JULIUS-MAXIMILIANS UNIVERSITY OF WÜRZBURG

From behavioral to neurobiological characterization of *Rsk2*-knockout mice as an animal model for Coffin-Lowry syndrome

„Vom Verhalten bis zur neurobiologischen Charakterisierung von Rsk2-defizienten Mäusen, einem Tiermodell für das Coffin-Lowry Syndrom“

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg, Section Neuroscience.

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Würzburg, 2018

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Date of Public Defence:

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Abstract

Coffin-Lowry syndrome is a rare syndromic form of X-linked mental retardation caused by heterogeneous loss-of-function mutations in the gene *RPS6KA3* that encodes the RSK2 protein. Clinical features are delayed motor development, small height, progressive skeletal malformations and mental retardation.

Rsk2 deficiency affects behavioral, cellular and molecular functions. To characterize and investigate how this deficiency affects these functions, we made a series of experiments using *Rsk2*-deficient mice as the animal model for Coffin-Lowry syndrome.

We applied a battery of behavioral tests and included the use of the IntelliCage for the first time as a behavioral paradigm to study anxiety-like behavior and depression-like behavior in *Rsk2*-deficient mice. Results from the conventional behavioral tests and from the IntelliCage indicate that *Rsk2*-deficient mice may have an anti-anxiety and anti-depressive phenotype.

We evaluated in *Rsk2* deficient mice the relative gene expression of a set of genes coding for proteins related to RSK2 which are involved in fear memory, synaptic plasticity, neurogenesis, learning, emotional behavior and stress. We found gene expression alterations in the prefrontal cortex and striatum. These results suggest that RSK2 may be involved in the expression of the genes.

RSK2 is known to be related to monoamine neurotransmitter function. We measured the levels of dopamine, serotonin and noradrenaline/norepinephrine and their metabolites in different brain regions of *Rsk2*-deficient mice. We found differences in the dopaminergic and noradrenergic systems suggesting an increased or decreased activity of these neurotransmission systems as a result of *Rsk2* deficiency.

Adult neurogenesis is a form of neuronal plasticity and a multi-step process of cell development. We explored if this form of neuronal plasticity was affected by *Rsk2*-deficiency. Our results indicate that adult hippocampal neurogenesis is not influenced by lifelong *Rsk2* deficiency. It would be worth to analyze in the future other aspects of neuroplasticity.

We have confirmed, that behavioral characteristics of *Rsk2*-deficient mice make them an interesting model to study the Coffin-Lowry syndrome by extending the behavioral characterization on the emotional level. Furthermore, we have extended the characterization of the model on a molecular level, opening new opportunities to study and understand the pathophysiological basis of the Coffin-Lowry syndrome.

Keywords: Coffin-Lowry syndrome, RSK2, behavior, gene expression, monoamines.

Abstrakt

Das Coffin-Lowry Syndrom ist eine seltene syndromale Form X-gebunden vererbter geistiger Behinderung, verursacht durch heterogene *loss of function* Mutationen im *RPS6KA3*-Gen, welches für das RSK2-Protein kodiert. Klinische Charakteristika sind eine verzögerte motorische Entwicklung, eine geringe Körpergröße, fortschreitende Skelett-Malformationen und geistige Behinderung.

Die *Rsk2*-Mutation hat einen Einfluss auf das Verhalten, auf zelluläre und molekulare Funktionen. Um zu charakterisieren und zu untersuchen, wie diese Defizienz diese Funktionen beeinflusst, führten wir eine Reihe von Experimenten durch und verwendeten *Rsk2*-defiziente Mäuse als Tiermodell für das Coffin-Lowry Syndrom.

Wir wandten eine Reihe von Verhaltens-Tests an, einschließlich erstmals den IntelliCage als ein Verhaltensparadigma, um Angst-ähnliches und Depressions-ähnliches Verhalten in *Rsk2*-defizienten Mäusen zu untersuchen. Ergebnisse konventioneller Verhaltenstests und aus dem IntelliCage sprechen dafür, dass *Rsk2*-defiziente Mäuse einen „anti-ängstlichen“ und „anti-depressiven“ Phänotyp haben.

Wir haben bei *Rsk2*-defizienten Mäusen die Expression einer Reihe von Genen untersucht, die für Proteine kodieren, die mit RSK2 in Zusammenhang stehen und darüber hinaus eine Bedeutung für das Angst-Gedächtnis, synaptische Plastizität, Neurogenese, Lernen, emotionales Verhalten und Stress haben. Im präfrontalen Kortex und Striatum konnten wir Genexpressionsunterschiede zwischen Rsk2-Wildtyp- und Knockout-Mäusen detektieren. Diese Ergebnisse sprechen dafür, dass RSK2 eine Rolle bei der Expression dieser Gene spielt.

Es ist bekannt, dass RSK2 eine Rolle für die monoaminerge Neurotransmitter-Funktion spielt. Deshalb haben wir die Konzentration von Dopamin, Serotonin und Noradrenalin/Norepinephrin und ihrer Metaboliten in verschiedenen Gehirnregionen von *Rsk2*-defizienten Mäuse untersucht. Wir haben Unterschiede im dopaminergen und noradrenergen System gefunden, was auf eine gesteigerte oder verminderte Aktivität dieser Neurotransmittersysteme als Folge der *Rsk2*-Defizienz hinweist.

Adulte Neurogenese ist eine Form neuronaler Plastizität und ein mehr-stufiger Prozess zellulärer Entwicklung. Unsere Untersuchungen der adulten Neurogenese im Hippocampus zeigten, dass sie nicht durch eine lebenslange *Rsk2*-Defizienz beeinflusst wird. In Zukunft wäre es jedoch sinnvoll, andere Aspekte der Neuroplastizität zu analysieren.

Durch unsere Verhaltensstudien wurde die Charakterisierung der *Rsk2*-defizienten Mäuse vor allem im emotionalen Bereich stark erweitert, wodurch wir bestätigen konnten, dass diese Mauslinie ein interessantes Modell zur Untersuchung des Coffein-Lowry Syndroms ist. Darüber hinaus haben wir die Charakterisierung des Modells auf der molekularen Ebene erweitert und damit neue Möglichkeiten eröffnet, die pathophysiologische Grundlage des Coffin-Lowry Syndroms zu studieren.

Übersetzung ins Deutsche: Dr. Dr. Matthias Fischer

Schlüsselwörter: Coffin-Lowry Syndrom, RSK2, Verhalten, Genexpression, Monoamine

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Register of Abbreviations

°C	:	celsius degree
µl	:	microliter
µM	:	micromolar
5-HIAA	:	5-hydroxyindoleacetic acid
5-HT	:	serotonin (5-hydroxytryptamine)
5-HT	:	serotonin
5htr1a	:	5-Hydroxytryptamine receptor 1A
ADHD	:	attention deficit/hyperactivity deficit
Adra2a	:	adrenoceptor alpha 2A
AMPA	:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AN	:	adult neurogenesis
Bdnf	:	brain-derived neurotrophic factor
Bdnf1	:	brain-derived neurotrophic Factor 1
BrdU	:	5-bromo-2`-deoxyuridine
BSA	:	bovine serum albumin
BT	:	behavioral tests
cDNA	:	complementary desoxyribonucleic acid
CE	:	cerebellum
CLS	:	coffin-Lowry syndrome
cm	:	centimeter
Comt	:	catecol-O-Metiltransferasa
Cq	:	quantification cycle
CREB	:	responsive-element-binding protein
Crh	:	corticotrophin releasing factor/hormone
Crhr1	:	corticotropin releasing hormone receptor 1
D1	:	dopamine receptor 1
D2	:	dopamine receptor 2
DA	:	dopamine
DAB	:	diaminobenzidin
DAT	:	dopamine transporter
DCX	:	doublecortin
DG	:	dentate gyrus
DNA	:	desoxyribonucleic acid
DOPAC	:	3,4-Dihydroxyphenylacetic acid
Drd2	:	dopamine Receptor D2
EPM	:	elevated plus maze
ERK	:	extracellular regulated kinase
F	:	forward
Fkbp5	:	immunophilin protein FK506 binding protein 5
GCL	:	granule cell layer
Gdi2	:	guanosine diphosphate dissociation inhibitor 2
gDNA	:	genomic desoxyribonucleic acid
gDNA	:	chromosomic DNA
h	:	hour
H3PO4-DTPA	:	diethylene triaminepentra acid buffer
HIAA	:	5-Hydroxyindoleacetic acid
HIPPO	:	hippocampus
HPA	:	hypothalamic-pituitary-adrenal axis
HPLC	:	high-performance liquid chromatography
HVA	:	homovanillic acid
HYP	:	hypothalamus
Igf1	:	insulin-like growth factor

IRC	:	inter-run calibrators
KO	:	knock out
LDB	:	Light dark box
MAOA	:	monoamine oxidase A
MAOB	:	monoamine oxidase B
Mapk1/Erk2	:	mitogen-activated protein kinase 1
Mapk3/Erk1	:	mitogen-activated protein kinase 3
MCM2	:	minichromosome maintenance complex component 2
MHPG	:	3-Methoxy-4-hydroxyphenylglycol
min	:	minute
mm	:	millimeter
mPFC	:	medial prefrontal cortex
mRNA	:	messenger ribonucleic acid
MTC	:	motor cortex
NE	:	noradrenaline/norepinephrine
ng	:	nanogram
NGS	:	normal goat serum
NHS	:	normal horse serum
NRT	:	samples lacking the enzyme reverse transcriptase
NTC	:	control sample lacking nuclei acid template
OF	:	open field
PBS	:	Phosphate-buffered saline
PCR	:	polymerase chain reaction
PFA	:	paraformaldehyde
PFC	:	prefrontal cortex
PST	:	Porsolt swim test
qRT-PCR	:	quantitative real-time polymerase chain reaction
R	:	reverse
RG	:	reference genes
RNA	:	ribonucleic acid
RSK	:	90-kDa ribosomal S6 kinase
RSK2	:	90 kDa ribosomal protein S6 kinase 3
RT	:	room temperature
s	:	second
S.E.M.	:	standard error of the mean
SGZ	:	sub granular zone
STR	:	striatum
Tbp	:	TATA box binding protein
TBS	:	tris buffered saline
TG	:	target gene
WAGR	:	Wilms tumor, Aniridia, Genitourinary anomalies, and Retardation syndrome mental
W-o-L	:	window of linearity
WT	:	wild type
ZEMM	:	Centre of Experimental Molecular Medicine of the University of Würzburg, Germany

1 Introduction

1.1 Coffin-Lowry syndrome

Coffin-Lowry syndrome (CLS) (MIM 303600) is a rare syndrome form of X-linked mental retardation (Delaunoy et al. 2001; Jacquot et al.1998; Pereira et al. 2009) caused by heterogeneous loss-of-function mutations in the gene *RPS6KA3* encoding 90-kDa ribosomal S6 kinase (RSK2) (Jacquot et al., 1998; Trivier et al. 1996). The syndrome was first described by Coffin et al. (1966) and Lowry et al. (1971) in parallel and based on the examination of two different families. Temtamy et al. (1975) found the clinical features described by these authors in three new families and distinguished the condition with the eponymous title “Coffin-Lowry-Syndrome” (Hanauer and Young, 2002; Hunter 2002). The incidence is not precisely known; nevertheless the actual prevalence is estimated to be 1:50000 to 1:100000 (Pereira et al. 2009). CLS affects various ethnic groups (Delaunoy et al. 2001). The life expectancy in males affected with CLS ranges from about 18 to 39 years and is reduced compared to affected females who can survive until old age (Hanauer and Young, 2002; Pereira et al. 2009).

1.1.1 Clinical features of CLS

Being an X-linked syndrome the clinical features are different in males and females. Heterozygous females are less severely affected. The clinical features in new-born affected males are hypotonia and hyper laxity of joints, tapering fingers, usually with normal range in growth parameters. In early childhood facial abnormalities start to become visible. Around the second year of life these facial characteristics are accentuated and a distinguishable facial phenotype for the syndrome is established. Growth retardation, psychomotor development retardation, sensor neural hearing deficit and microcephaly have been consistently reported in the first years of life, but are not always present (Delaunoy et al. 2001; Pereira et al. 2009).

Small height and stooped posture are among clinical features in affected adult males (Pereira et al. 2009) with an average height of 143 cm (range 115-158 cm) in 80% of the patients (Hanauer and Young, 2002). As discussed, the syndrome has a distinguishable facial appearance characterized by prominent forehead, large and prominent ears, thick lips (large mouth), high narrow palate, abnormal dental position and peg-shaped incisors (Hanauer and Young, 2002; Jacquot et al. 1998; Pereira et al. 2009).

A very typical feature and a clinical sign that supports diagnosis are soft, fleshy, large and hyper extensible hands with short puffy tapered fingers (Hanauer and Young, 2002; Pereira et al. 2009).

Progressive skeletal malformations are present including delayed bone development and spinal kyphosis/scoliosis in 80% of male patients A small number of patients has been reported to have cardiac mitral dysfunction (Hanauer and Young, 2002; Pereira et al. 2009).

With regards to neurological features in males, the motor development and walking age is delayed. From childhood to the teenage with approximately 20% of incidence, male patients present “drop attacks” or stimulus-induced drop episodes in form of brief collapses without loss of consciousness. Other features are epileptic seizures affecting around 5% of male patients. The personality of the patients has been reported to be cheerful, easy going, and friendly (Hanauer and Young, 2002; Pereira et al. 2009).

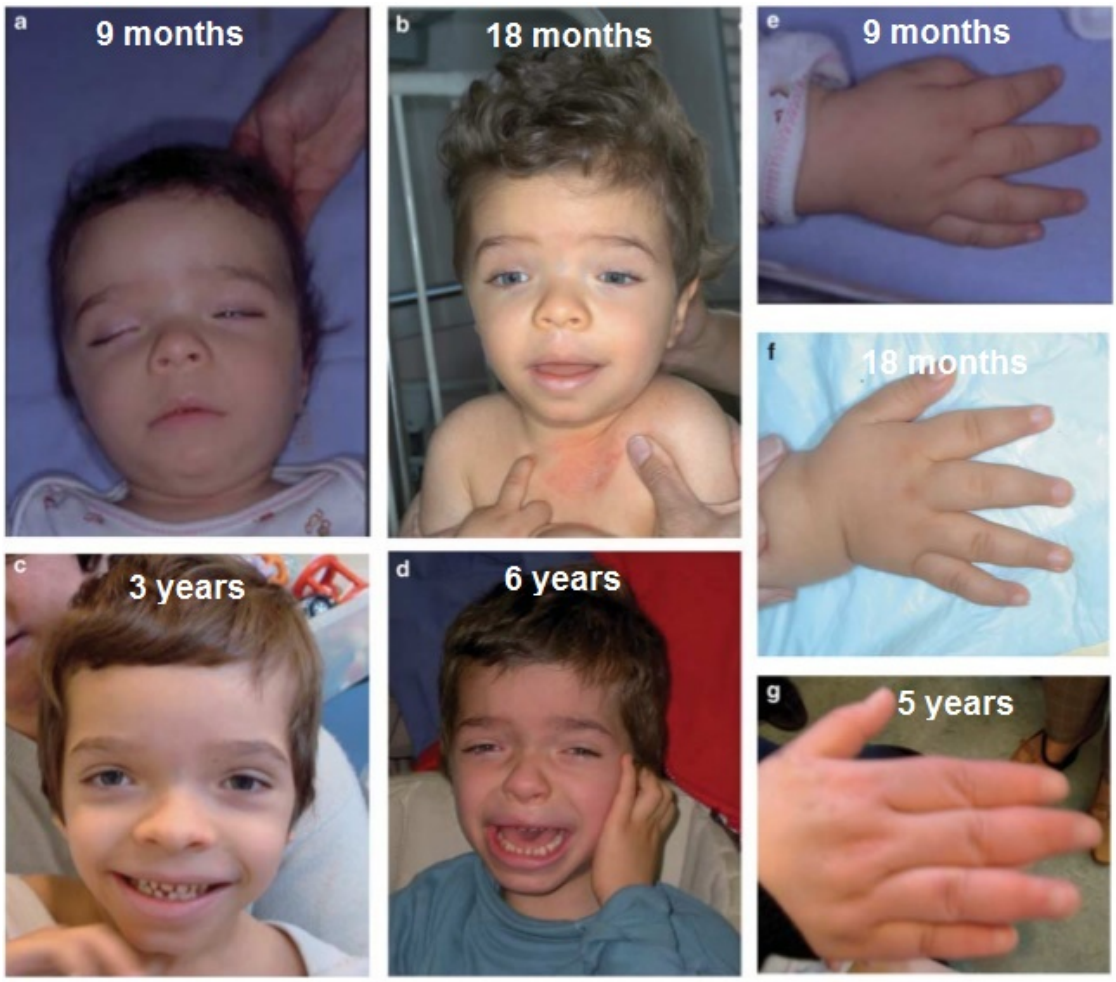


Figure 1: **Children affected with CLS.** Image taken from Pereira et al. (2009) and manually edited by using Paint software. The patient carries an *RPS6KA3* intragenic duplication. Facial evolution during infancy. At 9 (a) and 18 months (b), at 3 (c), and 6 years (d). Picture of the hand of the same patient, showing typical tapering fingers evolution at 9 (e) and 18 months (f), and at 5 years (g). It is possible to observe typical facial appearance.

A cardinal feature of this syndrome is the mental retardation. Most of the males diagnosed with CLS are severely affected with cognitive deficiencies (Zeniou et al. 2002) presenting IQ scores between 16 and 60 showing a moderate to profound mental retardation (Delaunoy et al. 2001; Hanauer and Young, 2002; Pereira et al. 2009). Due to this condition the development of the speech is impaired in most of the cases having a very limited vocabulary but when there is hearing impairment they never speak (Hanauer and Young, 2002; Pereira et al. 2009).

The manifestation of the syndrome in females is less severe than in males. The facial characteristics are milder and not always fully expressed (Hanauer and Young, 2002; Jacquot et al. 1998; Pereira et al. 2009). Females show less severe mental retardation with slightly reduced to normal intelligence (Simensen et al. 2002). It is possible to find affected females with short stubby hands, normal appearance and intelligence or with the typical facial dimorphism with moderate mental retardation.

