated in the absence of RSK2. This could contribute, in addition to a deregulation of C/EBP, to the decreased neurogenesis.

To understand the molecular mechanisms irresponsible for the decreased rate of neurogenesis in *Rsk2*-KO mice, further studies have to be performed, including analysis of C/EBP and CBP activities in neural precursors from our mouse model.

Conclusion

Conclusion:

To date, various studies have been conducted in order to understand the pathophysiology of CLS. Several assumptions have been made, but as yet, the molecular and cellular mechanisms leading to the different CLS clinical symptom are largely unknown. However, studies aimed at understanding the causes of the skeletal anomalies have revealed the important role of RSK2 in osteoblast differentiation, by regulating the activity of the transcription factor ATF4 (Yang et al., 2004). Interestingly, it was shown on the basis of these results, that a diet rich in proteins improves significantly osteogenesis in the mouse *Rsk2*-KO (Elefteriou et al., 2006). At the neuronal level, RSK2 has been implicated in exocytosis of pre-synaptic vesicles via phospholipase D1 (PLD1), in the synthesis of dopamine and in the formation of axons and synapses (Marques Pereira et al. 2008; Zeniou-Meyer et al., 2008; Fischer et al., 2009). These data suggested that alterations in the process of synaptic transmission and plasticity are involved in cognitive impairment associated with Intellectual disability. Our results confirm this hypothesis and provide new and important insights. We showed for the first time that RSK2 deficiency causes an impaired glutamatergic synaptic neurotransmission. Altered AMPA receptors transmission and GluR2 expression subunit has been identified in the hippocampus-*Rsk2*-KO. In addition, although the mechanism is not yet known, preliminary results show that NMDA neurotransmission is also severely altered. We have shown that the up regulation of the *gria2* gene expression is directly related to the up regulation of ERK/MAPK activity in RSK2 deficient cells. Our findings suggest an indirect role of RSK2 in modulating the expression of AMPA receptor GluR2 subunit and, therefore, in regulating glutamatergic neurotransmission and perhaps synaptic plasticity. Although highly likely, it is not yet known if synaptic plasticity is affected in the hippocampus of *Rsk2*-KO mice. LTP and LTD experiments will soon be performed in collaboration with Nathalie Rouach. Further investigations are also now necessary to determine contribution of this deregulation of GluR2 expression to the cognitive dysfunction.

Our results have also revealed alteration of expression of many other genes in the hippocampus of RSK2 deficient mice. The up regulated level of ERK1/2 activity is certainly the cause, directly or indirectly, for the deregulation of a fraction of them. The numerous abnormally expressed genes identified by our microarray analysis will help in

further unraveling the various functions of RSK2 in the hippocampus. It can also be speculated that some of these genes play a significant role in the pathogenesis of cognitive deficiency in Coffin–Lowry syndrome.

Our results are too preliminary to consider a therapeutic approach for cognitive impairment in CLS, but they suggest a critical role for ERK and GluR2 in the pathology. Further studies, including living animals, for example, by injecting stereotaxically in the hippocampus viral mediated shRNA vectors must be implemented now to confirm the involvement of GluR2 in cognitive impairment. If the implication is validated, the use of inhibitory molecules targeting the ERK pathway or GluR2 could be interesting. The MEK inhibitor (U0126) has been tested in clinical trials as anti-cancer molecule (Marampon et al., 2009, Ito et al., 2010) and Neuroprotection (Farrokhnia et al., 2008) and may be tested for its efficacy in mice. Du et al., in 2008 described the use of lithium and valproate to inhibit the expression of synaptic GluR1/GluR2 in hippocampal neurons in vitro and in vivo. However, there is no currently specific inhibitor of GluR2 available. The use of antagonists of type I mGluRs glutamate receptor have been used in clinical trials for XFRA (Krueger and Bear, 2011). In 1997, the first link between FMRP and mGluR has been established. Many studies have subsequently demonstrated the involvement of FMRP in the regulation of mGluR dependent protein synthesis. Mice doubly heterozygous for FMRP and mGluR targeted gene disruption allowed the restoration of some phenotypic manifestations associated with the FMRP mutation. These results prompted the researchers to study the consequences for animal models of XFRA of a treatment with a specific inhibitor of mGluR5, MPEP. This inhibitor, indeed, improves the cognitive phenotype in mice, Drosophila and "zebrafish" mutated in the FMRP gene. These encouraging data led to the establishment of clinical trials in humans. Several pharmacological molecules have been tested, such as acramprosate, lithium or the GABA inhibitor, baclofen. The results obtained with these studies are very encouraging. (Krueger and Bear, 2011).

If the importance of GluR2 in the pathogenesis of cognitive impairment in CLS is established, the FRAXA story could serve as an example for a therapeutic strategy for RSK2 deficiency.

In conclusion, together available data show that RSK2 acts in a broad variety of functions. However, investigations undertaken in our team in the recent years have mainly

focused on its involvement in the regulation of plasticity and synaptic transmission. Anne Schneider has shown, for example, that spinogenesis and the underlying cytoskeleton dynamic are altered in the hippocampus of *Rsk2*-KO mice.

The deregulation of cellular processes, such as neurotransmitter release, axonal and dendritic growth, functional maturation of synapses, is becoming increasingly evident to explain the cognitive impairment in CLS patients, but also in other cognitive dysfunctions or psychiatric disorders. The Results so far obtained do not answer to all the questions, but already allow us to make hypotheses.

Future Perspectives

Future prospects:

Further studies are needed to explore in more detail the mechanisms leading to up-regulation of the *Gria2* gene, and its consequences on synaptic transmission and plasticity. It is essential to further dissect the molecular mechanism of *Gria2* transcriptional control, including the identification by which *Sp1* activates the *Gria2* promoter. It is possible that *Sp1* phosphorylation may change its interaction with other transcription factors, as it was previously described for other genes. *Sp1* phosphorylation induced by the ERK pathway was also shown to cause the release of a histone deacetylase co-repressor complex from the luteinizing hormone receptor gene promoter, which may also be involved in the *Gria2* gene expression. On the other hand, it has been shown that, in addition to *Sp1*, *Gria2* expression is also influenced at the transcriptional level by another positive (Nrf-1) and a negative (Re1/Nrse-like silencer) regulatory element in the 5' proximal region of the promoter (Borges and Dingledine, 2001). It is clearly necessary to understand the roles of these different regulatory elements of the *Gria2* gene and their interplay in neurons.