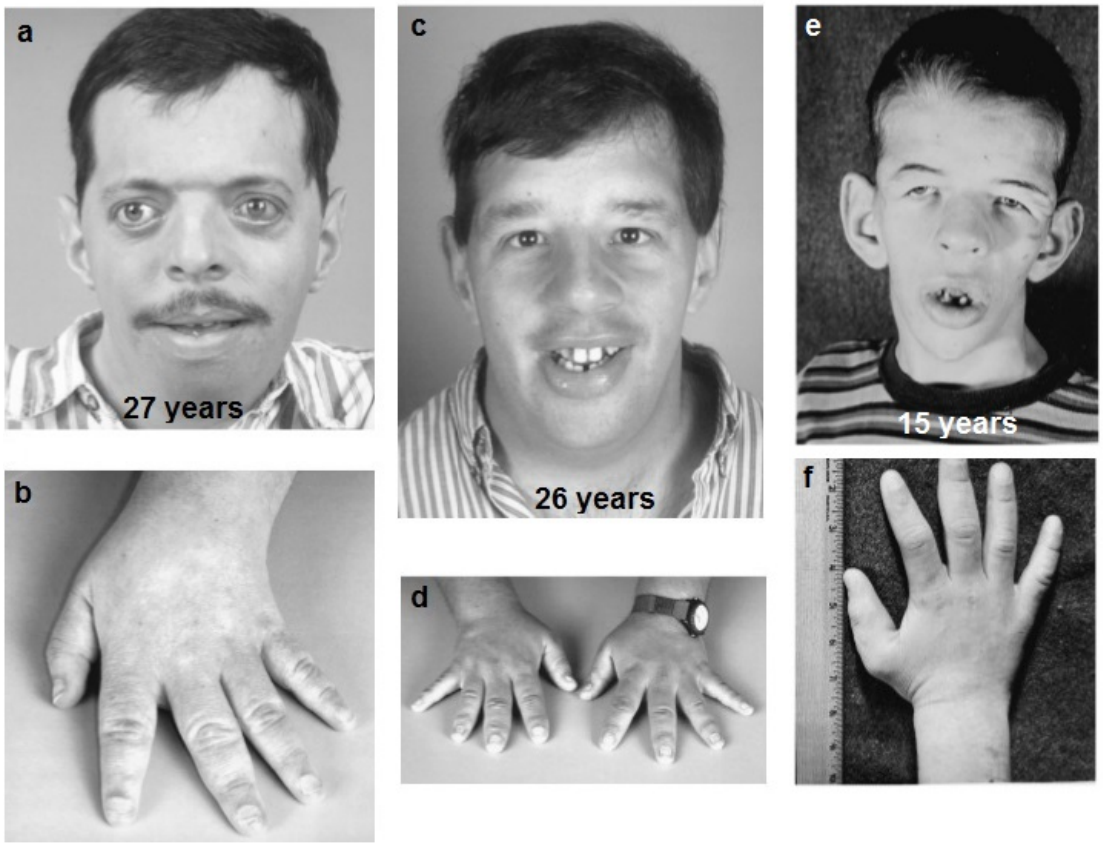


Figure 2: **Adults affected with CLS.** Image a, b, c and d taken from Hunter (2002) and manually edited by using Paint software. Image e) and f) taken from Coffin (2003) and manually edited with Paint software. The face of an affected 27 years old male (a) shows prominent eyes, full lips, small jaw, short nose, and large ears. Hands of the patient (b) showing full, fleshy, tapering hands, characteristics of the syndrome. Face of patient (c) at 26 years old presenting mild facial signs of the syndrome. His hands also present the characteristic form of the syndrome. Face of 15 years old patient (e) presenting an accentuated characteristic appearance of the syndrome, his hands are also typical of CLS.

Obesity and psychiatric disorders such as depression and schizophrenia have been found in female carriers (Hanauer and Young, 2002; Pereira et al. 2009; Sivagamasundari et al. 1994). However, the prevalence of emotional disorders in these patients as well as in males and females is presently unknown.

1.1.2 Genetic background of CLS

The loss of function mutations in the *RPS6KA3* gene which encodes the protein kinase RSK2 (ribosomal S6 kinase) are the cause of CLS (Trivier et al. 1996). The coding region of the gene which maps to Xp22.2 is split into 22 exons (Jacquot et al. 1998). The gene presents a strong allelic heterogeneity with more than 140 different inactivating mutations already found in patients with CLS (Delaunoy et al. 2006).

Around 80% of the male patients diagnosed with CLS do not have a family history of the syndrome and 30% present one or more family member affected with CLS (Pereira et al. 2009). CLS is X-linked and dominant in males whereas females will be carriers with an elevated propensity to present a mild phenotype of CLS (Pereira et al. 2009).

Mutations of *Rsk2* causing a reduction or a loss of the RSK2 kinase activity (Pereira et al. 2009) are present throughout all exons (except 1 and 2) not forming clusters and most mutations are unique and observed just in a single family. 2/3 of the *Rsk2* mutations cause a premature translation termination causing a complete loss of function of the aberrant allele and 1/3 of the *Rsk2* mutations cause the substitution of one amino acid for another (Hanauer and Young, 2002). Around 30-40% of the mutations correspond to missense mutations, 20-30% is short deletion or insertion events, 20% are splicing errors and 15-20% is nonsense mutations. Up to now only two large duplications and five intragenic deletion had been reported (Hanauer and Young, 2002).

With regards to the relation of the genotype and phenotype of patients with CLS a consistent relationship between specific mutations and severity expression of characteristic features has not been established (Hanauer and Young, 2002; Pereira et al. 2009; Simensen et al. 2002). Besides, there is not a correlation of the extent of protein defect with the severity of the expression of the disease. However, individuals carrying missense mutations have been related with mild phenotypes (Pereira et al. 2009) compared to the severity of symptoms in individuals having truncating mutations of *Rsk2* (Hanauer and Young, 2002).

These patients with missense mutation and mild symptomatic presence demonstrate that the residual enzymatic activity plays an essential role in the determination of the severity of the symptoms. Nevertheless, it is not possible to apply this observation to all missense mutations, because a high number of patients have not been properly clinically and genetically evaluated. Studies of families with many affected members reveal interfamilial variability of the severity of the syndrome or presence of rare features (Hanauer and Young, 2002).

1.1.3 Diagnosis of CLS

The diagnosis of patients with CLS is usually done based on the clinical and radiological features presented. However, the clinical features vary in severity causing difficulties in the diagnosis, which is worsened by the usual presence of uncommon associated features, the unclear presence of the typical characteristic in young children, the milder expression of the syndrome in females and high rate of sporadic cases (Pereira et al. 2009; Merienne et al.1999).

Western blot analyses are very useful to identify patients lacking the RSK2 protein product. Western blot samples are taken from lymphocyte protein extracted from fresh blood samples or from a lymphoblast or a fibroblast cell line. This test can detect up to 70% of the mutated RSK2 protein (Hanauer and Young, 2002). However, in vitro kinase assay can be used to detect mutations. This method also provides information about residual enzymatic activity which makes it a certain method for diagnose. Both tools can be used in the prenatal state for early diagnosis and can be performed using cultured amniocytes in which the RSK2 protein is detectable. In vitro kinase assay can also be used as a detection test for female carriers (Pereira et al. 2009). However, the *Rsk2* mutation analysis provides the confirmation of CLS (Pereira et al. 2009; Merienne et al. 1999).

Around 50% of the patients have been found to have no mutations in the *RPS6KA3* gene after mutation screening and even after Western blot as reported by Pereira et al. (2009) giving space to misdiagnosis. In patients screened by single-strand conformation polymorphism (SSCP) analysis no mutation was found in 66% of them,

even when they were clinically diagnosed with CLS. Nevertheless, it is possible that some mutations were missed by this analysis (Hanauer and Young, 2002). Genetic heterogeneity is very high among patients that phenotypically resemble CLS. In some cases these clinical features are present in patients without *Rsk2* mutation. Also, but not frequently *Rsk2* mutations do not cause the characteristic phenotype (Pereira et al. 2009).

The most reliable clinical feature of CLS is the presence of tapering fingers and is used as a cardinal feature to diagnose in infancy, but this feature is also present in other mental retardation syndromes that might lead to misdiagnosis: Borjeson syndrome (MIM 301900) and Prader-Willi syndrome (MIM 176270). Other syndromes often confused with CLS are: ATR-X syndrome (ATR-X; OMIM 300032) FG syndrome (OMIM 309550), Williams syndrome (OMIM 194050), Noonan syndrome (OMIM 163950) and Pitt-Hopkins syndrome (OMIM 610954) (Chen et al. 2014; Pereira et al. 2009).

Regarding the proper care and treatment, the early diagnosis is very important to proceed with an adequate management of the patients. There is not a specific treatment for patients with CLS at the time. However, the monitoring of hearing, dental, visual and skeletal aspects are very important to improve the quality of life and development of the patients and to determine the need of surgical corrections in time. In the case of drop attack episodes the use of medication (valproate, clonazepam and selective serotonin uptake inhibitors) and wheelchairs are recommended (Pereira et al. 2009).

1.2 RSK2 protein

The 90 kDa ribosomal S6 kinases (RSKs) belong to a family of Ser/Thr kinases located downstream of the mitogen-activated protein kinase/extracellular signal-regulated kinases MAPK/ERK pathway (Anjum and Blenis, 2008; Mehmood et al. 2011). In mammals there are four isoforms (RSK1, RSK2, RSK3 and RSK4) product of different genes with a very similar structure (De Cesare et al. 1998) and are widely expressed in various cell types. RSKs orthologs have been found in mouse, rat, chicken, *Xenopus laevis*, and *Drosophila* (Hauge and Frodin, 2006). Therefore, RSK proteins are highly conserved in evolution which suggests that they have an important role.

All the RSK proteins have two functional kinase catalytic domains: an N-terminal kinase that belongs to the AGC kinase family, and a C-terminal kinase that belongs to the CamK family connected by a 100-amino-acid linker region. Also the RSKs share 75–80% amino-acid identity. These protein kinases are directly activated by extracellular signal regulated kinase 1 and 2 (ERK1/2) in response to growth factors, neurotransmitters, hormones, polypeptides, phosphorylate cytosolic and nuclear targets associated to cell proliferation, cell survival, cell growth and motility (Anjum and Blenis, 2008; Hauge and Frodin, 2006; Pereira et al. 2009).

In the brain of both mice and humans RSK2 is found highly expressed in neocortex, hippocampus and cerebellar Purkinje cells, both areas and cells with an elevated synaptic activity (Pereira et al. 2009; Zeniou et al. 2002). An alteration of more than 100 genes implicated in many biological pathways of *Rsk2*-deficient mice in the hippocampus is reported, these data suggest a pivotal role of RSK2 in brain function (Mehmood et al. 2011).

RSK2 activates various cytosolic proteins, translation factors and nuclear proteins (Carriere et al. 2008; Hauge and Frodin, 2006). For instance, RSK2 modulates the

MAPK-pathway, exerts an inhibitory feedback on the ERK-pathway (Hauge and Frodin, 2006), directly activates CREB and histone H3 and is linked to the transcriptional coactivator protein CREB-binding proteins (CBP) and p300 (De Cesare et al. 1998). RSK2 is necessary for epidermal growth factor-induced phosphorylation of CREB protein and the transcriptional induction of the cFos gene (De Cesare et al. 1998).

The RSK2 protein contains a C-terminal sequence that binds PDZ (postsynaptic density fraction (PSD) 95discs large ZO-1) domains (Hanauer and Young, 2002) and the interaction of RSK2 with PDZ domain-containing proteins and the phosphorylation of these proteins or the binding partners is suggested to regulate excitatory synaptic transmission (Thomas et al. 2005). It has been reported that RSK2 has a regulatory function on the G protein-coupled receptors (GPCR) signaling acting as a ‘‘tonic brake’’ on second-messenger production of various GPCRs (Sheffler et al. 2006).

RSK2 has an important role in osteoblast differentiation and participates in the regulation of collagen type I synthesis (Yang et al. 2004). The aberrant activation or overexpression of RSK2 in humans has been associated with breast cancer (Maloney et al. 2005) and prostate cancer (Clark et al. 2005).

1.3 Animal model for CLS

Two lines of *Rsk2*-deficient mice have been generated by Dufresne et al. (2001) and Yang et al. (2004) and further used as animal models to study CLS.

Dufresne et al. (2001) produced a line of *Rsk2* KO mice by the disruption of the *Rsk2* gene with the aim of studying *in vivo* the RSK2 implication in the activation of glycogen synthase by insulin. They confirmed the lack of RSK2 in *Rsk2* KO mice by RSK2 immunoblotting of muscle lysates and showed that the expression of RKS1 and RSK3 in skeletal muscle was not affected in their *Rsk2* KO mice. *Rsk2*-deficient mice exhibit an alteration in ERK and glycogen metabolism in skeletal muscle, being 10% smaller and 14% shorter compared with *Rsk2* WT mice. When the animals were tested with behavioral batteries presented a poor motor coordination, deficient cognition and learning indicating an impaired spatial learning ability. The authors concluded that *Rsk2* KO may be a good animal model for CLS (Dufresne et al. 2001).

Yang et al. (2004) developed another line of *Rsk2*-deficient mice as a model to study skeletal defects in CLS patients. The mutation used was a null allele and did not interfere in the synthesis of other RSKs. The embryos and pups of *Rsk2*-deficient mice of both sexes presented a reduced size in the skull bone (frontal, parietal and intraparietal) creating wide cranial sutures and open fontanelles at birth because of delayed mineralization. At one month of age long bone length was significantly reduced, as well as, bone mass in the vertebrae. At older ages a reduction in the osteoblast function and thickness of trabeculae was visibly. Nerveless, the bone resorption was not totally affected by the *Rsk2* absence. *Rsk2*-deficient mice presented skeletal abnormalities, delay in bone formation and a phenotype of low bone mass. These defects resemble what can be observed in patients with CLS and are the result of a defect in osteoblast function (Yang et al. 2004).

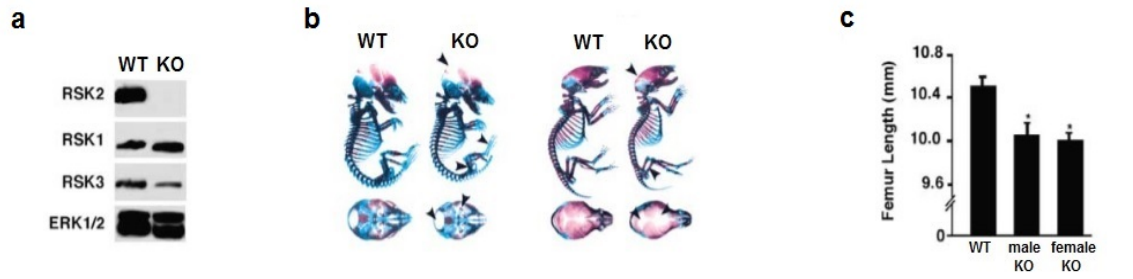


Figure 3: ***Rsk2*-deficient mice**. Image from Yang et al. (2004) and manually edited. a) Western blot analysis presenting lack of RSK2 but instead the presence of RSK1, RSK3 in Rsk2-deficient cells, and ERK1/2 as loading control. b) Skeletons staining (alizarin red/alcian blue) of embryos and at birth of *Rsk2* WT and *Rsk2* KO mice. Frontal, parietal, and interparietal bones of *Rsk2* KO mice (arrowheads in black) show a reduction compared with *Rsk2* WT. c) Significant difference in long bone length in *Rsk2* KO mice independent of the gender.

Low body weight and small size have been reported in the same *Rsk2*-deficient mice line (Dufresne et al. 2001; El-Haschimi et al. 2003). Nevertheless, the low body weight is caused by a progressively reduced accumulation of adipose tissue (El-Haschimi et al. 2003). Yang et al. (2004) found elevated fasting insulin and impair glucose tolerance.

In the hippocampus of *Rsk2*-deficient mice an elevated phosphorylation of ERK1/2 is reported. This aberrant ERK1/2 signaling is capable to affect various neural functions and having a significant role in the cognitive dysfunction of *Rsk2*-deficient mice (Schneider et al. 2011). Also, in the hippocampus of *Rsk2* KO mice a basal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated transmission was found to be decreased and this defect in AMPA neurotransmission and plasticity may cause the cognitive disabilities in *Rsk2*-deficient mice and possibly in patients with CLS (Mehmood et al. 2011).

In the dentate gyrus of the hippocampus of *Rsk2* KO multi-level alterations were found, as impaired synaptic transmission, decreased network excitability, decreased AMPA and (NMDA) conductance and dendritic spines morphology alteration (Morice et al. 2013).

In the cortex of *Rsk2*-deficient mice a significantly increased level of dopamine has been found together with the overexpression of the long dopamine receptor form (DrD2L) and the dopaminergic transporter (DAT). The elevated level of dopamine corresponds to an increased ERK activity on tyrosine hydroxylase. This hyperdopaminergia at the cortical level may be related to the cognitive alterations found in these mice according to Pereira et al. (2008). The normal development of cortical precursors in *Rsk2*-deficient mice was reported together with a reduction in the differentiation of cortical radial precursors into neurons (Dugani et al. 2010).

An extended battery of cognitive and non-cognitive behavioral tests was applied to *Rsk2*-deficient mice for the first time by Poirier et al. (2007) including the following areas: motor coordination (inverted grid test and wire suspension test), exploration and emotional reactivity (open field, elevated O-maze, elevated plus-maze, emergence test and object exploration) and learning and memory (spontaneous alternation in cross maze, open field activity and object recognition, conditioned taste aversion, spatial working memory procedure on the radial maze, hole-board task and water-maze).

Regarding results of the cognitive behavioral tests *Rsk2*-deficient mice showed a normal behavior and appeared to be physically healthy. *Rsk2* KO mice reveal an impaired acquisition of a place navigation task in the water-maze, reduced spatial reference memory (after an interval of 9 days), mild deficits in spatial working memory, preserved spatial reference memory (24 hours interval) and recognition memory for novel objects. Deficits in the acquisition in the water-maze were prominent but mice were able to learn the task with delay after additional training.

According to the results of the non-cognitive behavioral tests *Rsk2* KO mice showed normal results in sensorimotor tests and loco motor activity indicating that *Rsk2* KO mice do not present an increased anxiety-like behavior and are not hyperactive, but they could present a hyper-responsiveness to environmental stimuli changes. The authors also state that *Rsk2*-deficient mice are a good animal model to study CLS (Poirier et al. 2007).

In summary, *Rsk2* KO presented delayed acquisition of spatial reference memory task, mild impairment of spatial working memory, long-term spatial memory, sign of disinhibiting in exploratory activity and difficulties to adapt to new environments (Poirier et al. 2007).

Darcq et al. (2011) investigated the role of RSK2 in the ability to associate a context with an aversive stimulus by the application of lithium-induced conditioned place aversion learning. This learning was found impaired in *Rsk2* KO demonstrating that the capability to associate the lithium/aversive stimulus with the context is altered. Also, a high expression of RSK2 was found in the habenula indicating that RSK2 signaling in the habenula is pivotal for the association of the drug with the context.

Morice et al. (2013) tested *Rsk2*-deficient mice in different fear memory paradigms that engage hippocampal function. The *Rsk2* KO presented a selective deficit in the consolidation of trace fear memory and a mild deficit in reconsolidation of contextual fear memory. These results highlight the sensitivity of hippocampal-dependent memory in this genotype.

Object recognition memory is suggested to be subject to reconsolidation after recall. According to Davis et al. (2011) *Rsk2* KO mice present an accentuated deficit in post reactivation of long-term memory, demonstrating that object-place memory is *Rsk2*-dependent reconsolidation process followed by memory reactivation. And also, indicating that RSK2 is associated to object-place memory reconsolidation more than to object-place memory consolidation.

Knockout mice for other isoforms of RSK have been created *Rsk1* KO, *Rsk3* KO and *Rsk1*/*Rsk2*/*Rsk3* triple-KO mice seem to be viable (Anjum and Blenis, 2008) but even this triple-knockout does not display an clear phenotype (Dumont et al. 2005).

In *Drosophila* mutants for only one existing *Rsk* gene (*S6kII*) are available. *S6kII* is homologous to the human *RSK2* gene. The total deletion of this gene generates short term memory impairments in spatial learning and olfactory learning (Putz et al. 2008). RSK has an inhibitory role in the Ras-ERK pathway in *Drosophila* by anchoring ERK in the cytoplasm (Kim et al. 2006).

Fischer et al. (2009) investigated the function of RSK at synapse level on the neuromuscular junction in *Drosophila* larvae indicating that RSK has a negative effect on the button formation of neuromuscular junction in *Drosophila* larvae by the inhibition of ERK signaling.

In fly neuromuscular system loss of RSK function can induce defects in moto neurons and neuromuscular junction. It also affects ERK signaling indicating the essential role of RSK for normal synaptic morphology and function, as well as, in anterograde axonal transport (Beck at al. 2015).

1.4 Aim of the thesis

Coffin-Lowry syndrome is caused by loss of function mutations in the *RPS6KA3* gene that encodes the RSK2 protein. This mutation affects patients at physical and cognitive levels. Skeletal alterations and mental retardation are present in most patients. Information about clinical features has been extensively reported but information about the emotional abilities of the patients is not known.

Rsk2-deficient mice have been used as an animal model to study CLS. Reports indicate that the defective cognitive phenotype should be a result of molecular and cellular alterations (Mehmood et al. 2011). It has been observed that *Rsk2* deficiency affects behavioral, cellular and molecular mechanisms in *Rsk2*-deficient mice, but how these mechanisms are linked is not well known. We applied a series of experiments to investigate *Rsk2* deficiency, starting from behavioral to neurobiological studies.

Different data has provided evidence that RSK2 plays a similar role in the mental function of humans and mice (Poirier et al. 2007). Behavioral testing allows access to information about the mental functioning in mice and behavioral experiments had been done in *Rsk2*-deficient mice most of them focused on cognitive areas. Information regarding the emotional peculiarities in this animal model is lacking, as well as about CLS patients. To investigate this phenomenon, we applied a battery of different behavioral tests with the objective of study anxiety-like behavior and depression-like behavior in *Rsk2*-deficient mice. And finally, a glucocorticoid analysis was performed to explore if the behavioral battery applied was able to cause stress in the *Rsk2*-deficient mice.

Genes associated with exocytosis, mental retardation, apoptosis, cell differentiation, cytoskeleton organization, translation regulation and genes encoding ion channel sub-units have been analyzed in the hippocampus of *Rsk2*-deficient mice. The expression of these genes shows a significant alteration and these alterations may have a possible contribution to cognitive disabilities of patients with Coffin-Lowry syndrome (Mehmood et al. 2011). Our objective was to study genes coding for proteins activating RSK2, involved in fear memory, synaptic plasticity, neurogenesis, learning, emotional behavior and stress that may be possibly associated to intellectual disability in CLS. In order to achieve this goal, we applied quantitative real-time PCR (qRT-PCR) in five different brain regions in *Rsk2*-deficient mice.

In the cortex of *Rsk2*-deficient mice an increased level of dopamine and the expression of the long dopamine receptor form (DrD2L) were reported. Furthermore, RSK2 is known to be related to neurotransmitter functioning (Pereira et al. 2008). Therefore, we decided to measure the levels of dopamine, serotonin and noradrenaline/norepinephrine, and their metabolites in eight brain regions from *Rsk2*-deficient mice by using High-performance liquid chromatography (HPLC), with the objective of exploring if there where changes in the level of these monoamines in varied brain regions. This general view of monoaminergic systems functioning in the

animal model for the disease could help to understand possible underlying mechanisms in cognitive alterations in CLS patients. As these monoamines are involved in neuropsychiatric diseases, this analysis could also help to start exploring for the neurochemical basis for the mostly unknown psychiatric aspect of CLS.

Neural plasticity underlies processes like memory, learning, cellular growth and development, stress-reactivity and emotions, and adult neurogenesis is a form of neural plasticity. To understand if the neural plasticity is affected by *Rsk2* deficiency, we designed a study to analyze adult neurogenesis in *Rsk2*-deficient mice. Adult neurogenesis is the active generation of new functional neurons from adult neural stem cells. During this multi-step process, it is possible to find different states of development of the cells (Von Bohlen and Halbach, 2007). We analyzed three different cellular states by using immunohistochemistry and estimating the cellular density of each state.

All the studies were designed with the main goal to investigate how *Rsk2* deficiency affects the function of behavioral, cellular and molecular mechanisms by using *Rsk2*-deficient mice as an animal model for Coffin-Lowry syndrome.

2 Material and methods

2.1 Animals and general housing conditions

All animals used were chosen from the internal stock of *Rsk2* deficient mice bred at the Centre of Experimental Molecular Medicine of the University of Würzburg (ZEMM), Germany. These mice were generated in C57BL/6J background and genotyped by PCR as Yang et al. (2004) described. Only male mice were used to compare hemizygous y/- and y/+ *Rsk2* genotypes named *Rsk2* KO or deficient and *Rsk2* WT or wildtype mice. The genotype of the animals was blind in order to avoid bias in all the studies performed.

The mice were weaned at 21 days of age and maintained in same-sex litter-mate groups in cages with sawdust as bedding material, paper towel as nesting material, food and water were provided *ad libitum*.

A total of 85 naive mice were assigned to 4 different studies: behavioral: 12 WT and 12 KO mice with an average age of 7 months; gene expression: 11 WT and 11 KO mice with an average age of 3 months; neurotransmitters: 11 WT and 10 KO mice with an average age of 4 months and neural plasticity: 9 WT and 9 KO mice with an average age of 5 months.

In addition, glucocorticoid concentration was measured in blood plasma of the animals corresponding to the behavioral study (10 WT and 11 KO mice) and from a naïve group of mice (5 WT and 6 KO).

All behavioral tests were carried out in ZEMM, under standard laboratory conditions with 12/12 hours light/dark cycle (light on at 7 hours), 23±3°C room temperature and 55±5% humidity.

The gene expression, neurotransmitters and neural plasticity studies were carried out at the laboratory of the Department of Psychiatry, Psychosomatics and Psychotherapy at the University of Würzburg, Germany.

All animal protocols were performed after they had been approved by the Animal Ethics Boards of the University of Würzburg and of the Government of Lower Franconia (Permit number: 55.2-2531.01-47/12 and 55.2-2531.01-02/13). All procedures complied with the regulations covering animal experimentation within the EU (European Communities Council Directive 2010/63/EU).

2.2 Behavioral study

A battery of behavioral tests (BT) was applied to the experimental mice in the order as described below. An interval of 3-4 days of rest was given to the animals between the applications of the BT. These BT were recorded using an automated video tracking system (VideoMot2, TSE Systems Inc., Bad Homburg, Germany) with the exception of the Porsolt swim test. All efforts were made to minimize animal suffering and to reduce the number of animals used for the experiments.

2.2.1 Elevated plus maze (EPM)

The apparatus consists of two opposing open arms (30x5x0.25 cm) and two opposing closed arms (30x5x15 cm) extending from a central platform (5x5 cm) (Pellow et al. 1985). The device was made in black Perspex (TSE Systems), semipermeable for infrared light. The apparatus was elevated to a height of 60 cm above the floor. Mice were placed in the center area facing the open arm and the behavior was recorded for 5 min. Behavioral parameters measured are: distance travelled in open arms, distance travelled in closed arms, distance travelled in both arms (cm), time spent in the open arms, closed arms and in the center (sec), number of visits to the closed and open arms and latency to enter to the closed and open arms.

2.2.2 Light dark box (LDB)

The LDB is a transparent box divided in two compartments: a light and a dark compartment. The light area consists of a disclosed compartment (40x40x27 cm) and the dark area is a smaller enclosed compartment (40x20x27 cm) with a gate (5x5 cm) in the center to allow the animals to move between the two compartments (Bourin and Hascoet, 2003). Mice were placed in the dark compartment and the behavior was recorded for 5 min. Behavioral parameters measures considered were: time spent in the light and dark area (sec), walk distance in the light and dark area plus the total walking distance (cm), velocity (cm/sec), number of visits to each area and latency to move from one compartment to the other.

2.2.3 Open field (OF)

Square-shaped box (50x50x40 cm) made of Perspex XT, a black opaque material semi-permeable for infrared (IR) light (TSE Systems Inc., Bad Homburg, Germany). The arena was divided in the center area (25x25 cm) and the peripheral area or surrounding zone (Walsh and Cummins, 1976). Mice were placed close to the wall of the box and the behavior was recorded for 20 min. Behavioral parameters measured are: time spent in the center area (sec), time spent in the peripheral area (sec), distance travelled in the center area, distance travelled in the periphery, total distance travelled (cm), and number of vertical rears in the center area and peripheral area.

2.2.4 Porsolt swim test (PST)

This test consists of a transparent cylinder (height 20 cm, diameter 15 cm) filled with water (25°C, height 12 cm) (Porsolt et al. 1977). The mice were placed in the water and their behavior was recorded for 5 min. Behavioral parameters include: latency to start floating, floating time, climbing time and swimming time. The floating time was considered as the time they spent in physical immobility, performing minimal movements to keep the head above water.

2.2.5 Glucocorticoid study

Blood samples were taken from a group of 21 *Rsk2* mice (11 KO and 10 WT) after the performance of Porsolt swim test, a behavioral test considered as a stressor in animals. This group formed the stressed group of animals. From a naïve group of 11 *Rsk2* mice (6 KO and 5 WT), animals without any intervention, blood samples were

collected by intracardial puncture under deep anaesthesia, as the first group. The last group, formed by the non-stressed or naïve group of animals was used to determine baseline corticosterone values. The blood samples were transferred to EDTA coated blood collection tubes (GK 150 EDTA 200 µl, KabeLabortechnik, Germany) and centrifuged at 3000×g for 10 min at 4°C. After this process the plasma was separated, extracted and placed in new tubes to be stored at -80°C. This process was performed at the laboratory of the Department of Psychiatry, Psychosomatics and Psychotherapy at the University of Würzburg. The Plasma corticosterone concentrations were determined by radioimmunoassay (RIA) using the ImmuChemTM Double Antibody Corticosterone 125I RIA Kit for mice (MP Biomedicals). All standards, samples, and controls were run in duplicate concurrently. The intra and inter assay coefficients of variation were between 3.3% and 6.0%. This study was carried out with the help of co-operation partners of Dr. Angelika Schmitt-Böhrer in Maastricht.

2.2.6 IntelliCage system

All experiments using the IntelliCage (TSE-Systems GmbH, Bad Homburg) were performed by Dr. Dr. Matthias Fischer.

The cage is made of polycarbonate with the following dimensions: 20.5 cm high x 58 cm x 40 cm at top, 55 cm x 37.5 cm at bottom. It has a conditioning chamber in the four corners and each chamber contains 2 bottles for drinking on the left and right sides. The access to the bottles is restrained by a closable round opening called “door”. The pokes at these doors are registered by a light beam sensor and termed “nose pokes”. A transponder reader antenna is inserted in mice to allow a registration of every entry or “visit” to each corner. Lickometers indicate licking at bottle caps. IntelliCage Plus controlling software (NewBehavior AG, Zürich) identifies visits to a corner, registers nose poking and licking and can perform conditioning schemes based on reward by door opening, punishment by air puff application, and conditional LED stimuli. For further details please see Fischer et al. 2017.

The IntelliCage permits to tests the animals in social group and perform simultaneous and long-term experiments. As no human handling is involved the animals are not exposed to stress-induction. Also adaptation to new environment does not interfere as the animals live in the cage.

2.3 Gene expression study

The study of the comparative expression levels of different genes in diverse brain regions was performed with a group of WT and another group of KO naive mice by applying quantitative real-time PCR (qRT-PCR). qRT-PCR amplifies and simultaneously quantifies (in real time) a fragment of DNA and was performed in a thermocycler TProfessional (Biometra GmbH, D-37079 Göttingen). This procedure consists on a series of temperature fluctuations or cycles and the final product is the amplification of numerous cycles with different reaction temperatures. Measurements can be performed after each amplification cycle and the template used corresponds to a cDNA obtained by Reverse-transcription of RNA.

2.3.1 Brain tissue

Brains from naïve mice were removed from the skull to immediately be frozen in precooled isopentane and stored at -80°C until dissection procedure. The dissection process was done under a stereo microscope (Olympus) on a pre-cooled plate at - 6°C approximately. Brain tissue samples from prefrontal cortex (PFC), motor cortex (MTC), striatum (STR), hippocampus (HIPPO) and cerebellum (CE) were quickly dissected and kept frozen at -80°C until the RNA extraction procedure.

2.3.2 Quantitative real-time PCR

2.3.2.1 RNA extraction

RNA was extracted from different brain regions using the RNA isolation kit RNeasy Mini Kit (Qiagen). All the tubes (2 ml) containing the tissue samples were placed on ice and stainless-steel beads with a diameter of 5 mm (Qiagen) were inserted to each tube. An amount of 300 µl QIAzol Lysis Reagent (Qiagen) was added immediately to each sample and the tubes were shaken at 20 Hz for 60 second (sec) in the TissueLyserII (Qiagen) at 4°C. After incubation at RT for 5 min, 60 µl of chloroform (Carl Roth) were added to the samples and were shaken for 15 seconds by hand. The samples were then incubated for 10 min on ice. During this incubation process, 50 µl RNase-free H2O (Ambion) were added to the tubes. The content was carefully mixed and transferred to MaXtractHigh Density Tubes (Qiagen), previously centrifuged. The samples were centrifuged at 12000 g and 17°C for 5 min. Later, the upper liquid phase in the tube was transferred to a new 1.5 ml tube and 1.5 volumes of 100% pure ethanol (Carl Roth) were added to each sample. The solution was then mixed and transferred to an RNeasy Mini Spin Column (silica-membrane) and centrifuged at 12000 g and RT for 20 sec. The flow-through in the collection tubes was discarded after each centrifugation step. The RNeasy Mini Spin Columns were washed with 350 µl of buffer RWT (Qiagen) and then re-filled with 80 µl of DNase 1 solution (RNase-Free DNase Set, Qiagen), incubated for 15 min at RT and centrifuged at 12000 g for 20 secs to then be washed again with 350 µl of buffer RWT. RNeasy Mini Spin Columns were washed twice again with 500 µl of buffer RPE and centrifuged at 12000 g for 2 min. The RNeasy Mini Spin Columns were transferred to 1,5 ml tubes and centrifuged at 12000 g for 1 min. The RNeasy Mini Spin Columns were transferred again to a tube and 45 µl of RNase-free H2O were added, incubated for 1 min and centrifuged for 1 min, to finally store the samples at - 80°C.

2.3.2.2 Quantification of isolated RNA and purity check

The calculation of the amount of extracted RNA and revision of possible contaminations (e.g. DNA, proteins, chloroform, ethanol or chaotropic salts) of all the samples were performed by using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE 19819, USA) a full micro volume spectrophotometer for DNA, RNA and dyes quantification. The measurement parameters for RNA/DNA concentration, ratio 260/ 280 and ratio 260/ 230 served to evaluate an estimation of RNA/DNA purity and the calculation basis for the amount of RNA template further used to cDNA synthesis. A few samples were outside of the accepted ratio variation range due a possible contamination. These “contaminated” samples were excluded. (ng/uL:

sample concentration in ng/uL based on absorbance at 260 nm and the selected analysis).

The Experion™ Automated Electrophoresis System (Bio-Rad Laboratories Inc., 80939 Munich) was used for the evaluation of RNA integrity and concentration. All RNA samples were tested with the help of the Experion™ RNA Analysis Kit (Bio-Rad Laboratories Inc., 80939 Munich). The calculation of RQI (RNA quality indicator), a quantitative integrity assessment for each sample, was used as a decisive parameter to include or not the RNA samples for the qRT-PCR analysis. RQI on a scale of 1–10, 1 is highly degraded and 10 is highly intact total RNA. A RQI higher than five is accepted as good total RNA quality, as and higher than eight as perfect total RNA qualities (Fleige and Pfaffl, 2006).

2.3.2.3 Reverse transcription

By the use of the iScript™ kit (Bio-Rad, Munich, Germany) cDNA synthesis was performed on each sample of the obtained RNA following manufacturer's protocol. An amount of 1000 ng RNA per sample was synthesized as cDNA for MTC, STR, HIPPO and CE. For the PFC 800 ng RNA per sample was used for the synthesis of cDNA. In addition to all the samples, another cDNA synthesis was performed to generate five IRC controls (inter-run calibrators) for each brain region, NRT controls (samples without the enzyme reverse transcriptase) and NTC controls (samples without any nucleic acid) to verify the absence of genomic DNA. A master mix with the 5 x iScript reaction mixes with the enzyme reverse transcriptase was added to all the samples. To equilibrate all samples with the same volume at 20 µl Nuclease-free H2O was inserted. Finally, cDNA samples were stored in a freezer at -20°C.