Our finding of an abnormally increased level of GluR2 expression in RSK2 deficient neurons could lead to therapeutic strategies for CLS, e.g. by modulating the glutamatergic system. Indeed, in the Fragile X syndrome (FXS), observations suggesting increased metabotropic glutamate receptor (mGluR5) led to preclinical studies showing that inhibition of mGluR5 can ameliorate multiple mutant phenotypes in mouse and drosophila models of FXS, and clinical trials based on this therapeutic strategy are underway (Krueger and Bear 2011). However, the extent of glutamate transmission alteration, and whether synaptic plasticity is modified remain first to be explored. Alteration of NMDAR transmission has to be confirmed, and the mechanism involved, as well as its contribution to cognitive impairment, to be investigated. Levels of synaptic and extrasynaptic glutamate using pharmacology (with low affinity AMPA and NMDA receptors competitive antagonist (γ-DGG and D-AA, respectively), should also be assayed to investigate whether the decrease in AMPA receptor-mediated transmission identified in *Rsk2* knockout mice is also associated to alterations in extracellular glutamate levels. Density of functional surface, and extra-synaptic AMPARs, using local application of AMPA in whole-cell recordings and in somatic outside-out patches from CA1 pyramidal cells, respectively should also be measured. Since the GluR2 subunit is involved in LTP, LTD and plays an important role in

learning and memory, short and long-term plasticity in the hippocampus of *Rsk2* knockout mice should be investigated in detail. These data should give insights into the altered hippocampal cellular physiology accounting for the psychomotor impairments in the *Rsk2*-KO mice. Studies designed to understand the contribution of GluR2 over expression in the cognitive dysfunction of *Rsk*2-KO mice have already been undertaken. The goal is to determine whether the RSK2 deficient phenotype can be restored by a partial inactivation of GluR2 in mice. No specific inhibitor of GluR2 being currently available, AAV-mediated shRNA are used to knockdown partially GluR2 in *Rsk*2-KO mice. Viral particles will be injected stereotaxically into the hippocampus.

The contribution of other pathways to the AMPA receptor dysfunction remains also to be investigated. Among the deregulated genes identified by our transcriptome analysis at least one other participates in regulation of AMPAR function: Cacng8. This gene encodes a synaptic protein, TARPc-8 that participates in consolidation phase of memory and is involved in modulating neurotransmitter release. Evidence was provided that TARPc-8 is critical for basal AMPA receptor expression and localization at extrasynaptic sites in the hippocampus (Rouach et al. 2005). Up-regulation of Cacng8 may thus contribute to AMPA receptor dysfunction.

Indeed we expect that our studies will provide approaches for pharmaceutical intervention in Coffin-Lowry Syndrome.

Experimental Procedure

Material and Methods:

6.1. Ethics statement:

All experimental procedures for care and use of mice were performed according to agreements with the Departmental Direction of Veterinarian Services (Prefecture du Bas-Rhin, France No. 67-225) and IGBMC Animal Welfare Insurance (NIH, PHS No. A5100-01). Mice were housed 4 per cage in a 12h dark/light cycle (light on from 7 am to 7 pm), under controlled conditions of temperature and humidity. Euthanasia of the mice used to derive primary cell cultures was carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/EEC), and the study was specifically approved by the Ethics Committee in Animal Experiments (permit number 17) of the Institute of Genetics and Molecular and Cellular Biology.

6.2. Primary hippocampal neuronal Cultures and Cobalt staining:

Primary cultures of hippocampal neurons were derived from one day old WT and *Rsk2*-KO male mice, with a C57Bl/6x genetic background. After treatment of the hippocampi with Papain, dissociated cells were plated onto 24-well plates containing poly-d-lysine-coated cover slips (Sigma), at a density of ~100 000 neurons/well. Growth media consisted of Neuro-Basal (GIBCO, Invitrogen) supplemented with 1x B27 (GIBCO, Invitrogen), 0.5mM L-Glutamine and 1x penicillin/streptomycin. The cultures were stored in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 for 8 days.

The cobalt staining procedure was essentially performed as described previously (Jensen et al., 1998), with small modifications. The initial medium was removed from the wells after 8 days, and cells were washed twice with HEPES buffer (146mM NaCl, 4.2mM KCl, 0.5mM MgCl2, 0.8mM CaCl2, 55.6mM glucose, 20mM HEPES, pH 7.4) at 37°C. Cells were then incubated with 5mM $CoCl_2$ in HEPES buffer.

Three Conditions were then used; i) without AMPA treatment (control); ii) addition of AMPA (to a final concentration of 100 µM) to activate AMPA receptors and allow the influx of Co^{++} into the neurons iii) addition of AMPA (100 µM final) and cyclothiazide (30

µM final) which act as non-desensitizing agent on AMPA receptors in the presence of AMPA.

Following 30-35 min incubation the cells were washed twice with HEPES buffer supplemented with 2mM EDTA (10 min each) in order to remove the excess of extra cellular $Co^{++.}$ The intracellular Co^{++} was then precipitated by using HEPES buffer containing 0.12% Na_2S, for 5 min. The cells were then washed once with HEPES buffer and fixed with 4% paraformaldehyde and 4% sucrose in phosphate-buffered saline (PBS; 8 g/l NaCl, 0.2 g/l KCl, 2.9 g/l Na2HPO4.12 H2O, 0.2 g/l KH2PO4, pH 7.4), for 30 min at room temperature. The cells were then washed with development solution (292mM sucrose, 15.5mM hydroquinone, 42mM citric acid) at 50ºC. The development was performed in a dark room in the developmental solution containing 1 mg/ml AgNO3 at 50°C for 50–60 min. When the Co^{2+}-positive neurons appeared dark colored the development was stopped by removing the developing solution and washed once with warm development solution. To preserve the stain, the neurons were incubated with 5% sodium thio-sulfate at room temperature for 10 min (Malva et al., 2003). Finally, the neurons were air dried and mounted with KAISER's glycerol gelatin (Merck). The stained slides were visualized under bright field microscope to monitor the intensity of cobalt chloride staining.