2.3.2.4 Primer pairs for quantitative real-time PCR

The primer pairs chosen for this study were purchased as QuantiTect Primer assays from Qiagen or were self-designed as is show in the table legend 1. The sequence for design was taken from UCSC genome browser and oligonucleotide primers were synthesized by Metabion International AG. The primer pairs tested that showed an irregular melt curve or the presence of genomic DNA were excluded and replaced by other primer pair. Primer pairs were designed as intron-spanning to avoid the detection of gemonic DNA in the cDNA samples. The list of primer pairs used for qRT-PCR is shown in the table below.

Table legend 1: **Primer pairs.** The name of the gene, its abbreviation and the GenBank accession numbers are indicated. The amplicon length accepted was between 70 to 150 bp. The intron-spanning is indicated (yes/no). Finally the primer sequence (direction 5′ to 3′) forward and reverse is given, with the exception of the purchase primers pairs, which are not declared. Forward: F; reverse: R.

Primer pairs			
Gene: Name and GenBank accession	Ampli-con length	Intron-spanning	Primer Sequence: 5′ to 3′
5-Hydroxytryptamine Receptor 1A (5htr1a); NM_008308.4; Target gene	115 bps	No	F: AACCAGTTTTGTGTCCTCTCA R: AGCACCTAAATAATTTTCTTCTCTG
Adrenoceptor Alpha 2A (Adra2A); NM_007417.4; Target gene	112 bps	No	F: TTCTTTTTCACCTACACGCTCA R: TGTAGATAACAGGGTTCAGCGA
Brain-derived neurotrophic factor transcript variant 1 (Bdnf1); NM_007540.4; Target gene	94 bps	Yes	F: AGTCTCCAGGACAGCAAAGC R: TGCAACCGAAGTATGAAATAACC
Catechol-O-Methyltransferase (Comt); NM_001111063.1; Target gene	109 bps	Yes	F: ACCGCTACCTTCCAGACACA R: GCCAGGAAGTCAGGGGTTC
Corticotrophin Releasing Hormone (Crh); NM_205769.3; Target gene	90 bps	Yes	F: GAGGCATCCTGAGAGAAGTCCC R: CAGCCGCATGTTAGGGGC
Corticotrophin Releasing Hormone Receptor 1 (Crhr1); NM_007762.5; Target gene	115 bps	Yes	F: GCCTTTTTCTACGGTGTCCG R: CGTTGAGAATCTCCTGGCACT
Dopamine Receptor D2 (Drd2); NM_010077.2; Target gene	113 bps	Yes	F: TGAATTTCCACTCACCCACC R: GCAGACCACCACCAACTACC
FK506 binding protein 5 (Fkbp5); NM_010220.4; Target gene	118 bps	Yes	F: AACCCAAACGAAGGAGCAAC R: TCGGAATGTCGTGGTCTTCT
Insulin-like growth factor-1 (Igf1); NM_010512; Target gene	115 bps	Yes	F: CTGAGCTGGTGGATGCTCTT R: TCATCCACAATGCCTGTCTG
Monoamine oxidase B (MaoB); NM_172778.2; Target gene	124 bps	Yes	F: GCTGATCTCTCGTGTGCCTT R: TGCAATTGGAGCTTCCTCTC
Mitogen-activated protein kinase 1 (Mapk1/Erk2); NM_011949.3; Target gene	111 bps	Yes	F: CTGCGCTTCAGACATGAGAA R: AAGGTCCGTCTCCATGAGGT
Mitogen-activated protein kinase 3 (Mapk3/Erk1); NM_011952; Target gene	102 bps	Yes	F: ACCCTGGAAGCCATGAGAG R: GCAGATGTGGTCATTGCTCA
Guanosine Diphosphate Dissociation Inhibitor 2 (Gdi2); NM_008112.4; Reference gene	126 bps	No	F: GTCAGAATTGGTTGGTTCTGTT R: AGCTCTTGGATCACACAATCG
Ribosomal protein, large P0 (Rplp0); NM_007475; Reference gene	125 bps	No	Not declared; QuantiTect primer assay: QT00249375
TATA box binding protein (Tbp); NM_001172085; Reference gene	114 bps	Yes	Not declared; QuantiTect primer assay: QT00198443
Ubiquitin-C (Ubc); NM_019639; Reference gene	75 bps	No	Not declared; QuantiTect primer assay: QT00245189

2.3.2.5 Quantitative real-time polymerase chain reaction

This process is divided into 3 steps. In the first step or denaturation, the DNA is melted into two single strands by the disruption of the hydrogen bond between the complementary bases and occurs at 95º C. In the second step or annealing, the primers anneal and hybridize to the complementary DNA template and it occurs between 50-60ºC. During the third step or extension or elongation, the DNA polymerase enzyme synthetizes a new DNA strand complementary to the DNA template strand, adding deoxynucleotide triphosphates to the template strand and it occurs at 68-72ºC. In case the procedure works optimal after one cycle the number of PCR product is duplicated.

The gene expression level of each respective target gene (TG) was measured in PFC, MTC, STR, HIPPO and CE.

Each well (Life technology, Gaithersburg, USA) was filled with a total of 10 µl sample volume (per well). Each plate of TG and RG contained: cDNA of the corresponding brain region, IRCs, NRT and NTC. All of samples were run in triplicates. The cDNA samples were diluted with 1x TE buffer (1:5 ratio) adding 100 µl per sample. Thereafter, this solution was diluted again with Nuclease-Free H_2O (1:4) and was used as cDNA master mix.

For every target gene the F and R primer was diluted in Nuclease-Free H_2O to a 10x reaction mix of 5.5 µM. Primers purchased as QuantiTect Primer Assays were delivered in a lyophilized powder that was diluted in 1x TE buffer as the manufacturer indicated and received a 10x ready-to-use solution of 5 µM for both the F and R primer.

A second master mix that contained the SYBR Green I-based reaction chemistry (SYBR Select Master Mix for CFX, Life Technologies) and the previous diluted F and R primer was prepared for each plate. With the help of a 10 µl-multichannel pipette the two-master mixes were inserted into the wells of the 384-well plate, according to the distribution previously planned for each plate. Immediately after the plate was covered with Microseal® 'B' PCR Plate Sealing Film (adhesive sealing films for all thermal cycling) (Bio-rad) it was centrifuged at 2200 g for 60 sec and stored 24 h at 4°C. Then, the plate was centrifuged at 2200 g for 60 secs and placed into the Bio-Rad CFX384™ Thermal Cycler. The temperature protocol used for qPCR measurements consisted on: initialization step (95ºC for 2 min), denaturation step (95ºc for 5 sec), annealing and elongation step (60ºC for 30 sec, repeated in 50 cycles each), default dissociation (95ºC for 10 sec) and melt curve (65°C to 95°C; increment 0.5°C for 5 sec). Afterwards the plate was left to cold down and storage at 4º C.

2.3.2.6 Data analysis.

Analysis of the qRT-PCR data was performed following different approaches (Derveaux et al. 2009) by using different software and steps recommended. The first software used was LinRegPCR (Heart Failure Research Centre, Academic Medical Centre, Amsterdam, The Netherlands) used to calculate the mean PCR efficiency (maximum amplification obtained from a given number of cycles) of the RG and TG and also to find possible outliers in the samples. This software analyses qRT-PCR data defining baseline fluorescence by doing a baseline subtraction. The amplification is

represented by a curve divided in two phases: exponential phase and a non-exponential one. In the exponential phase the PCR product is duplicated in each cycle and the fluorescence can be detected. The quantification cycle (Cq) or Ct (Threshold point) indicates the point in the time when the fluorescence detected in the sample exceeds the level of background fluorescence (cycle 18). The reaction “decreases” and shifts to a non-exponential plateau phase. The amplified product is proportional to the fluorescence and is measured as fluorescence units (RFU). The program takes the upper and lower limits of the log-linear phase of each sample creating a window of linearity (W-o-L) and computes a linear regression. Thereafter, the set a W-o-L and the PCR efficiencies per sample are calculated. The samples with irregular amplification pattern were automatically excluded. All data samples calculated by LinReg may follow some parameters and can be used for further analysis: each PCR efficiency should range between 1 and 2; whereby 2 indicates a perfect efficiency and data obtained should present more than 4 points within the W-o-L or an R2-value (correlation coefficient square) more than 0.9995 to be accepted for further steps.

Thereafter, a manual analysis of the Cq variation of the triplicated data of each sample was performed. Triplicates within a 0.5 Cq internal variation were accepted and used for further analyses. An outlier sample was defined as a Cq value with a difference of more than 0.6 Cq to the closest value. Outliers were excluded from subsequent analyses. The remaining Cq values were used as duplicates only if the distance between them was not higher than 0.3 Cq. When a Cq value presented a substantial difference to the other values but within a 0,6 Cq interval, the remaining duplicates were accepted only if the variation was in the range of 0.15 Cq. Samples with acceptable variations in Cq values were used for the following analysis in qbaseplus (Biogazelle NV, Technologiepark 3, B-9052 Zwijnaarde, Belgium).

The software qbase is used for further analysis of qPCR data and is based on the geNorm and qBase technology (Hellemans et al. 2007). The software is intended for relative quantification of gene expression and for gene copy number analysis. The geNorm is used for determine the stability of reference genes and the optimal number of reference genes for normalization. qBase calculates the normalization of the data, the specific variation of samples corrected by means of reference genes. Finally, it was performed a statistical analysis of the obtained relative gene expression levels/data in different brain regions of WT and KO mice. Statistical calculation done is described in more detail in Chapter 2.6.

2.4 Neurotransmitters study

Tissue levels of monoamines and their related metabolites were assessed in different brain regions of WT and KO mice by using High-performance liquid chromatography (HPLC).

2.4.1 Brain tissue

The brains from naïve mice were removed from the skull, frozen in precooled isopentane and stored at -80°C. Then, brain tissue samples from PFC, MTC, STR, hypothalamus, amygdala, HIPPO, raphe nuclei, and CE were quickly dissected with

the help of a stereo microscope (Olympus) and an ice-cold plate and kept frozen at -80°C until sample preparation.

2.4.2 Sample preparation neurotransmitters measurement at HPLC

Tissue samples were weighed and then stored at -80°C until the sample preparation. The day of preparation the tissue samples were transferred to a tube for sonication (Stardest) and were placed on ice as well as the diethylene triaminepentra acid buffer (H3PO4-DTPA: 150 mM H3PO4, 500 µM DTPA). A 1:20 homogenate was prepared: weight of the sample (in mg) x 19 = number of microliters of H3PO4-DTPA buffer added to each tissue sample. Immediately after the addition of buffer to the tissue sample, this was placed in a small beaker with ice and inserted in the sonicator (Branson Digital Sonifier 250, Branson Ultrasonic Corporation). The sonication tip and also the small tip with Argon gas needed to be inserted in the tube at the same time to proceed with the sonication for 20 sec. Sonication treatment is performed as follows: amplitude 15%, 10 sec = 0,5 sec pulse + 0,5 sec pause per 10 times. Afterwards, the tube was placed on ice until centrifugation. Samples were transferred to the centrifuge (SM-24-rotor, Sorvall, Boston Laboratory Equipment) and placed with the most similar amount of content opposite to each other to then proceed with the centrifugation at 19000 rpm, for 20 min at 4°C. After this centrifugation step, tubes were placed in ice and the content of the surface was extracted with a pipette and transferred to a fresh tube. The content of the bottom of the tube was discarded. Samples were then stored at -80°C until they were used to perform the HPLC analysis. Before sonication the sonicator was cleaned by sonifying a tube with distilled water and also in beaker with ice. The sonication tip was cleaned with 70% ethanol in between the tubes.

2.4.3 HPLC analysis

Levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), 5-hydroxyindoleaceticacid (5-HIAA), noradrenaline/norepinephrine (NE) and 3-Methoxy-4-hydroxyphenylglycol (MHPG) were analyzed by HPLC using electrochemical detection at +0.75 V using EC 150/4.6 Nucleodur 100-3-C18 reversed-phase chromatography columns (Machery-Nagel, Düren, Germany), with mobile phase consisting of 84% 0.1 M NaH_2PO_4, pH 3.35, 16 % MeOH, 0.65 mMoctanesulfonic acid, 0.50 mM triethylamine and 0.1 mM EDTA. Monoamines and metabolites were quantified relative to standard injections of known amounts and tissue levels calculated as ng per mg wet tissue weight.

2.5 Adult neurogenesis study

2.5.1 5-bromo-2`-deoxyuridine administration

Cell labelling using the exogenous marker 5-bromo-2`-deoxyuridine (BrdU) makes it possible to determine survival neuronal cells, due to BrdU is incorporated into the new synthesized DNA during the adult neural progenitor cell cycle in the mammalian (Kuhn et al. 1997). By using immunohistochemistry against anti-BrdU it is possible to identify and count the stained neurons.

To analyze the possible effect of *Rsk2* genotypes on Adult neurogenesis (AN), a form of neural plasticity, WT and KO mice were treated with intraperitoneal (i.p.) BrdU

injections. BrdU labelling was performed following the paradigm called differentiation/maturation (Sun et al. 2015). This protocol consists of the application of BrdU (Roche Diagnostics, Mannheim, Germany; dissolved in PBS) (50 mg/kg) i.p. injections 4 times every 2 hours for one day, as it is shown in the table below. Then, mice were kept under normal housing conditions for 28 days before sacrifice.

<table>
<tr><th colspan="5">BrdU differentiation/maturation paradigm:</th></tr>
<tr><td colspan="4">day 1</td><td>day 2-29</td><td>day 30</td></tr>
<tr><td>n°1
BrdU
i.p.</td><td>n°2
BrdU
i.p.</td><td>n°3
BrdU
i.p.</td><td>n°4
BrdU
i.p.</td><td rowspan="2">animals under normal housing/no interventions</td><td rowspan="2">Sacrifice</td></tr>
<tr><td colspan="4">each i.p was applied every 2 h</td></tr>
</table>

Figure 4: **Scheme of BrdU differentiation/maturation paradigm.** i.p.= intraperitoneal.

2.5.2 Brain tissue

The brains were dissected and fixed by immersion for 48 hours (h) in freshly produced 4% Paraformaldehyde (dissolved in Phosphate-buffered saline,PBS,pH 7.5) and this solution was changed once after 24 h. Fixed brains were washed 4 times every 15 min in PBS to then successively be treated for 24 h with 10% and 20% sucrose (dissolved in PBS), respectively. Finally, brains were frozen in precooled isopentane and stored at -80°C.

Each brain was cut on a freezing cryostat at a box temperature of about -23 °C (Microm, Germany). The hippocampus was sectioned in coronal sections of 50 µm thickness. The sections were placed in a 24-well plate filled with 1x Tris-buffered saline (TBS). Free-floating sections were collected in a one-in-sixth series. Each series contains 8-12 sections.

All brain sections not used for staining were stored in cryoprotectant solution (30% ethylenglycol and 25% glycerin in 1x PBS) at –20°C until further use.

2.5.3 Quantitative immunohistochemistry

The number of cells immunopositive for minichromosome maintenance complex component 2 (MCM2, an endogenous marker of proliferation), doublecortin (DCX, a marker of immature neurons), and BrdU (5-bromo-2`-deoxyuridine, marker of proliferating cells), were quantified in the dentate gyrus (DG) of the HIPPO by using immunohistochemistry.

2.5.4 Immunodetection of MCM2 and DCX

The procedure was performed according to Karabeg et al. (2013) for the detection of MCM2 and DCX: sections were placed in a 24 well-plate and rinsed twice for 10 min in TBS (Tris buffered saline, 0,15 M NaCl and 0,1 M Tris-HCl, pH 7.5). Then, sections were incubated in 0,6% hydrogen peroxidase diluted in TBS for 30 min at room temperature (RT). After rinsing the sections 3 times for 5 min in TBS this buffer was replaced for 10 mM Sodium Citrate Buffer pH 8.5 using 1 ml per wells. The 24 well-plate were placed in a pre-heated water bath at 80°C and the tissue sections were

incubated for 30 min for antigen retrieval process. After the sections were cooled down at RT they were rinsed again three 3 to 5 min in TBS. The tissue sections were incubated with a blocking solution containing normal horse serum [5% normal horse serum (NHS); 0,25 % Triton X-100; 2% bovine serum albumin (BSA)] in TBS at RT for 1,5 h. Thereafter, the tissue sections were incubated in a blocking solution containing the primary antibodies: polyclonal anti-MCM2 antibody (1:1000) or the polyclonal anti-DCX antibody (1:500) (the two of them were made from goat and obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA.) at 4°C overnight. After 24 h the sections were rinsed 3 times for 10 min in TBS and incubated for 2 h at RT in biotinylated anti-goat IgGs made in horse. The secondary antibody was diluted 1:1000 (Vector Laboratories, Burlingame, CA, USA) in a solution containing 2% NHS (2% NHS; 0,25 % Triton X-100; 2% BSA in TBS). Another rinsing step of 3 times for 10 min in TBS was performed, to then incubate the tissue sections with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) in 1x TBS for 1.5 h. This solution had to be prepared 30 min before its use. Afterwards, tissue sections were rinsed 3 times for 10 min in TBS and incubated with Diaminobenzidin (DAB) (DAB substrate kit; Roche Diagnostics, Mannheim, Germany) substrate of the peroxidase. The incubation with DAB was among 5-10 min. This reaction was stopped by rinsing the tissue sections at least 3 times for 10 min in TBS. Finally, tissue slices were carefully mounted on glass slices, left dry overnight and the next day they were covered with a cover slip and VitroClud (R. Langenbrinck; Emmendingen).

2.5.5 Immunodetection of BrdU

This technique was performed according to the procedure described by Kuhn et al. (1997) using a monoclonal anti-BrdU antibody (1:400 dilution; Roche Diagnostics, Mannheim, Germany). The protocol used replicates the same steps of the protocol to stain MCM2 and DCX with the following differences: after blocking endogenous peroxidases by incubation with H2O2 tissue sections were incubated in 50% Formamid / 2x SSC at 65°C for 2 h. Thereafter, tissue sections were incubated in 2N hydrochloric acid 37°C for 30 min. These two incubation steps result in DNA denaturation which is necessary to allow the antibodies to bind to BrdU. Another rinsing step was performed in 0.1 M Boric acid (pH 8.5) for 10 min to then incubate the sections in a blocking solution containing normal goat serum (5% normal goat serum (NGS); 0,25 % Triton X-100; 2% BSA in TBS) at RT for 1 h. Thereafter, sections were incubated overnight at 4°C in the blocking buffer containing the polyclonal Anti-BrdU. After overnight incubation the tissue sections were rinsed 3 times for 10 min in TBS and incubated for 2 h at RT in biotinylated goat anti-rat IgGs (secondary antibody diluted 1:600; Vector Laboratories, Burlingame, CA, USA) in a solution containing 2% NGS (2% NGS; 0,25 % Triton X-100; 2% BSA in TBS). Tissue sections were rinsed 3 times per 10 min and incubated with avidin-biotinylated horseradish peroxidase complex in 1x TBS (Elite Kit; Vector Laboratories) for 2 h, the following steps were described in the staining of MCM2 and DCX (see 2.5.4).