Summary and Results in French

Identification des déficits moléculaires, cellulaires et cognitifs chez le modèle souris du retard mental causé par la mutation du gène *Rsk2*

Résumé

Le retard mental (RM), le trouble humain le plus fréquent, est présent dès l'enfance et se caractérise par une limitation de l'intelligence et des capacités d'adaptation: la déficience cognitive affecte environ 3% de la population dans les pays industrialisés. Des mutations dans les gènes liés à l'X sont la cause génétique la plus fréquente de déficience mentale. Le syndrome de Coffin-Lowry (CLS), une forme de retard mental syndromique liée à l'X, est caractérisé par un retard psychomoteur sévère, une dysmorphie faciale, des anomalies chiffres et progressive des déformations du squelette. Le CLS est causé par des mutations perte de fonction dans le gène *RPS6KA3*, localisé en Xp22.2, qui code pour la protéine S6 ribosomale kinase (RSK2). La déficience cognitive est habituellement sévère chez les patients masculins avec un QI allant de 15 à 60 et un retard dans l'acquisition du langage, la plupart des patients ayant un vocabulaire très limité. Le développement moteur est également toujours retardé, et environ un tiers des patients présentent des crises de cataplexie. Les mutations affectant le gène de *Rsk2* chez les patients CLS sont extrêmement hétérogènes et conduisent à l'arrêt prématuré de la traduction et / ou à la perte de l'activité phosphotransférase. A un niveau macroscopique, aucune anomalie cérébrale spécifique n'a été signalée chez les patients CLS, suggérant que RSK2 n'est pas requis pour les principaux aspects développementaux du cerveau. RSK2 appartient à une famille de quatre membres très homologues chez les mammifères, RSK1-4, codées par des gènes distincts. Les RSKs sont des sérine/thréonine kinases dont l'activité est régulée par les facteurs de croissance et certains neurotransmetteurs, et qui agissent dans la voie Ras-MAPK. Les substrats nucléaires des RSK2 incluent des facteurs de transcription (dont CREB et ATF4) et des histones, suggérant un rôle dans la régulation de l'expression génique. Les souris invalidées pour le gène *Rps6ka3* (souris *Rsk2*-KO) montrent des déficits d'apprentissage et de mémoire spatiale à long terme. RSK2 est fortement exprimé dans l'hippocampe une structure impliquée dans ces processus cognitifs.

Pour comprendre les conséquences de l'absence d'expression de RSK2 dans l'hippocampe nous avons effectué une comparaison des profils d'expression génique d'hippocampe de souris *Rsk2*-KO et de souris WT de mêmes fratries. Elle a révélé une altération d'expression de 100 gènes (l'expression de 75 était anormalement augmenté et celle de 25 diminué chez les souris *Rsk2*-KO) codant pour des protéines agissant dans divers processus biologiques. Nous avons analysé les conséquences de la dérégulation de l'un de ces gènes, *Gria2* (**Figure 29**), qui code pour la protéine GluR2, une sous-unité du récepteur glutamate (ionotropique) AMPA. Dans l'hippocampe des souris *Rsk2*-KO, le niveau d'expression de GluR2 était le double de celui observé chez les souris WT. C'était également le cas de la protéine GluR2 exprimée à la surface des synapses des neurones d'hippocampe en culture. Les récepteurs AMPA, des canaux cationiques activés par des ligands, interviennent dans la composante rapide des courants postsynaptiques excitateurs dans les neurones du système nerveux central. Ils jouent également un rôle important dans la plasticité synaptique. Les études électrophysiologiques ont révélé une réduction de 25% de la transmission basale des récepteurs AMPA dans l'hippocampe de souris KO-*Rsk2* (**Figure 30**). Etant donné que la sous-unité GluR2 contrôle la perméabilité au Ca^{2+} des récepteurs AMPA, nous avons aussi utilisé une approche par coloration au cobalt (un ion divalent, Co^{2+}, comme le calcium) pour tester la fonction des récepteurs AMPA. La coloration au chlorure de cobalt, après stimulation des récepteurs AMPA, était diminuée dans les cultures primaires de neurones d hippocampe de souris *Rsk2*-KO par comparaison à des neurones de souris WT. Ces résultats ont montré que la fonction des récepteurs AMPA était affectée dans l'hippocampe des souris *Rsk2*-KO. C'est la première fois qu'une telle dérégulation est démontrée dans le modèle murin du syndrome de Coffin-Lowry. Cette neurotransmission AMPA anormale est susceptible de contribuer à la dysfonction cognitive des souris *Rsk2*-KO et des patients CLS. Nous avons aussi montré que le glutamate active ERK1/2 dans des cultures primaires de neurones hippocampiques et que le niveau d'activation de ERK1/2 est nettement plus élevé dans les neurones dérivés des souris *Rsk2*-KO que dans ceux dérivés de souris WT, en conformité avec une fonction de rétroaction inhibitrice de RSK2 sur l'activité de ERK1/2 dans les cellules WT.