2.5.6 Quantification of immune-labelled cells

The Neurolucida imaging system (MicroBrightField Bioscience Inc., Willsiton, VT, 05495 USA) is composed by: Olympus BX51 polarization microscope (Olympus), CX9000 digital camera (Olympus), motorized substage (MicroBrightField Bioscience Inc., Willsiton, VT, 05495 USA), and the StereoInvestigator software (Microbrightfield Inc., Williston, VT, USA) enabling unbiased stereological quantification was used to quantify immuno-reactive cells detected in the DG of the hippocampus.

Cells immune-reactive for MCM2, DCX and BrdU were counted in both hippocampi in every sixth 50 μm thick section. These immuno-reactive cells were quantified in two areas of the DG: the subgranular zone (SGZ) and granule cell layer (GCL). In every section immuno-positive cells were counted using a special variant of the optical fractionator method with counting frame and sampling grid of 150 by 150 μm size with ratio 1:1 done as a two-dimensional evaluation using the 10x 1600 magnification.

The Stereo Investigator program provided different parameters as the volume and the number of the estimated population of positive cells. These parameters were used to calculate the density. Further statistical analyses were performed by using the density of positive cells.

2.6 Statistical analysis.

Statistical analyses were performed using GraphPad Prism® Version 5 (GraphPad Inc., La Jolla, CA, USA). Differences between data of WT and KO mice were analyzed using nonparametric test: the Mann–Whitney U test. Statistical significance was set at (*) $p \leq 0.05$ and (**) $p \leq 0.01$. Statistically highly significant set at (***) $p \leq 0.001$. A non-significant trend (#) was considered at $0.05 < p \leq 0.1$. All results are expressed as mean values ± standard error of the mean (S.E.M.).

Statistical analyses performed in the IntelliCage were conducted using R (R CoreTeam 2015) and the differences between groups were calculated by repeated measures ANOVA, linear mixed models, sequential probability ratio statistics and F-tests.

3 Results

3.1 Behavioral studies

Behavior of *Rsk2* WT and KO mice was primarily studied to investigate affective behaviors like spontaneous activity, anxiety-like behavior and depression-like behavior by performing some conventional behavioral tests and -for the first time- using the IntelliCage as a behavioral paradigm.

3.1.1 *Rsk2*-deficient mice show increased spontaneous activity, no change in trait anxiety and an anti-depressive phenotype

The behavioral battery consisted of EPM, LDB, OF and PST. These behavioral tests have as main objective to evaluate anxiety-like behavior, loco motor activity levels and depression-like behavior.

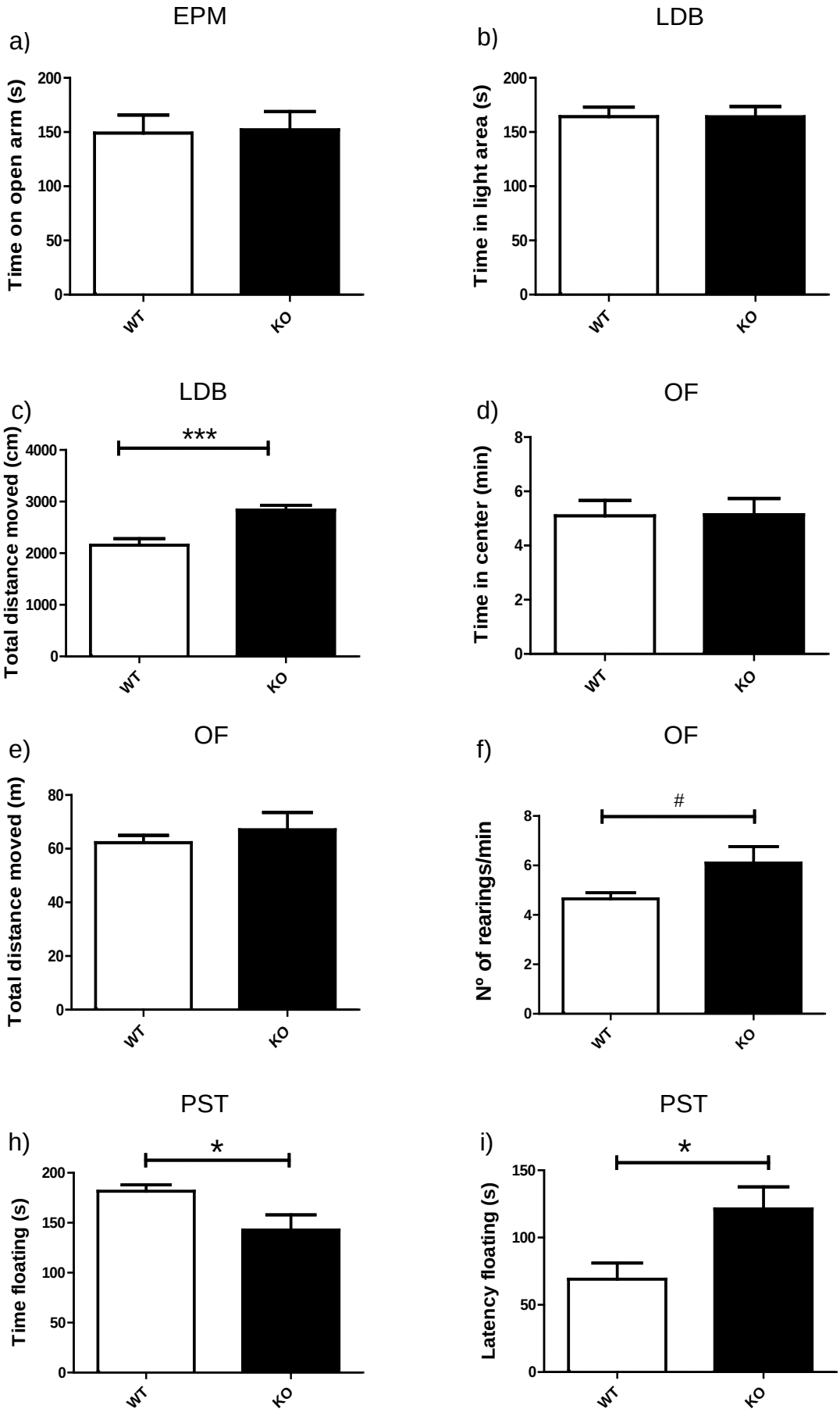
Results of EPM did not reveal any significant difference between *Rsk2* genotypes. There was no difference in the time spent on the open arms (p=0,9310; figure 5a), the number of entries to open arms (p=0,7285), and explorative behavior as walking activity (p=0,9770).

In the LDB KO mice showed a highly significant increase in walking activity (p=**0,0003**; figure 5c) compared to WT mice. The walking activity or total distance moved corresponds to the sum of the time walking in the light and dark chamber. The time spent in the light chamber (p=0,8294; figure 5b) as well as the number of entries to the light chamber (p=0,4020), situations aversive to mice, did not show significant differences between *Rsk2* genotypes.

Regarding OF all mice showed a significant avoidance of the central area (p=0.001) of the arena and spent more time in the periphery (p=0.001) or close to the walls. No significant differences were found between mice of different genotypes concerning the time spent in the center area (p=0,9264; figure 5d) and the number of entries to the center area (p=0,6890). Also, no significant differences for total walking distance or total distance moved (p=0,8777; figure 5e) were found. A trend-level of an increased average number of rearing (p=0.072; figure 5f) as an augmented “vertical” activity of KO mice compared to WT mice was shown.

PST was evaluated analyzing time frames of 5 min. KO mice spent significantly less time in an immobile state or floating (p=**0,0376**; figure 5h) and had a longer latency to start floating (p=**0,0208**; figure 5i) than WT mice. In line with this, KO mice spent more time in an active state by trying to climb up the walls (p=**0,0440**; figure 5j). Time swimming was not significantly different (p=0,8690; figure 5k) between KO and WT. A second evaluation analyzing time frames of 1 min revealed, that KO mice were longer active (time spent swimming plus climbing) during the second min (p=**0,0111**; figure 5l) than WT mice.

A resume table with the data corresponding to this experiment can be found in the supplement.


EPM
a)
Time on open arm (s)
200
150
100
50
0
WT
KO
LDB
b)
Time in light area (s)
200
150
100
50
0
WT
KO
LDB
c)
***
Total distance moved (cm)
4000
3000
2000
1000
0
WT
KO
OF
d)
Time in center (min)
8
6
4
2
0
WT
KO
OF
e)
Total distance moved (m)
80
60
40
20
0
WT
KO
OF
f)
#
Nº of rearings/min
8
6
4
2
0
WT
KO
PST
h)
*
Time floating (s)
200
150
100
50
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WT
KO
PST
i)
*
Latency floating (s)
150
100
50
0
WT
KO

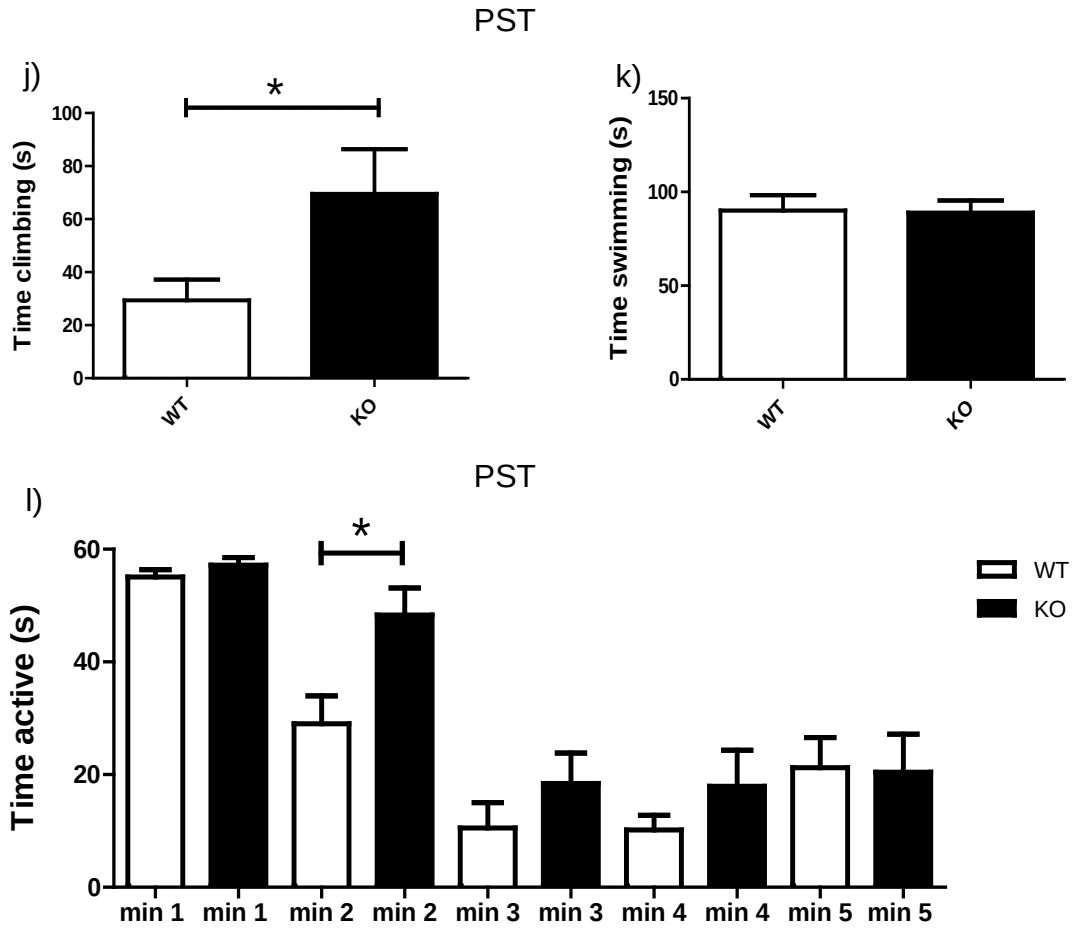


Figure 5: *Rsk2*-deficient mice show increased spontaneous activity, no changes in trait anxiety and an anti-depressive genotype. EPM showed no significant difference with regards to the time spent in the open arm (a). LDB revealed no significant difference in the time spent in the light chamber (b) and a significantly increased walking activity or total distance moved (c) in KO mice. OF did not present significant differences in the time spent in the center (d) and in the total distance moved/walking distance (e) but a trend level was found in the nº of rearing (f) KO mice compared to WT. Statistical calculations reveal in PST a significant decrease in the time floating or being immobile (h) in KO mice. KO mice showed a significantly increased latency to start to float (i) and in the time climbing (j), but no difference in the time swimming (k) compared to WT mice. I: graph of analysis of time frames of 1 min revealed that KO mice spent more time active (time swimming plus climbing), a significant difference was observed during the second min. Bars represent arithmetic means ± standard error of mean (SEM). Pair-wise comparisons were performed using the Mann-Whitney U- test. #: p-value <0.1 and >0.05; *: p-value<0.05; ***: p-value<0.001. WT= Wild type (n=12) and KO= Knock out (12).

3.1.2 Behavioral tests performed in the IntelliCage reveal that *Rsk2*-deficient mice present no changes in anxiety state and a high sucrose seeking behavior

The mice were tested in groups of 10-16 (similar numbers of KO and WT mice) per cage and were kept in groups at least 30 days before were inserted in the IntelliCage. A period of adaptation of 6 days with all doors opened and free access to the drinking bottles was given to the mice before starting the experiments. Additional 2 days were assigned to let the animals to adapt to nose poking for door opening; after the animal

poked at the door, the door was kept opened for 7 sec. per each visit to the corner. The place learning schemes and anxiety tests were applied using two drinking sessions during the night phase with the goal of increasing learning motivation. The conditioning experiments were conducted during drinking sessions and the doors were shut after the drinking sessions.

During the period of free adaptation spontaneous activity was measured by recording the time till the animals made the first visit, nose pock and lick, number of visits till first nose poke/lick, nose poking or drinking in all the four corners. The results of spontaneous behavior were significantly different between genotypes during the free adaptation periods. The measure that most discriminates between genotypes appeared to be repetitive (sum of observed returns to same corner) ($F(1,77.76) = 7.71$, $p<0.01$). The visits with nose poke and licking activity were not significantly different. Also, no differences were observed in general preference for one corner instead on another during the six days of free adaptation.

With regard to place learning, the learning performance and correct responses did not show any significant difference between genotypes. The same happened in the other places learning experiments: place preference, place 75, place learning reversal and place change. In the chaining and patrolling tests, mice were able to learn the tasks progressively over the days but no significant differences were found in KO mice compared to WT.

Motor Impulsivity testing shows that both genotypes were able to learn the task. The proportion of correct responses declined with delay during training and test phase (Interaction phase: delay, $p<0.001$). (KO: n= 13; WT: n=16).

Cognitive impulsivity was tested by a delayed discounting paradigm. All mice showed a highly significant preference for sucrose ($p<0.001$) which declined with the addition of a delay to get the sucrose. The decline in KO mice was significantly later than in WT mice (KO: n=20; WT: n=17) indicating that *Rsk2*-deficient mice do not present a defect with regard to cognitive impulsivity and may have a sucrose-seeking genotype.

In the anxiety test KO mice showed a lower anxiety-like behavior than WT mice because after receiving an air puff (aversive stimuli) they returned to the corner were the air puff was generated significantly earlier than WT mice($p<0.01$). However, during the inter phases of the experiment, where no air puffs were delivered no significant difference between genotypes was reported. The number of mice used to test the anxiety paradigm: Sucrose: KO: n=7; WT: n=6 and water: KO: n=6; WT: n=5.

For methods and graphs please see Fischer et al. (2017).

3.2 *Rsk2* genotype does not affect corticosterone concentrations in blood plasma

The analysis of the blood plasma corticosterone concentrations measured in a group of naïve mice (without stress exposition) and in a group of stressed mice (after performing PST) shows significantly increased corticosterone levels in stressed WT (**p=0,0013**) and KO (**p=0,0002**) mice. This result proves the efficacy of PST as a stressful experience yet was not genotype dependent. No differences between genotypes were

found in the blood plasma concentrations of naïve mice (p=0,7922) neither in stressed mice (p=0,9118; figure 6a).

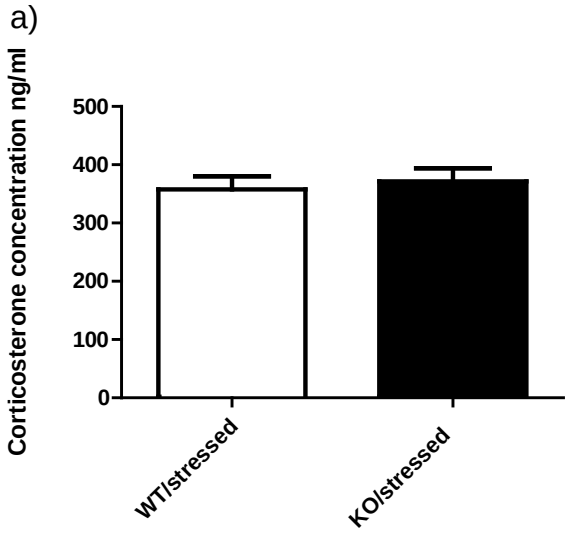


Figure 6: ***Rsk2* genotypes do not affect concentration of corticosterone after exposure to stress.** As is shown in the figure a) no difference between WT and KO was found after the application of PST. Bars represent arithmetic means ± standard error of mean (SEM). Pair-wise comparisons were performed using performed using the Mann-Whitney U- test. Naïve: without PST exposition WT= Wild type (n=5) and KO= Knock out (n=6) and stressed: with PST exposition WT= Wild type (n=10) and KO= Knock out (n=11).