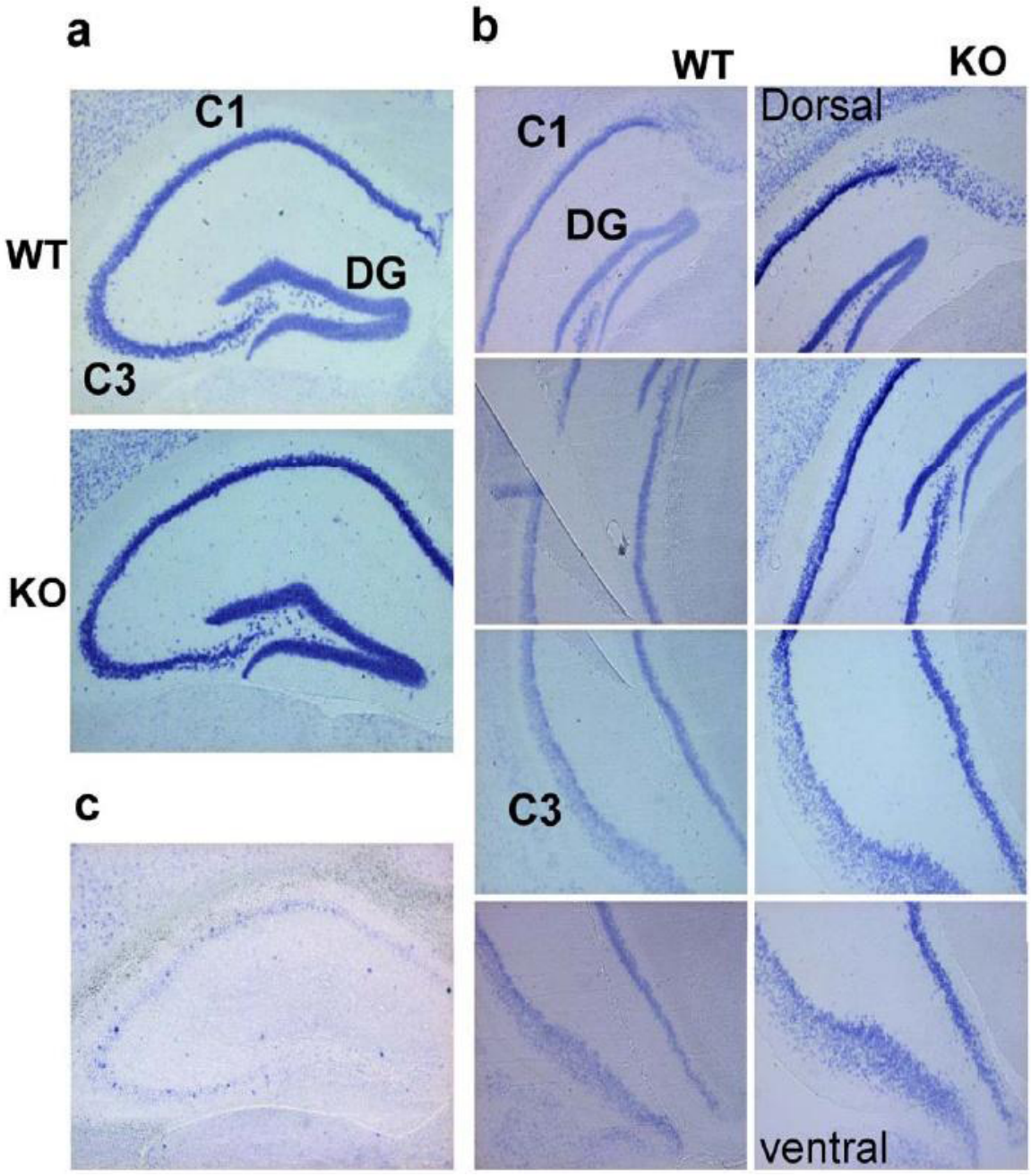

Figure 29 : Hybridation in situ.

Des ARN *Gria2* sens et antisens marqués à la digoxigénine (Dig) ont été hybridées à des coupes coronales de 25 mm de trois cerveaux de trois souris *Rsk2*-KO et trois souris WT adultes. Une photo est montrée pour chaque génotype. (a) On observe une augmentation significative de l'expression dans tous les domaines de l'hippocampe antérieur de *Rsk2*-KO chez la souris. (b) Dans l'hippocampe postérieur le niveau du mARN de *Gria2* est surtout augmenté dans le gyrus denté, dans CA1 et dans la région CA3 ventrale. (c) Aucune coloration n'a été observée avec l'ARN sens.

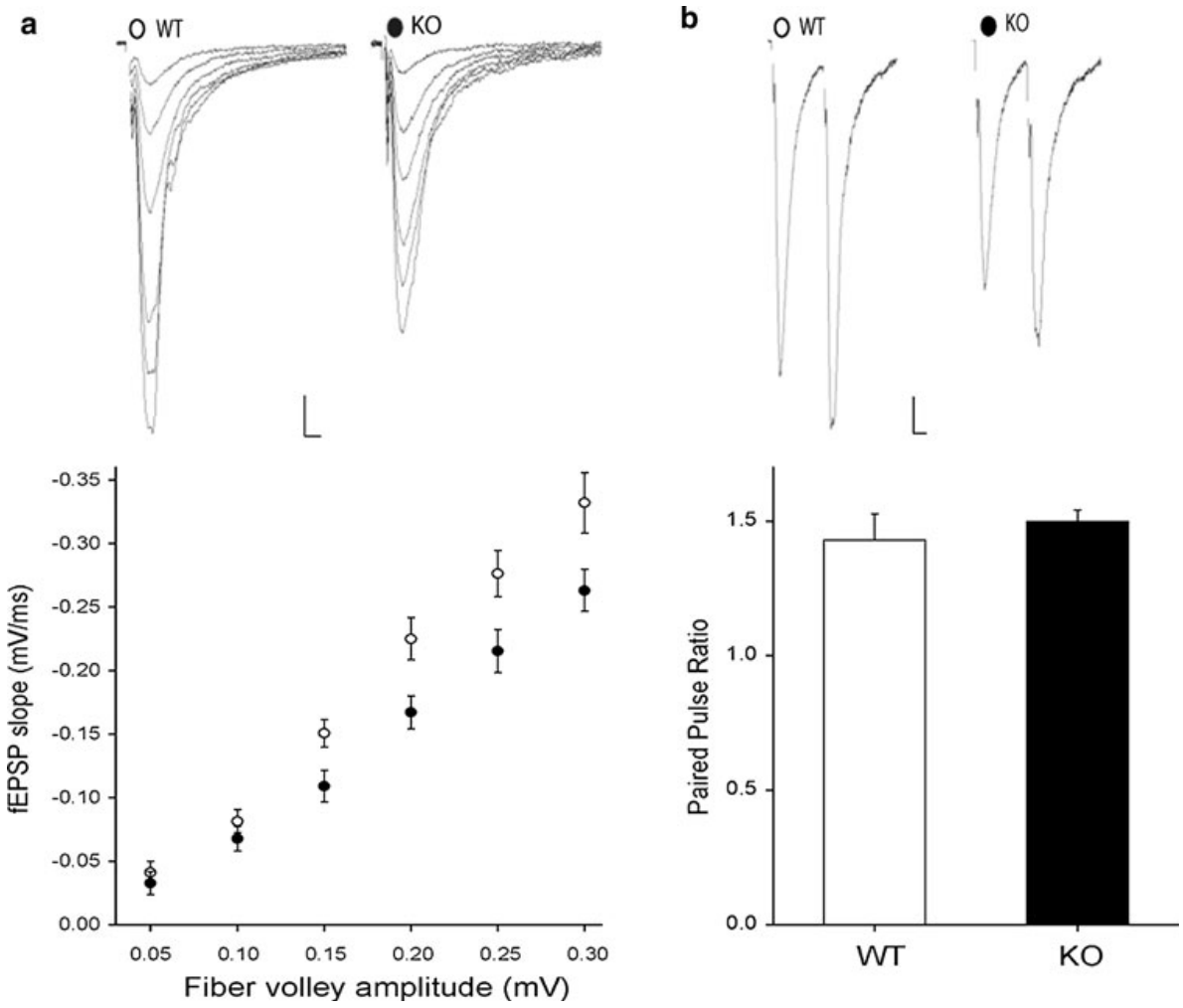

Figure 30 : Diminution importante de la transmission synaptique basale AMPAR dépendante.

Analyse par Patch-clamp. (a) Courbes d'entrées-sorties pour la transmission synaptique basale dans des tranches d'hippocampe. Comme illustré par les tracés et graphes, pour chaque entrée (fiber volley : C 0,15 mV), la sortie (fEPSP) est réduite de 25% dans les tranches RSK2-KO (p B 0,05, n = WT 10; KO n = 9). Barre d'échelle de 0,1 mV, 5 ms. (b) La paired-pulse facilitation (PPF) ne diffère pas entre les souris RSK2-KO (n = 9) et WT (n = 6). Des exemples de tracés sont représentés au-dessus des graphes. Barre d'échelle :0,05 mV, 10 ms (Expérience réalisée Nathalie Rouach, Collège de France).

Nous avons montré que le niveau d'activité d'ERK1/2 était également anormalement élevé dans l'hippocampe adulte de souris *Rsk2*-KO (**Figure 31**).

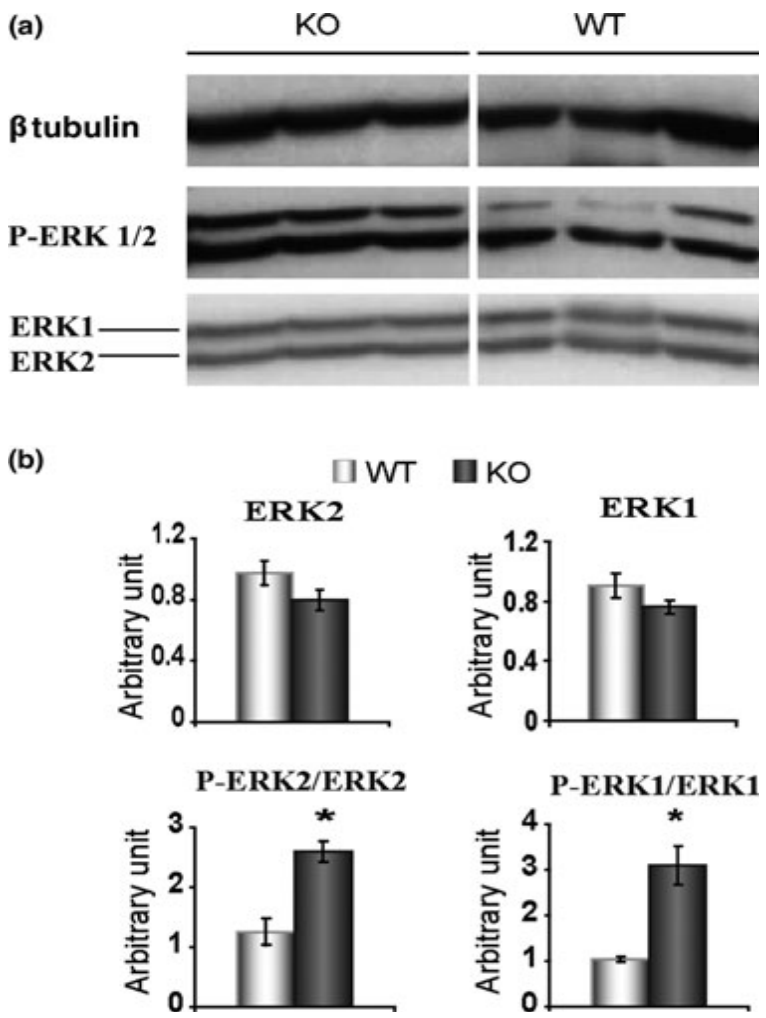

Figure 31 : Augmentation de la phosphorylation d'ERK dans l'hippocampe *Rsk2*-KO.

(a) les protéines détectées (ERK1 / 2, P-ERK1 / 2 et b-tubuline) dans trois souris *Rsk2*-KO et trois souris WT sont montrés. (b) Les données représentées sont les valeurs moyennes normalisées avec ERK1/2 et la b-tubuline ± SEM pour six souris de chaque génotype. Les niveaux de P-ERK1/2 sont significativement augmentés dans l'hippocampe de souris *Rsk2*-KO. * p <0,05 par t-test de Student.

En outre, le niveau de phosphorylation des deux facteurs de transcription nucléaire CREB et Elk1, des cibles nucléaires de ERK1/2, est aussi plus élevé dans les neurones déficients en activité RSK2. Ces résultats suggèrent que le niveau anormalement élevé de ERK1/2 dans les neurones *Rsk2*-KO peut-être impliqué, directement ou indirectement, dans

la dérégulation de certains des gènes identifiés dans notre précédente étude de comparaison de transcriptomes.

Mon but fut ensuite d'essayer de comprendre les mécanismes de signalisation impliqués dans l'augmentation du niveau de transcription du gène GluR2 dans les neurones déficients pour RSK2. Pour répondre à cette question, nous avons utilisé la technologie d'interférence ARN pour bloquer l'expression de RSK2 dans les cellules PC12. Nous avons testé l'efficacité de trois plasmides produisant différents ARN en épingle à cheveux de petite taille (shRNA) ciblées contre différentes régions codantes de l'ARNm de *Rsk2*. Un de ceux-ci a induit une diminution drastique de l'expression de la protéine RSK2 et de son ARNm (*Rsk2*-Knockdown). Nous avons observé dans les cellules RSK2-knockdown une augmentation importante de l'expression GluR2 au niveau de l'ARN et de la protéine ainsi qu'une augmentation du niveau de phosphorylation de ERK1/2, de manière similaire à ce qui avait été observé dans les neurones hippocampiques de souris *Rsk2*-KO. L'inhibition de la voie ERK, dans les cellules *Rsk2*-knockdown avec l'inhibiteur de MEK U0126, résulta en une diminution dramatique du niveau d'expression de GluR2, montrant que ERK1/2 joue un rôle crucial dans la surexpression de GluR2. Il avait été montré précédemment que l'expression de GluR2 est influencée au niveau transcriptionnel par le facteur de transcription *Sp1*. Cependant nous n'avons trouvé aucune différence dans le niveau d'expression de la protéine *Sp1* entre cellules RSK2-knockdown et cellules non transfectées. Comme il avait été aussi montré que deux sites de *Sp1* sont phosphorylés par ERK1/2, nous avons analysé les niveaux de phospho-*Sp1* (**Figure 32**). Nous avons ainsi observé un niveau anormalement élevé de phospho-*Sp1* dans les cellules PC12 RSK2-knockdown par rapport aux cellules PC12 non transfectées (WT). Le traitement par U0126 abolit la phosphorylation de *Sp1*, confirmant ainsi le rôle spécifique de ERK1/2 dans cette phosphorylation. Le blocage partiel de l'expression de *Sp1* dans les cellules PC12 *Rsk2*-knockdown avec un siRNA entraîna une diminution dramatique du niveau d'expression de GluR2, confirmant ainsi le rôle crucial que joue *Sp1* dans le mécanisme de surexpression de GluR2 dans les cellules déficientes pour RSK2 (**Figure 33**). J'ai par la suite montré que dans l'hippocampe des souris *Rsk2*-KO le niveau de pospho-*Sp1* est également plus élevé que chez les souris WT de mêmes fratries, supportant ainsi nos résultats dans les cellules PC12.