3.3 *Rsk2*-deficiency affects the expression of various genes in prefrontal cortex and striatum– a quantitative real time-PCR study

Our goal was to analyze genes coding for proteins that are upstream activators of RSK2 like Mapk1/Erk2 and Mapk3/Erk1 since it is known that the MAPK/ERK pathway plays an important role in mammalian synaptic plasticity and learning (Sweatt, 2001). Also, we focused in extracellular activators of RSK2 as Bdnf1 and Igf1 genes that encode grow-factors and are associated to neural plasticity, depression and learning (Han et al. 2013; Shirayama et al. 2002). Furthermore, we studied the expression of genes linked to emotional behavior like receptors and enzymes involved in the function of monoamines like *5Htr1a*, *Adra2a*, *Comt*, *Drd2* and *MaoB*. We also searched for genes linked to stress-related proteins like *Crh*, *Crhr1*, *Fkbp5* for the role that neurotransmitters and stress play in human and animal behavior.

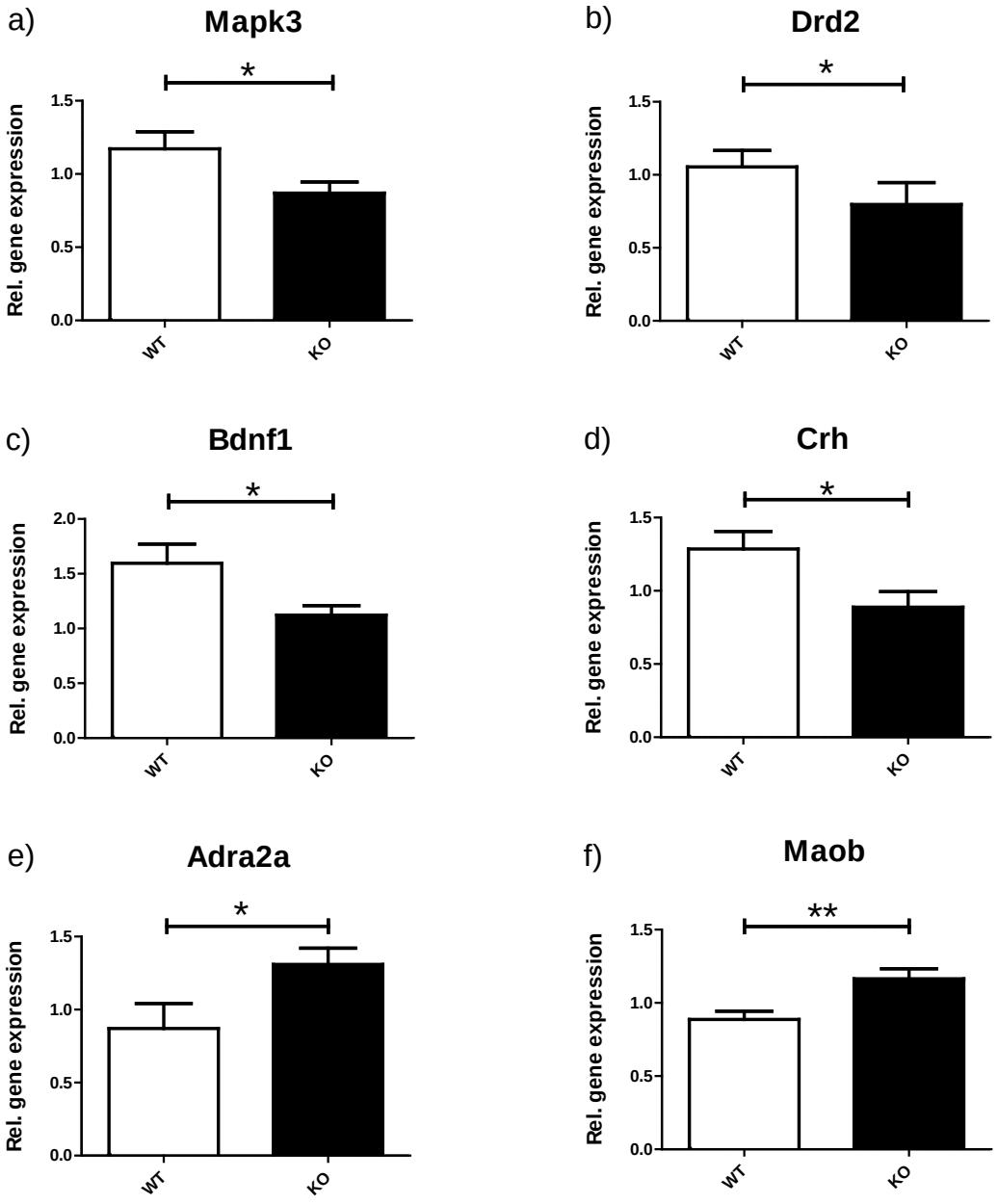
The analysis of the expression of the twelve target genes: *5htr1a, Adra2a, Bdnf1, Comt, Crh, Crhr1, Drd2, Fkbp5, MaoB, Igf1, Mapk 1/Erk2, Mapk3/Erk1* and four RG *Gdi2, Rplp0, Tbp and Ubc*, was performed in five brain regions: PFC, MTC, STR, HIPPO and CE of WT and KO mice.

Significant differences between WT and KO groups were found only in two brain regions: PFC and STR. As shown in Figure 7, relative gene expression of *Mapk3, Drd2, Bdnf1* and *Crh* were significantly reduced in the PFC and the relative gene expression of *Adra2a* and *Maob* were significantly increased in the STR of KO mice.

The relative expression levels of the *Mapk3* (p=**0,044**; figure 7a), the *Drd2*; (p=**0,043**; figure 7b), *Bdnf1* (p=**0,044**; figure 7c) as well as the *Crh*; (p=**0,035**; figure 7d) gene in the PFC was significantly reduced in KO compared to WT mice. In the STR of *Rsk2*-deficient mice the relative expression level of *Adra2a* (p=**0,022**; figure 7e) was significantly increased and of *Maob* (p=**0,005**; figure 7f) was increased with high significance compared to WT controls. Also in the STR a trend-level for the relative expression of *Fkbp5* (p=0,075; figure 7g) and the *Igf1* gene (p=0,075; figure 7h) were found showing a tendency of increased expression in KO mice compared to WT mice. In HIPPO a trend for increased expression of *Crhr1* (p=0.065) was found in KO mice compared to WT mice.

These results indicate that *Rsk2* deficiency affects the expression of the following investigated genes: PFC and/or STR: *Mapk3, Drd2, Bdnf1, Crh, Adra2a* and *Maob*.

A summary table with the data corresponding to this experiment can be found in the supplement.



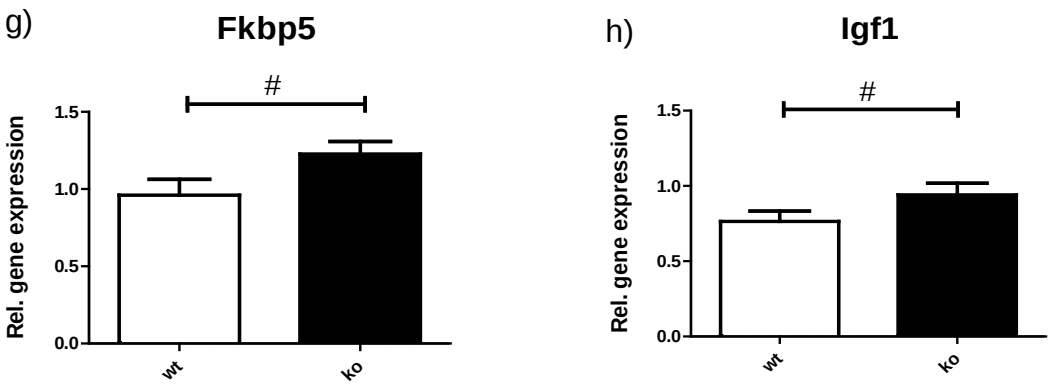


Figure 7: Expression of various genes in prefrontal cortex and/or striatum of mice *Rsk2* WT and KO mice. Graphs of the relative gene expression of *Mapk3/Erk1* (*Mitogen-activated protein kinase*; a), *Drd2* (*the dopamine receptor 2*; b), *Bdnf1* (*brain-derived neurotrophic factor variant 1*; c), *Crh* (*corticotrophin releasing hormone*) show a significantly reduced expression in the PFC of KO compared to WT mice. The relative gene expression of *Adra2a* (*alpha-2-adrenergic receptor 2a*; e), *Maob* (*monoamine oxidase b*; f) and trend-level of *Fkbp5* (*immunophilin protein*; g) and *Igf1* (*insulin growth factor 1*; h) was detected to be significantly increased in the striatum of KO compared to WT mice. Bars represent arithmetic means of relative gene expression levels ± standard error of mean (SEM). WT= Wild type (n=11) and KO= Knock out (n=11). #: p-value <0.1 and >0.05; *: p-value <0.05; **: p- value < 0.01.

3.4 Tissue levels of metabolites related to the dopaminergic, serotonergic and noradrenergic system were found to be altered in prefrontal cortex, hippocampus and striatum in *Rsk2*-deficient mice- a HPLC study

Our aim was to analyze the levels of monoamine transmitters to search for a possible neurochemical correlate of found behavioral differences. The brain regions studied were selected due to the important role that they play for emotion regulation, memory, loco motor activity, cognitive flexibility and stress-reactivity, among others.

The analysis of the concentration of the monoamines dopamine, serotonin, noradrenaline and their respective metabolites DOPAC, HVA, 5-HIAA and MHPG was performed applying the HPLC method and using tissue samples of PFC, MTC, STR, hypothalamus, amygdala, HIPPO, raphe nuclei and CE of KO and WT mice.

Significant differences were uniquely found in the metabolites but not in the neurotransmitters themselves as is shown in the figure 8. Significant differences were found related to the dopaminergic system. DOPAC tissue concentrations were significantly increased in PFC (p=**0,0424**; figure 8a) and HIPPO (p=**0,0435;** figure 8b). A trend level of a higher HVA tissue concentration was detected in MTC (p=0,072; figure 8c) in KO compared to WT mice. The serotonin metabolite 5-HIAA was increased at a trend-level in PFC (p=0.073; figure 8d), STR (p=0.083; figure 8e) and raphe nuclei (p=0.081; figure 8f) in KO compared to WT mice. The noradrenaline metabolite MHPG (p=**0,0220** figure 8g) was shown to be significantly decreased in HIPPO of KO compared to WT mice.

A resume table with the data corresponding to this experiment can be found in the supplement.

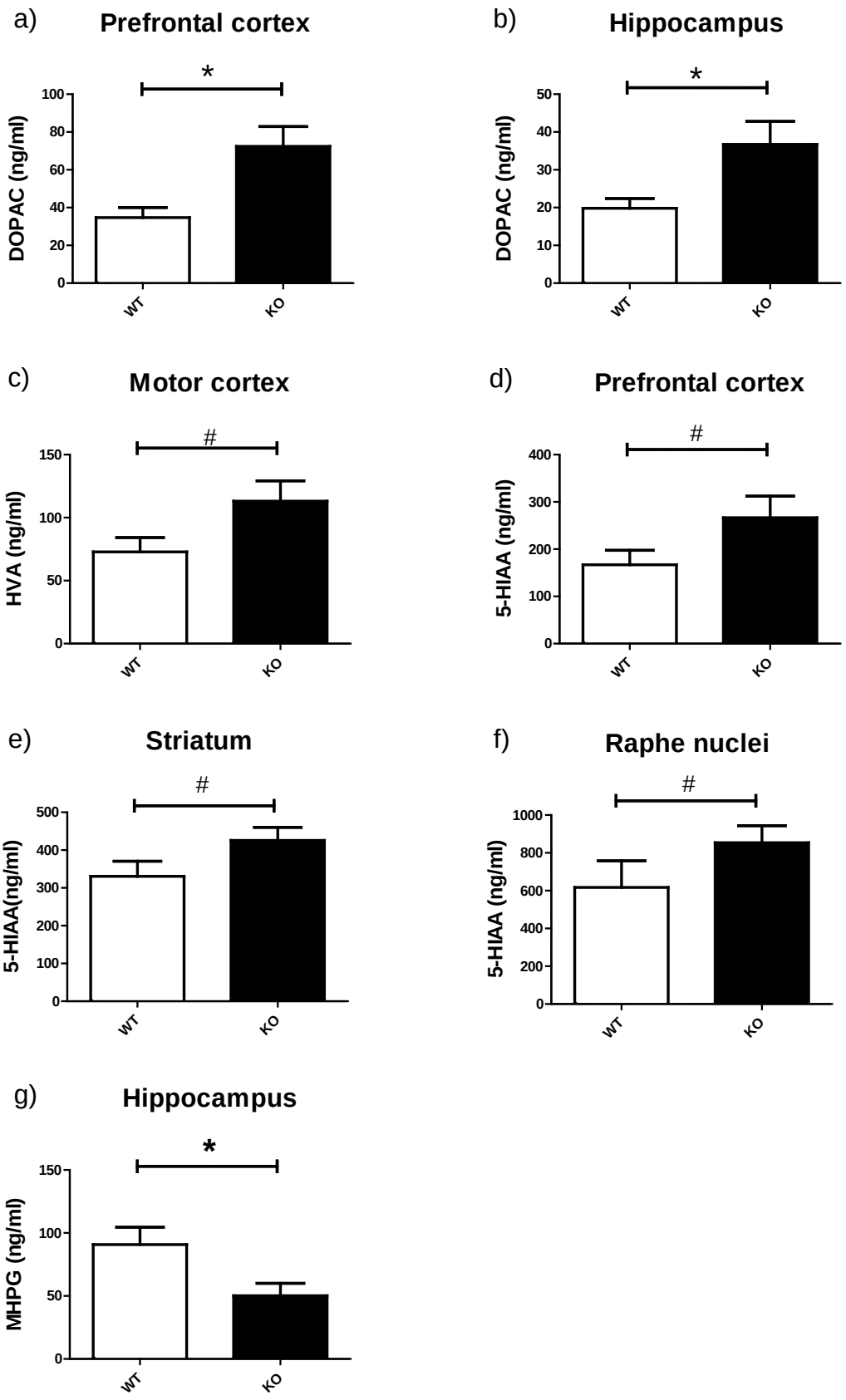


Figure 8: **Tissue concentrations of the metabolites of various monoamines in different brain regions present different alterations**. Graphs related to the dopaminergic system present the result of increased DOPAC concentrations in prefrontal cortex (a) and hippocampus (b), also increased HVA concentrations in the motor cortex of KO compared to WT mice. Regarding the serotonergic system a trend towards increased HIAA tissue concentrations in prefrontal cortex(d), striatum (e) and raphe nuclei (f) was revealed in KO to WT mice. In the hippocampus of KO mice the NE metabolite MHPG (g) was found to be decreased compared to WT mice. Bars represent arithmetic means of tissue concentrations (ng/g wet tissue weight) ± standard error of mean (SEM). Pair-wise comparisons were performed using performed using the Mann-Whitney U- test. #: p-value <0.1 and >0.05; *: p-value <0.05. WT= Wild type (n=11) and KO= Knock out (n=10) mice.

3.5 Adult hippocampal neurogenesis was not influenced by lifelong *Rsk2* deficiency – a quantitative immunohistochemistry study

In order to study a neuronal plasticity phenomenon in *Rsk2*-deficient mice we examined adult neurogenesis (AN), the birth of new neurons in the dentate gyrus of the hippocampus (Von Bohlen und Halbach, 2011) by analyzing different AN stages, either quantifying two endogenous AN marker or by applying BrdU. AN is a complex multi-stage process during which proliferating stem cells and their new-born progeny express different proteins such as MCM2 that can be used as marker proliferation of stem cells (Zhang and Jianwei, 2015) and DCX that labels mitotic active neuroblasts and post mitotic immature neurons (Brown et al. 2003). Moreover, we studied AN through the exogenous Thymidine analog Bromodeoxyuridine (BrdU) which was administered by intraperitoneal injections and can be detected by using immunohistochemistry, a noninvasive method and efficient means of labeling population of newly generated cells. BrdU has been extensively used to analyze cell proliferation and survival (Kuhn et al. 1997) and its localized into the nucleus of the cell. During DNA replication BrdU is replaced for thymidine and is incorporated into the newly synthesized neural stem cells or neuronal progenitor cells (Imayoshi et al. 2009; Von Bohlen und Halbach, 2011). The combination of BrdU with immature neuronal markers like DCX and MCM2 makes possible to determine the early morphological features linked to BrdU-labeled cells using immunohistochemistry. Adult neurogenesis was assessed by performing an immuno staining of MCM2, DCX and BrdU to evaluate their integration in the SGZ and GCL. In the figure 8a and 8c it is possible to observe a higher density of cells immnuno-labeled by DCX and BrdU antibodies in the GCL, and a lower density of cells immnuno-labeled by MCM2 in the figure 8c.

Statistical analysis of the density of immune-reactive cells as shown in the figure 8 revealed that neither the density of MCM2 (p=1,0000; figure 8a) and DCX (p=0,5457; figure 8b) nor the density of BrdU (p=0,4174; figure 8c) was significantly different between mice of the two *Rsk2* genotypes. The quantification of DCX was performed by the bachelor student Michaela Groma.

A summary table with the data corresponding to this experiment can be found in the supplement.

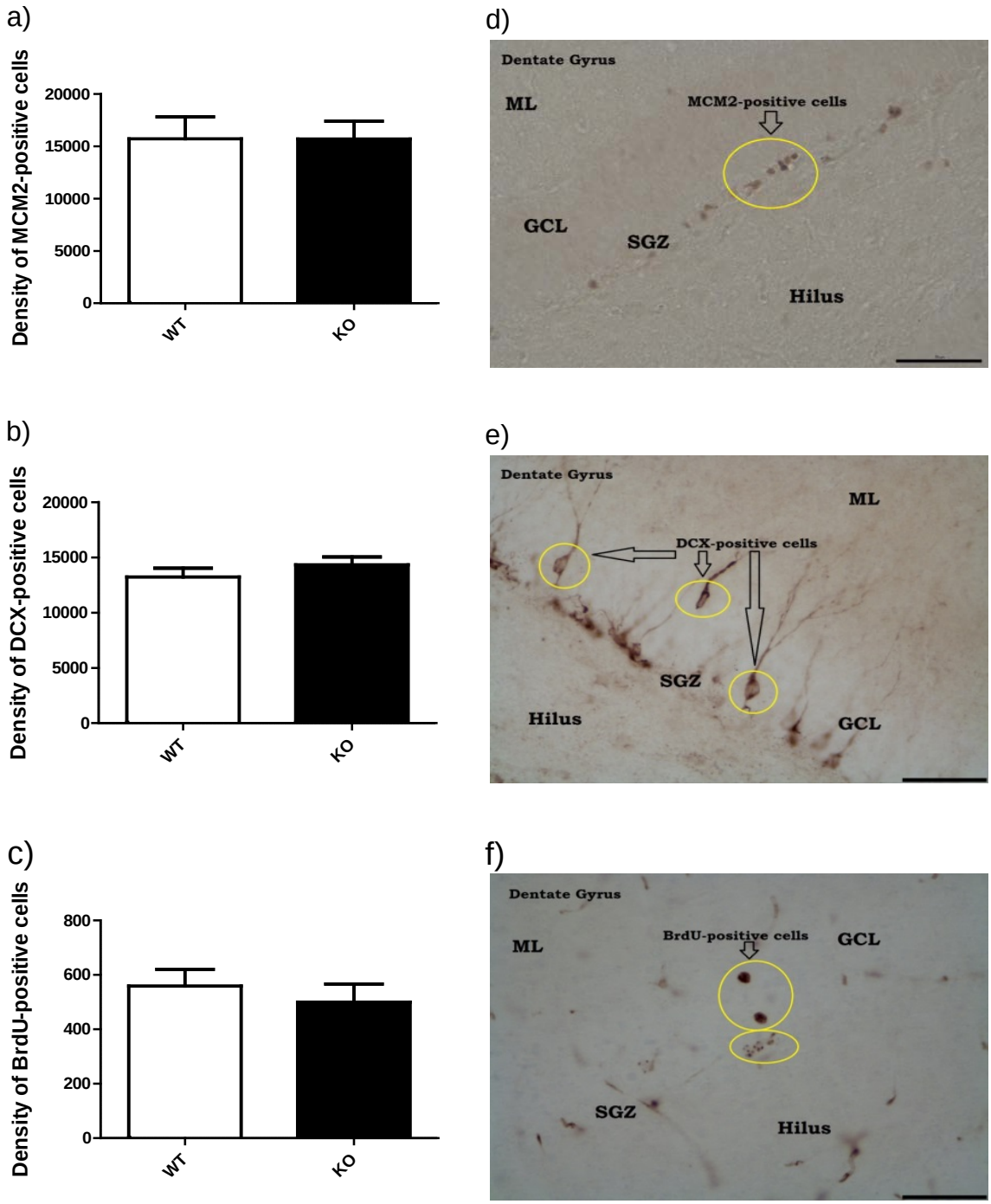


Figure 9: **Density of MCM2, DCX and BrdU-immuno-reactive cells is not different in the dentate gyrus of KO and WT mice.** Results of the statistical evaluation of the density of immune-reactive cells of MCM2 (a), DCX (b) and BrdU (c) did not show any significant difference. Pictures of the DG show immune-reactive cells of MCM2 (d), DCX (e) and BrdU (f) indicated by arrows. Hilus: Hi; Molecular layer: ML; granule cell layer: GCL; sub granular zone: SGZ. Scale bars in d, e, f represent 50 µm. WT= Wild type (n=9), KO= Knock out (n=9) mice. Bars represent arithmetic means of the density of immuno-positive cells of both hippocampi ± standard error of mean (SEM).

4 Discussion

The present experiments were designed to investigate whether and how *Rsk2* deficiency impacts affective behavior and underlying neurobiological mechanisms in the brain in order to characterize *Rsk2* deficient mice as an animal model for the CLS. In our behavioral battery including the results shown in the IntelliCage we found that KO mice may have an anti-anxiety, anti-depressive and sucrose seeking phenotype. With regards to our gene expression study the expression of various genes is affected by *Rsk2* deficiency in PFC and STR. Concerning the neurotransmitter study the tissue levels of metabolites related to the dopaminergic and noradrenergic system are shown to be altered in PFC and HIPPO in KO mice. Moreover, AN is not influenced by lifelong *Rsk2* deficiency.

4.1 *Rsk2*-deficient mice show increased spontaneous activity, no change in trait anxiety and an anti-depressive phenotype

It has been reported that KO mice present several symptoms that resemble the clinic description of humans diagnosed with CLS, e.g. skeletal abnormalities and cognitive defects. Concerning the expression of neurological and psychiatric defects male patients show a severe intellectual disability while female carriers may have a higher prevalence of psychiatric diseases (Hanauer and Young 2002). Cognitive deficits in these mice are mild compared with the severe cognitive deficits observed in human male patients. This is the reason why we decided to apply a broad behavioral battery to characterize this animal model choosing tests associated with psychological and emotional problems such as depression and anxiety. For the first time, PST and IntelliCage experiments where applied to animals with this genotype.

The tests used to evaluate trait anxiety, EPM, LDB and OF did not reveal any significant difference in trait anxiety. Here is important to note that anxiety is a basic emotion that prepares the body and the brain to confront potentially detrimental situations and must be distinguished from trait anxiety, which is a more stable situation not just elicited by potential harms and that can result from chronic stress and or genetic predisposition, and is associated to other psychiatric alterations.

In KO mice spontaneous/exploratory activity is increased in the LDB. Intriguingly, this was not the same in the OF where KO mice traveled as much as WT animals. The increment in exploratory activity is consistent with the findings of Poirier et al. (2007) that reported similar results in the “emergence test”. This could be explained because in LDB the mice have a dark area where they can find refuge and also access to an open area where they can freely explore in contrast with OF where there is only an open area. This contrast could induce to explore the new environment more in KO than in WT. We found a trend of more rearing, considered as “vertical” activity in OF. Nevertheless, no difference in activity was observed in EPM even when these tests share similar spaces with open and close areas. Other signs of increased activity were reported in KO mice, e.g. more visits to the arms in cross-maze, Poirier et al. (2007). In the IntelliCage no increased activity rate was found in KO mice besides a trend for more licks.

The increased floating time in the PST is generally accepted as a measure of depression-like behavior. The test was originally created to assess the effect of anti-depressive drugs (Can et al. 2012). We have applied it for the first time to *Rsk2*-deficient mice showing in our results a reduced floating time in KO mice which can be interpreted as an anti-depressive phenotype of KO mice in this test. Additionally, KO mice showed an increased activity in this test that was observed in various parameters measured. KO mice presented a reduced floating time, reduced latency to start to float and increased time climbing the walls. The floating parameter is an indicator of immobility which indicates that the animal is "depressed" and presents a possible learned helplessness and the latency to start to float indicates that KO was active for a longer time than WT whether climbing or swimming. With regards to the climbing parameter it is a clear attempt to escape from the aversive situation rather than the swimming parameter which was not significantly different and could also be an activity parameter in general. These results indicate reduced behavioral despair excluding a possible interpretation of hyperactivity and argue for an anti-depressive phenotype of *Rsk2* deficient mice in concordance with the increased sucrose seeking behavior observed in the IntelliCage.

In the water maze KO mice spent more time close to the wall. Poirier at al. (2007) argued that the wall hugging may be the result of an adaptation defect to the new environment. Following Poirier's idea in the PST the behaviors to confront this aversive situation could be divided in two: a high energy expenditure behavioral display (climbing and swimming) and a low energy expenditure behavior (floating). Normally, animals start with the high energy behavior and switch to a phase of predominantly low energy expenditure behavior; this switch can be considered an adaptive behavior. We found a reduced floating time and an increased latency to float in KO mice. Our animals spent more time in the high energy behavior and were delayed in switching to the low energy behavior. This retarded switching could be interpreted as cognitive inflexibility but our animals performed well in reversal task learning, suggesting that this is not the case. Thus, this high energy expenditure behavioral display could be interpreted as an anti-depressive phenotype. In summary, we can observe a clear increase in activity in KO mice that could be seen as none- or anti-depressive phenotype and we show for the first time a change in depression-like behavior in *Rsk2* KO mice.

4.2 Behavioral tests performed in the IntelliCage reveal that *Rsk2*-deficient mice present lower state anxiety and a high sucrose seeking behavior

To explore more about depression-like behavior in a social context, which is not possible to do in a conventional behavioral test, we tested anhedonia in *Rsk2* deficient mice by using a paradigm of delayed discounting test with sucrose as a positive reinforce. We found that there is no difference in basal sucrose preference between mice of different *Rsk2* genotypes confirming the results reported by Poirier et al. (2007) in a paradigm of taste aversion learning.

In this delayed discounting test in the IC, which is a cognitive impulsivity test, when a delay is applied the preference for sucrose declines in mice of both genotypes but faster in WT mice than in KO mice. This can be interpreted as a higher motivation for

sucrose reward that may reflect a greater tendency to engage in pleasurable behaviors or “pro-hedonic” phenotype. This supports our hypothesis that the results from PST reflect an anti-depressive phenotype.

Rsk2 KO mice presented elevated glucose levels, mild diabetes and insulin resistance, and defective glucose tolerance (Dufresne et al. 2001; El-Haschimi et al. 2003). These conditions could also explain the high motivation for sucrose in KO rather than a higher hedonism. Tests for the preference for other rewards, as for example a sexual reward in a sexual motivation test, could help to discriminate between these hypotheses. Nevertheless, favoring the motivational explanation our results show in two different paradigms, single testing and social testing, that *Rsk2* deficient mice may have an anti-depressive phenotype.

Further to study emotional behavior an air puff was used as an aversive stimulus in the IntelliCage. After receiving the air puff KO mice returned in a shorter time to the air puff corner than WT mice. This result is in accordance with previous reports using different aversive memory tests. Morice et al. (2013) found that KO mice presented a selective deficit in the consolidation of trace fear memory and a mild deficit in reconsolidation of contextual fear memory, highlighting the sensitivity of hippocampal-dependent memory in this genotype. Darcq et al. (2011) showed lithium-induced conditioned place aversion learning impaired in the KO demonstrating that association of the lithium/aversive stimulus with the context is affected in KO mice. It was also found that RSK2 signaling in the habenula is pivotal for the association of the drug with the context. It is noteworthy that habenula has been shown to code negative reward-related stimuli in primates (Matsumoto et al. 2007). RSK2 in this area could have a role in negative information processing that is lost in KO mice.

Therefore, our results confirm previous findings but in a social context. Interpreting these results is not trivial since they could indicate a reduced anxiety-like behavior or memory impairments. It is important to notice that just the return to the air puff corner after the air puff-“shock” is changed but the general avoidance of this corner over 24 h is not different to the WT. This shows that the animals can identify and remember the place as aversive, suggesting that KO mice do not have a place learning defect. These results support the idea that these animals have a reduced state anxiety while the results from EPM, LDB and OF indicate a normal trait anxiety.

Poirier et al. (2007) suggested that an increase in the acceleration during progressive movements or darting behavior is an indicator of impulsivity. Given that, we found increased activity in LDB; motor impulsivity was tested in the IC showing no signs of increased impulsivity in KO mice. Mice could withhold nose pokes for a certain time to get access to water reward and waited for longer time than WT for the sucrose reward (data not shown) which also could respond to a sucrose seeking phenotype.

Poirier et al. (2007) argued that altered adaptation ability to a new test environment could induce learning deficits. In the IC all the tests are performed in the “home-cage”. Thus, the learning is not affected by such adaptation.

Cognitive abilities were tested in the IC by applying different paradigms of place learning for several weeks. No differences between genotypes were present even when the difficulty of the task was increased, for example by changing the rewarded

corner. Poirier et al. (2007) reported deficits in KO mice in a serial reversal experiment in the water maze with changing platform locations. The authors indicate that in long term experiments or training periods as we did in the place learning paradigm, the learning deficit diminishes when the animals have more time to learn the task. But we found in the IC that initial learning speed did not present any difference.

Chaining and patrolling tests have been used in the IC to study working memory. For example, Kobayashi et al. (2013), and they did not found differences between genotypes. In opposition to our results Poirier et al. (2007) found memory deficits in water maze and 8-arms radial maze. As argued before, in the IC the animals do not need adaptation to the new environment and this could explain the absence of learning deficits observed in our animals.

According to Voikar et al. (2010) air puff avoidance, chaining and patrolling are hippocampus-dependent tasks, as well as reported by Morice et al. (2013) with regards to consolidation of trace fear memory and reconsolidation of contextual fear memory in KO mice. Also, water maze and 8-radial maze are hippocampus-dependent tasks. Nevertheless, the difference in learning performance of KO mice in the IC compared with conventional tests is not possible to be explained by hippocampus-dependency of the tasks, and in object recognition, hole board task and taste aversion learning the performance of KO mice is not different compared with WT mice (Poirier et al. 2007).

4.3 *Rsk2*-deficiency affects the expression of various genes in prefrontal cortex and striatum

Statistical analyses of qRT-PCR data revealed significant differences of the expression of various genes in the PFC and STR. The expression of *Mapk3/Erk1, Drd2, Bdnf1* and *Crh* is shown to be significantly reduced in the PFC, and the expression of *Adra2a* and *Maob* is significantly increased in the STR of KO compared to WT mice. Moreover, we found a tendency of increased expression of *Fkbp5* and *Igf1* in the STR of KO mice. We did not find any differences in the relative gene expression of *5htr1a, Comt* and *Mapk 1/Erk2* in all different brain regions studied.

Abnormal axonal innervation was found in mutants defective in *Drosophila rolled*, a gene homologous to human MAPK3. Dysfunction of this gene can cause alterations in the neural circuit connectivity that may lead to alterations in neural function predisposing individuals to present autism spectrum disorder (Park et al. 2017). The role of MAPK3 seems to be essential for the correct functioning of the central nervous system and as we found, a reduced expression of *Mapk3/Erk1* in the PFC could suggest an alteration in the neural function of KO mice. Nevertheless, only one brain region was analyzed.

Furthermore, the two MAPK/ERK isoforms, ERK1 and ERK2 are highly expressed in mature neurons of the central nervous system. It has been reported that they are implicated in mammalian synaptic plasticity and learning. ERK activation is a pivotal part of the biochemical cascades to establish behavioral plasticity according to Sweatt (2001). In addition, the ERK/MAPK pathway plays an essential role strengthening contextual fear memory consolidation and reconsolidation and enhancing extinction (Cestari et al. 2014).

As the ERK signaling pathway was found to be impaired in depressed animals (Qi et al. 2009) and it may be a major element of depression-related molecular mechanisms. The inhibition of the ERK pathway in the medial prefrontal cortex causes anhedonia and loco motor deficits in rats and a decreased phosphorylation of the cyclic AMP-responsive-element-binding protein (CREB) in the same region (Qi et al. 2009). Our results indicate a reduced relative expression of *MapK3/Erk1* in the PFC. Nevertheless, our animals did not show signals of anhedonia in the sucrose test in the IntelliCage and, on the contrary, they seemed to be hyper-hedonic and were hyperactive. However, we did not measure MAPK-phosphorylation that could be pivotal for the interpretation of the results.

Drd2 gene encodes the D2 subtype of the dopamine receptor. We had special interest in investigating this gene due to a hyperdopaminergia reported in the cortex of *Rsk2*-deficient mice (Pereira et al. 2008; Poirier et al. 2007). Pereira et al. (2008) found increase level of DA in the brain cortex and a high level expression of DrD2 receptor for the long form and dopamine transporter. Nevertheless, our results indicate that the relative expression of *Drd2* is decreased in the prefrontal cortex of *Rsk2*-deficient mice suggesting that there is an alteration of *Drd2* expression in the cortex.

The function of the *Drd2* receptors in the PFC is associated to cognitive flexibility in tests that involve reversal rules (Rinaldi et al. 2007). In our results, the relative expression of *Drd2* is decreased in the PFC. Nevertheless, in the tests applied in the IntelliCage that included reversal tasks we did not find significant differences between genotypes. In other words, the low expression of this gene does not seem to affect the reversal learning in KO.

When D1 and D2 receptor antagonists are injected in the PFC, these selectively affect spatial learning in mice (De Steno and Schmauss, 2009). No difference between genotypes with regarding spatial learning was demonstrated in the experiments carried out in the IntelliCage.

Reduction in *Bdnf1* expression is congruent with a reduced cognitive function given the role of *Bdnf* in neural plasticity and central neural system development. Haplo-insufficiency of *Bdnf* is linked to reduced cognitive abilities in human subjects with WAGR (Wilms tumor, Aniridia, Genitourinary anomalies and mental Retardation) syndrome (Han et al. 2013). However, our findings showed a reduction in *Bdnf1* expression in the PFC together with a possible anti-depressive phenotype observed in behavioral tests.

It has been reported that the chronic exposure to high levels of CRH and corticosterone can cause neurological impairments and anxiety in mice (McGill et al. 2006). Acute restraint stress increases *Crh* mRNA in the PFC and glucocorticoid may inhibit the expression of *Crh* mRNA in the PFC (Meng et al. 2011). We detected decreased levels of *Crh* in the PFC and similar concentrations of glucocorticoids in the blood plasma, ruling out the possibility that increased basal glucocorticoids levels maintain decreased levels of *Crh*. This could suggest that *Rsk2*-deficient mice have an innate resilience to develop stress-reaction and no influence of glucorticoids on stress-reactivity.

Fear extinction is mediated by a complex brain circuitry that includes the amygdala, hippocampus, and medial prefrontal cortex (mPFC), among others. Intracellular signaling associated with this phenomenon may be impaired in deficient fear extinction as it happened with reduced ERK activity in the brain areas mentioned (Singewald et al. 2015). The mPFC seems to be involved in the retrieval of extinction memories. D2 receptor in the PFC supports extinction but the administration of a less selective D2 receptor antagonist accelerated the extinction (Singewald et al. 2015). Our findings of reduced *Drd2* in the same region could partially explain the impairment of KO in the consolidation of trace fear memory and a mild deficit in reconsolidation of contextual fear memory found by Morice et al. (2013).

According to our results, *Adra2A* is increased in the STR of *Rsk2*-deficient mice. This is the first time that an alteration in the noradrenergic system of *Rsk2*-deficient mice is reported. α-2A-receptors are widely distributed in cortocolimbic structures, thalamus and basal ganglia. Adrenergic pathways have been suggested to play an important role in the control of motor behavior, mood, cognition and attention (Arnsten et al. 1998; Millan et al. 2001). Therefore, an alteration in adrenergic transmission is likely to partially explain behavioral alterations found in our animal model. It is known that activation of α2A receptors in the central nervous system produces a loco motor inhibition probably by inhibiting dopamine release (Philipp et al. 2002). Nevertheless, our animals showed an increase in loco motor activity and this difference could be explained by the other neurotransmission alterations found in our model.