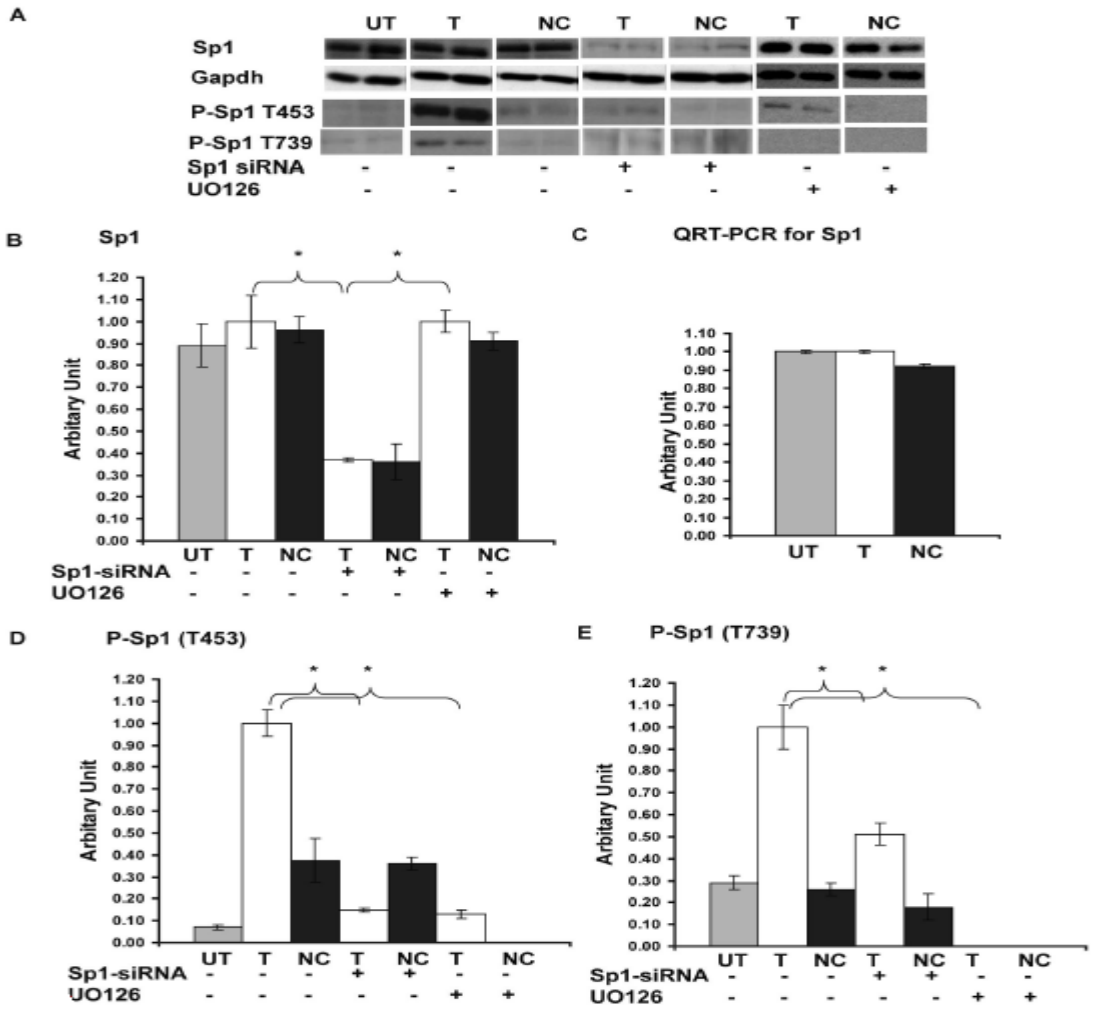

Figure 32 : Les niveaux d'expression de *Sp1* et de phospho-*Sp1* (P-*Sp1*) dans les cellules PC12 non transfectées (UT), transfectées avec le *rsk2*-shRNA (T) ou un shARN de séquence au hasard (contrôle négatif, NC).

(a). Les résultats du Western blot. (b) Quantification des niveaux d'expression de la protéines Sp1. Les données, normalisée avec la GAPDH, sont représentées comme la moyenne ± SEM (UT: n> 3, T: n> 3, NC: n> 3). L'expression de la protéine *Sp1* est fortement diminuée dans les cellules transfectées avec le *Sp1*-siARN, alors que dans toutes les autres conditions, il est très similaire. (c). Quantification de l'expression du mARN de *Sp1* par QRT-PCR et normalisée avec l'expression du gène *gapdh* (UT: n> 3, T: n> 3, NC: n> 3). La barre représente la moyenne ± SEM. * p <0,05 par t-test de Student. (d, e). Les niveaux de P-*Sp1* (T453 et T739) sont beaucoup plus élevés dans les cellules transfectées avec le *Rsk2*-shARN (T), que dans toutes les autres conditions. Le traitement par U0126 ou Sp1-siARN réduit la phosphorylation de Sp1 de façon drastique aux deux sites, T453 T739, dans les cellules appauvri en RSK2 (T). (UT: n> 3, T: n> 3, NC: n> 3). Les différences avec * p <0,05 entre les groupes UT, T et NC ont été considérés comme significatives par t-test de Student.

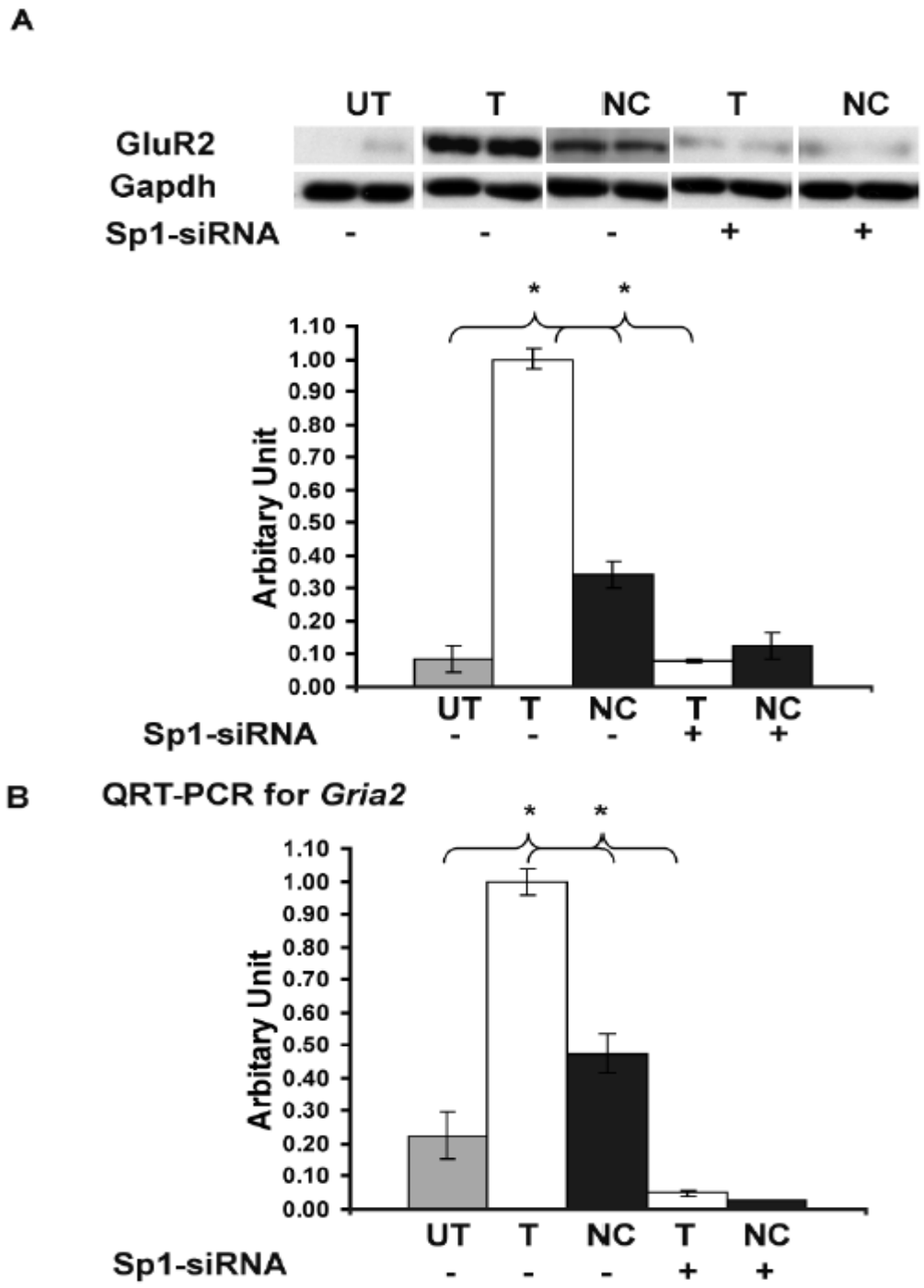

Figure 33: L'expression de GluR2 est considérablement diminuée dans les cellules *Rsk2*-knockdown traitées avec le *Sp1*-siARN.

(A) Les résultats de Western blot ont révélé une forte diminution de l'expression GluR2 dans les cellules *Rsk2*-knockdown, par rapport aux cellules *Rsk2*-knockdown non traitées avec le *Sp1*-siARN. (B) Quantification de l'expression de l'ARNm de *Gria2* par QRT-PCR. Les données sont normalisées avec l'expression du mARN *gapdh* et sont représentées comme la moyenne ± SEM (UT: n> 3, T: n> 3, NC: n> 3) (* p <0,05 par t-test de Student.

Ensemble, mes résultats ont suggéré que la surexpression de GluR2 dans les cellules déficientes pour RSK2, était causée par une activation accrue de la transcription du gène *Gria2* par *Sp1*, conséquence d'un niveau accru d'activité de ERK1/2.

Nos résultats sont très prometteurs, car ils pourraient conduire à des stratégies thérapeutiques pour le syndrome de Coffin-Lowry, par exemple, en modulant la neurotransmission glutamatergique. Toutefois, d'autres investigations sont encore nécessaires pour déterminer la contribution de la surexpression de GluR2 au déficit d'apprentissage et de mémoire spatiale chez les souris *Rsk2*-KO. À cette fin, l'équipe a l'intention de normaliser la fonction du récepteur AMPA dans les souris *Rsk2*-KO en utilisant des vecteurs d'expression lentiviral-shRNA injectée par stéréotaxie dans l'hippocampe, afin de déterminer si cela permet d'améliorer le déficit cognitif.

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Summary

Intellectual disability (ID) is the most frequent human disorder that begins in childhood and is characterized by limitation in both intelligence and adaptive skills: it affects approximately 3% of the population in industrialized countries. Mutations in X-linked genes are the most common genetic cause of ID. Coffin-Lowry syndrome (CLS) is a syndromic form of ID that is caused by mutations in the *RPS6KA3* gene located in Xp22.2 and encoding the ribosomal S6 kinase 2 protein (RSK2). *Rsk2* belongs to a family of four highly homologous members in mammals, RSK1-4, encoded by distinct genes. RSKs are widely expressed growth factor-regulated serine/threonine protein kinases, acting in the Ras-MAPK signaling pathway. *Rsk2*-KO mice display spatial learning and memory impairment. RSK2 is highly expressed in the hippocampus a structure involved in these cognitive processes. To understand the consequences of RSK2 deficiency in the hippocampus we performed a comparison of the hippocampal gene expression profiles from *Rsk2*-KO and WT littermate mice. It revealed differential expression of 100 genes, encoding proteins acting in various biological pathways. We further analyzed the consequences of deregulation of one of these genes, *Gria2*, which encodes the GluR2 protein, a subunit of the glutamate AMPA receptor. An abnormal two-fold increased expression of GluR2 at the protein and RNA levels was found in the hippocampus of *Rsk2*-KO mice and also at synapses surface in *Rsk2*-KO hippocampal cultures. Electrophysiology studies showed a 25% reduction of basal AMPA receptor-mediated transmission, in the hippocampus of *Rsk*2-KO mice. Less cobalt chloride staining upon AMPA stimulation was observed in *Rsk2*-KO cultured primary hippocampal neurons compared to WT cells. These results provided evidence that the AMPA receptor function is affected in the hippocampus of *Rsk2*-KO mice. Indeed, abnormal AMPA neurotransmission may contribute to the cognitive dysfunction of *Rsk2*-KO mice and CLS patients. This is the first time that such deregulations have been demonstrated in the mouse model of the Coffin-Lowry syndrome. Preliminary results show also a 40% reduction of NMDA receptor-mediated transmission.

We showed also that glutamate activates ERK1/2 and RSKs in primary hippocampal neuronal cultures and that ERK1/2 activation is significantly stronger in *Rsk2*-KO than in WT neurons, in accordance with a feedback inhibitory function of RSK2 on ERK1/2 activity in WT cells. ERK1/2 activity is also abnormally increased in the adult hippocampus of *Rsk2*-KO mice. In addition, two nuclear transcription factors CREB and ELK1 were shown to be over activated by the abnormal nuclear activity of ERK1/2 in RSK2 deficient neurons. These results suggest that ERK1/2 over activation may account, directly or indirectly, for some of the deregulated genes identified in our transcription profiling study through over activation of transcription factors or over induction of IEGs.

My subsequent goal was to try to unravel the signaling mechanisms involved in the increased levels of transcription of the *Gria2 gene* in RSK2 mutant neurons. To address this issue, we used the RNA interference technology to knockdown the *Rsk2* gene in PC12 cells. ShRNA, caused a 90% decrease of the RSK2 protein expression. We observed in *Rsk2*-knockdown PC12 cells a strong increase of GluR2 expression at the RNA and protein levels as well as, an increased level of ERK1/2 phosphorylation, similar to our previous observations in *Rsk2*-KO adult mouse hippocampal neurons. Inhibition of the ERK pathway, in *Rsk2*- knockdown PC12 cells with the MEK inhibitor U0126 resulted, in a dramatic decrease of GluR2 expression, showing that ERK1/2 plays a crucial role in GluR2 up-regulation. Since it was previously reported that GluR2 expression is influenced at the transcriptional level by several transcription factor, including Sp1, we assayed the expression of *Sp1*. We observed no difference in total Sp1 expression between *Rsk2*-knockdown and untransfected PC12 cells. Since it was also shown that two sites of Sp1 are phosphorylated by ERK kinases, by using Sp1 specific phospho-antibodies raised against these sites, we analyzed levels of phospho-Sp1. We observed an abnormally increased level of phospho-Sp1 in *Rsk2*-knockdown PC12 cells. Treatment with U0126 abolished phosphorylation of Sp1, confirming the specific role of ERK1/2 in this phosphorylation. Partial silencing of *Sp1* expression in *Rsk2*-knockdown PC12 cells with siRNA resulted in a dramatic decrease of GluR2 expression, confirming further that Sp1 plays a prominent role in the mechanism of GluR2 up-regulation in RSK2 deficient cells. I subsequently showed that in the hippocampus of *Rsk2*-KO mice the level of pospho-Sp1 is, indeed, significantly higher than in WT littermate mice, supporting our results in PC12 cells. Together, my results suggested that over expression of GluR2 in RSK2 deficient cells, is caused by increased Sp1 transcriptional activity on the *Gria2* gene, which, itself, is the consequence of ERK1/2 increased signaling.

Tahir MEHMOOD

Unraveling Molecular, Cellular and Cognitive Defects in the Mouse Model for Mental Retardation Caused by *Rsk2* Gene Mutation

Résumé

Le syndrome de Coffin-Lowry (CLS), une déficience intellectuelle liée à l'X, est causée par des mutations du gène RPS6KA3 codant pour la kinase RSK2 régulée par les facteurs de croissance. Pour comprendre les conséquences du déficit en RSK2 dans l'hippocampe nous avons effectué une comparaison des profils d'expression génique d'hippocampes de souris *Rsk2*-KO et WT. Elle a révélé l'expression différentielle de 100 gènes, codant pour des protéines agissant dans divers processus biologiques. Nous avons analysé les conséquences de la dérégulation de l'un de ces gènes Gria2 codant pour GluR2, une sous-unité du récepteur glutamate AMPA. Un niveau d'expression doublé de GluR2 a été relevé dans l'hippocampe des souris *Rsk2*-KO et les études électrophysiologiques y ont révélé une réduction des transmissions AMPAR et NMDAR. L'activité de ERK1/2 était aussi anormalement augmentée dans l'hippocampe des souris *Rsk2*-KO, ainsi que le niveau de P-Sp1. Ensemble, mes résultats ont suggéré que la surexpression de GluR2 dans les neurones déficients en RSK2, était causée par une augmentation de l'activité transcriptionnelle de Sp1 sur le gène *Gria2*, qui, elle-même, est le résultat de l'augmentation anormale de l'activité de ERK1 / 2.

Mots-clés: Coffin-Lowry Syndrome, RSK2*, Gria2,* glutamate receptor*, ERK, CREB, PC12,* Sp1

Résume en anglais

Coffin–Lowry Syndrome (CLS), an X-linked form of intellectual disability, is caused by mutations of the *RPS6KA3* gene encoding the growth factor regulated kinase RSK2. To understand the consequences of RSK2 deficiency in the hippocampus we performed a comparison of the hippocampal gene expression profiles from *Rsk2*-KO and WT mice. It revealed differential expression of 100 genes, encoding proteins acting in various biological pathways. We further analyzed the consequences of deregulation of one of these genes, *Gria2* encoding GluR2, a subunit of the glutamate AMPAR. An abnormal two-fold increased expression of GluR2 was found in the hippocampus of *Rsk2*-KO mice. Electrophysiology studies showed a reduction of basal AMPAR and NMDAR mediated transmission, in the hippocampus of *Rsk2*-KO mice. Activity of ERK1/2 was also abnormally increased in the adult hippocampus of *Rsk2*-KO mice. P-Sp1 level was also significantly higher in RSK2 deficient cells. Together, my results suggested that over expression of GluR2 in RSK2 deficient cells, is caused by increased Sp1 transcriptional activity on the *Gria2* gene, which, itself, is the result of ERK1/2 increased signaling.

Key Words: Coffin-Lowry Syndrome, RSK2*, Gria2,* glutamate receptor*, ERK, CREB, PC12, Sp1*