It has been reported that neurotransmission signals that arrive to the PFC were enhanced by norepinephrine acting on α-2A-receptors (Kim at al. 2010). Moreover, α2A adrenoreceptors signaling weakening in the PFC may underlie the symptomology of attention deficit/hyperactivity deficit (ADHD) (Arnsten, 2006). And α-2A-receptors are tonically active inhibitory auto receptors on adrenergic neurons (Millan et al. 2001). Also, α-2A-receptors may be implicated in the inhibition of front cortical and subcortical dopaminergic pathways, as well as in corticolimbic serotonergic projections.

MaoB is increased in the STR of KO mice. Monoamine oxidases have two isoforms, A and B, and are the enzymes that catalyze the oxidative deamination of serotonin, dopamine and noradrenaline in the central and peripheral nervous system. MAOB is mainly localized in glial cells and linked to Parkinson's disease, as dopamine was found to be decreased in the nigro-striatal area of patients (Nagatsu and Sawada, 2006). In different species MAOA and B act differently. Dopamine in mice is metabolized by MAOA in basal state but under high concentrations of dopamine it is metabolized by MAOA and MAOB, and in rats dopamine is always metabolized by MAOA regardless the dopamine concentration (Fornai et al. 1999). In humans dopamine is primarily metabolized by MAOB in the cortex and caudate (Garrick and Murphy, 1980). This increased *MaoB* expression in the STR could be related to the dopamine dysregulation in the central nervous system of *Rsk2* KO mice.

We also found a trend of increased expression of *Igf1* and *Fkbp5* in the STR. Increased expression levels of *Igf1* are in line with the study of Mehmood and coworkers (2011) who presented increased *Igf1* expression in the hippocampus of *Rsk2* KO mice. It is known that FKBP51 plays an essential role in human response to stress (O'Leary et al. 2013). Nevertheless, there is no information about the link of this protein and the association to stress response in our mice model.

Our results showed a trend level for increased *Crhr1* in the hippocampus of KO mice. According to Meng et al. (2011) in primary cell culture CRH induces an increase in CRHR1 expression through the MEK/ERK1/2 pathway. This could indicate that this pathway is altered in our mice model.

In summary, the findings offer interesting opportunities to further explore possible mechanisms underlying the behavioral phenotype observed in the mice model that is likely to resemble the mechanisms underlying the clinical manifestation of the CLS.

4.4 Tissue levels of metabolites related to the dopaminergic and noradrenergic system were found altered in prefrontal cortex and hippocampus of *Rsk2*-deficient mice

We found differences in the tissue concentration in PFC and HIPPO only of the metabolites but not of the respective neurotransmitters measured. We did not find any changes in the amygdala and hypothalamus, neither change of neurotransmitter tissue concentrations nor of their metabolites.

The most notorious differences were found in the dopaminergic system. DOPAC was shown to be significantly increased in PFC and HIPPO of KO compared to WT mice, and HVA concentrations were decreased at trend level in the MTC of KO. Also, increased concentrations 5-HIAA at trend-level was detected in the PFC, STR, and raphe nuclei of KO mice and MHPG was significantly decreased in the HIPPO of KO.

The dopaminergic system appears to have an important role in the PFC with relation to the control of loco motor activity. Destructions of the mesocortical dopaminergic projections in rats result in increased motor activity indicating that one function of dopamine in the PFC is to suppress loco motor activity (Suzuki et al. 2010). Nevertheless, in spite of the increased DA turnover in the PFC we did not find a decrease in spontaneous loco motor activity. On the contrary, we observed increased loco motor activity in LDB.

DA is a powerful player in the regulation of PFC-dependent functions, is widely expressed in the central nervous system and the different subtypes have a laminar-specific distribution within the PFC. Alterations of DA transmission in the PFC has been linked to diseases like schizophrenia, ADHD and PTSD (Xing et al. 2016). DA plays an important role in executive functions like working memory and inhibitory response control (Robbins and Arnsten, 2009).

Also, the mesocortical DA system can be selectively activated by stress (Thierry et al. 1976), whereas a rise of DA in the PFC is suggested to be rather a coping strategy than a stress response (Robbins and Arnsten, 2009). According to Suziku et al. (2010) DA metabolites are increased in the PFC in response to prenatal exposure to low concentration of suspended particulate matter from diesel as a stressor. However, levels of serum corticosterone were not altered in this experiment which indicates that the dopaminergic response to stress is independent from the pituitary-adrenal-cortical axis. The increased DA turnover in the PFC in our non-stressed animals should not be interpreted as if they are basally stressed because corticosterone levels are normal.

A trend level of decreased HVA was found in the MTC of KO suggesting that DA turnover is reduced in KO motor cortex. DA in the MTC is involved in neural plasticity

and acquisition of motor skills (Rioult-Pedotti et al. 2015). Therefore, motor skills acquirement could be impaired in our model which could have affected some of our results but this has to be further tested.

We found increased concentrations of the 5-HT metabolite 5-HIAA as a trend-level in the PFC, STR, and raphe nuclei of KO mice. Interestingly, it has been reported that autistic children have significantly higher serum serotonin levels than healthy controls (Mostafa and Al-Ayadhi, 2011). Also, research has detected an association between serum 5-HT levels and severity of autism. It is known that in autism motor control and learning are affected, mechanisms also affected in CLS. Nevertheless, is difficult to determine whether the hyperserotonemia (large amount of serotonin in the circulating blood) is consequence of autism or if it has a pathogenic role in the disease (Mostafa and Al-Ayadhi, 2011).

It has been suggested that changes in DOPAC levels may respond to alterations in the noradrenergic activity (Winstanley et al. 2006). Our results show that the noradrenalin metabolite MHPG is significantly decreased in hippocampus of KO mice. Male *translin* KO, a model that possibly shares several features with mice lacking fragile X mental retardation protein (FMRP), has a decreased anxiety-like behavior and hyperactivity, among others. NE was detected to be significantly reduced in the HIPPO and cortex and the NE metabolite MHPG was significantly reduced in both brain regions resembling what we found in our KO animals. Also *translin* KO presented a decreased anxiety-related behavior, exaggerated fear responses and defensive behaviors like freezing, jumping and escape when threatened. This behavioral and neurochemical resemblances are worthy to study because NE has an important role with regards to selective attention and behavioral flexibility, and a reduction in the cortical and hippocampal NE may contribute to increase escape latency in the Morris water maze observed in male *translin* KO (Stein et al. 2006) in a similar way that reduced NE turnover in RSK2 deficient mice HIPPO could be possible related to their altered behavior in the PST. Moreover, reduced levels of NE may predispose *translin* KO mice to present seizures (Stein et al. 2006). Interestingly, CLS patients have also been reported to be predisposed to seizures.

In KO mice the metabolites of monoamines neurotransmitters where altered suggesting an increased or decreased activity of this neurotransmission systems that could be implicated in altered behavior observed in these animals and possibly resembling what happens in this transmission system in CLS patients.

4.5 Adult hippocampal neurogenesis was not influenced by lifelong *Rsk2* deficiency

According to the stereological quantification of MCM2-, DCX- and BrdU-immunoreactive cells with subsequent statistical analysis of the density of these immuno-positive cells, we studied AN in the hippocampus. As we did not find significant differences between Rsk2-KO and WT mice analyzing all three AN markers, AN does not seem to be influenced by lifelong *Rsk2* deficiency. Hippocampal AN plays a key role in structural plasticity and network adaption and it possibly contributes to learning and memory processes (Aimone et al. 2010; Kempermann et al. 2004). Thus, the lack of effect of *Rsk2*-deficiency was unexpected. However, we used naïve mice for our AN study, therefore, we cannot rule out that hippocampal AN is altered in Rsk2 KO

mice experiencing stress or after performing a learning task. Furthermore, RSK2 is highly expressed in mouse brain in the hippocampus suggesting a pivotal role of RSK2 in hippocampal function (Pereira et al. 2009, Mehmood et al. 2011, Zeniou et al. 2002). RSK2 regulates cell survival through transcription-dependent mechanisms and more specifically, RSK2 promotes the survival of primary neurons by the increasing of CREB-dependent transcription of survival promoting genes (Xing et al. 1996). This indicates that the survival of primary neurons may be altered *in Rsk2*-deficient mice; however, the survival paradigm applied of BrdU treatment allowed us to analyze survival of newborn neurons yet the neuronal phenotype of these newborn cells was not analyzed. Reduced levels of RSK2 in cultured cortical precursor disrupt embryonic neurogenesis and the expression and activity of RSK2 in precursor cells indicates the essential role of RSK2 for embryonic neurogenesis (Dugani et al. 2010). By contrast, we did not find alterations in AN in KO mice. We did not analyze the neural plasticity phenomena in depth and additional experiments could have been helpful. In addition to this basic investigation, to analyze other aspects such as dendrite arborization, dendrite spine number and structure may help to study more in detail the effect of *Rsk2* deficiency in neural plasticity.

In summary, we may confirm that *Rsk2* deficiency affects behavioral and molecular functions. We have confirmed that behavioral characteristics of *Rsk2*-deficient mice made them an interesting model to study the Coffin-Lowry syndrome. Also, we have extended the behavioral characterization of the model showing that these animals present alterations at emotional level, mainly a possible anti-depressive and hyper hedonic phenotype. This is relevant because little is known in terms of emotional and psychiatric aspects of the patients, and also because this model could be useful in the study of depression. Furthermore, we have extended the characterization of the model at a molecular level, opening new opportunities to study and understand the pathophysiological basis of the Coffin-Lowry syndrome.

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Supplement

Behavioral study

Result data of the battery test, statistical calculation of EPM, LDB, OF and PST are presented as arithmetic means ± SEM. Pair-wise comparisons were performed applying the Mann-Whitney U- test. #: p-value ≤0.1 and >0.05; *: p-value ≤0.05 (indicated by arrows).

Elevated plus maze (EPM)			
Behavioral parameter measured	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P. Value
Distance CA (cm)	419,3 ± 43,78	495,2 ± 60,07	0,4025
Distance center (cm)	232,6 ± 18,38	332,0 ± 117,8	0,7950
Distance OA (cm)	679,6 ± 73,87	616,1 ± 65,80	0,5067
Global distance moved (cm)	1332 ± 74,78	1313 ± 101,7	0,9770
Time CA (ms)	86159 ± 12118	101608 ± 14310	0,4357
Time center (ms)	64685 ± 10600	46958 ± 4693	0,2145
Time OA (ms)	149157 ± 16671	152105 ± 16730	0,9310
Total nº visit arms	23,00 ± 1,728	23,75 ± 2,802	1,0000
Nº visit CA	9,000 ± 1,435	10,08 ± 1,559	0,6221
Nº visit OA	14,00 ± 1,365	13,67 ± 1,940	0,7285

CA: close arm, OA: open arm, cm: centimeter, nº: number, ms: millisecond.

Light dark box (LDB)			
Behavioral parameter measured	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P. Value
Distance moved in dark (cm)	1008±75,47	1296±113,0	0,1166
Distance moved in light (cm)	1148±99,40	1541±108,5	0,0210↑
Total distance moved (cm)	2156±125,7	2837±92,78	0,0003↑
Time spent dark (ms)	135700±8857	135826±9478	0,8294
Time spent light (ms)	164301±8861	164044±9517	0,8294
Nº visit dark	8,545±0,8672	9,417±0,7330	0,4742
Nº visit light	8,273±0,8538	9,333±0,7213	0,4020

cm: centimeter, ms: millisecond, nº: numb

Open field (OF)			
Behavioral parameter measured	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P. Value
Center entries n°	4,068 ± 0,2955	4,175 ± 0,3636	0,6890
Distance center (cm)	89,73 ± 7,290	91,85 ± 10,29	0,9755
Distance periphery (cm)	221,3 ± 13,30	243,5 ± 27,50	0,9264
Global distance (cm)	311,1 ± 13,83	335,3 ± 32,09	0,8777
Nº rearing center/min	0,7682 ± 0,1982	1,255 ± 0,2315	0,0871↓
Nº rearing periphery/min	3,882 ± 0,2027	4,845 ± 0,6246	0,2596
Time center (ms)	15300 ± 1691	15408 ± 1798	0,9264
Time periphery (ms)	44294 ± 1679	44296 ± 1786	0,9264
Time rearing center (ms)	1145 ± 336,5	1813 ± 357,2	0,0980
Time rearing periphery (ms)	6914 ± 388,3	8201 ± 1177	0,7513
(total) Average nº rearings/min	4,650 ± 0,2491	6,100 ± 0,6650	0,0723↑

ms: milisecond, cm: centimeter, nº: number

Porsolt swim Test (PST)			
Behavioral parameter measured	WT	KO	Mann-Whitney test
5 min time of evaluation	Mean ± SEM	Mean ± SEM	P. Value
Time floating (s)	181,5 ± 6,483	142,8 ± 15,22	0,0376↓
Time swimming (s)	90,00 ± 8,250	88,92 ± 6,482	0,8690
Time climbing (s)	29,40 ± 7,847	69,50 ± 16,89	0,0440↑
Latency floating (s)	69,10 ± 12,01	121,3 ± 16,32	0,0208↑
1 min time of evaluation			
Active time(s)/ min 2	29,00 ± 4,996	48,33 ± 4,809	0,0111↑

Second:s

Gene expression study

Data are presented as arithmetic means ± SEM. Pair-wise comparisons were performed applying the Mann-Whitney U- test. #: p-value ≤0.1 and >0.05 (indicated by arrows); *: p-value ≤0.05 (indicated by arrows in bold). *5htr1a (5-Hydroxytryptamine Receptor 1A) Adra2A (Adrenoceptor alpha 2A), Bdnf1 (Brain-Derived neurotrophic factor 1), Comt (Catechol-O-methyltransferase), Crh (Corticotropin releasing hormone), Crhr1(Corticotropin releasing hormone receptor 1), Drd2 (Dopamine receptor D2), Fkbp5 (FK506 binding protein 5), MaoB (Monoamine oxidase B), Igf1 (Insulin-like growth factor 1), Mapk1/Erk2 (Mitogen-activated protein kinase 1)* and *Mapk3/Erk1 (Mitogen-activated protein kinase 3).* WT=Wildtype and KO=knockout.

Prefrontal Cortex			
Gene name	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
5htr1a	1,09 ± 0,08	1,17 ± 0,08	0,393
Adra2A	1,20 ± 0,12	1,14 ± 0,16	0,578
Bdnf1	1,59 ± 0,17	1,12 ± 0,08	**0,044** ↓
Comt	1,05 ± 0,04	0,99 ± 0,05	0,352
Crh	1,28 ± 0,11	0,88 ± 0,10	**0,035**↓
Crhr1	1,16 ± 0,11	0,98 ± 0,07	0,307
Drd2	1,05 ± 0,11	0,79 ± 0,15	**0,043**↓
Fkbp5	1,04 ± 0,10	0,93 ± 0,08	0,646
MaoB	1,18 ± 0,12	0,96 ± 0,06	0,324
Igf1	1,14 ± 0,06	1,02 ± 0,05	0,204
Mapk1/Erk2	1,08 ± 0,08	0,94 ± 0,04	0,361
Mapk3/Erk1	1,17 ± 0,11	0,86 ± 0,07	**0,044**↓

Motor Cortex			
Gene name	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
5htr1a	1,06 ± 0,08	1,21 ± 0,07	0,167
Adra2A	1,01 ± 0,11	1,13 ± 0,12	0,742
Bdnf1	1,18 ± 0,09	1,10 ± 0,10	0,511
Comt	0,97 ± 0,04	1,01 ± 1,12	0,645
Crh	1,44 ± 0,16	1,42 ± 0,15	0,879
Crhr1	1,03 ± 0,09	0,95 ± 0,09	0,554
Drd2	0,80 ± 0,09	0,78 ± 0,09	0,973
Fkbp5	0,90 ± 0,08	0,96 ± 0,09	0,645
MaoB	1,09 ± 0,06	1,00 ± 0,08	0,393
Igf1	1,01 ± 0,09	0,98 ± 0,06	0,895
Mapk1/Erk2	1,00 ± 0,07	0,94 ± 0,06	0,470
Mapk3/Erk1	0,96 ± 0,08	0,81 ± 0,07	0,167

Hippocampus			
Gene name	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
5htr1a	1,18 ± 0,05	1,24 ± 0,09	0,549
Adra2A	1,42 ± 0,17	0,97 ± 0,10	0,052
Bdnf1	1,21 ± 0,05	1,29 ± 0,10	0,378
Crh	1,32 ± 0,09	1,10 ± 0,13	0,105
Crhr1	0,93 ± 0,09	0,69 ± 0,05	0,065↓
Drd2	0,93 ± 0,09	0,80 ± 0,04	0,356
Fkbp5	1,05 ± 0,08	1,14 ± 0,05	0,503
MaoB	1,01 ± 0,04	1,01 ± 0,06	0,860
Igf1	1,00 ± 0,03	0,98 ± 0,03	0,698
Mapk1/Erk2	1,04 ± 0,04	0,98 ± 0,04	0,503
Mapk3/Erk1	1,03 ± 0,06	0,93 ± 0,03	0,113

Striatum			
Gene name	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
5htr1a	0,90 ± 0,17	0,98 ± 0,11	0,352
Adra2A	0,87 ± 0,17	1,31 ± 0,11	**0,022**↑
Bdnf1	1,19 ± 0,23	0,93 ± 0,21	0,474
Comt	1,01 ± 0,06	1,08 ± 0,03	0,352
Crh	0,84 ± 0,09	0,84 ± 0,03	0,888
Crhr1	0,94 ± 0,16	1,11 ± 0,12	0,694
Drd2	2,38 ± 0,15	2,26 ± 0,17	0,578
Fkbp5	0,96 ± 0,10	1,22 ± 0,08	0,075↑
MaoB	0,88 ± 0,05	1,16 ± 0,06	**0,005**↑
Igf1	0,76 ± 0,06	0,94 ± 0,07	0,075↑
Mapk1/Erk2	0,98 ± 0,05	1,04 ± 0,03	0,315
Mapk3/Erk1	0,99 ± 0,08	0,98 ± 0,04	0,911

Cerebellum			
Gene name	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
5htr1a	0,63 ± 0,33	3,15 ± 2,58	0,234
Adra2A	0,93 ± 0,13	1,35 ± 0,37	1,000
Bdnf1	0,54 ± 0,11	1,50 ± 1,07	0,612
Comt	0,94 ± 0,12	1,01 ± 0,05	0,955
Crh	0,37 ± 0,07	3,18 ± 2,72	0,315
Crhr1	1,56 ± 0,25	1,42 ± 0,13	0,536
Drd2	0,88 ± 0,34	2,50 ± 1,92	0,730
Fkbp5	0,76 ± 0,17	1,07 ± 0,34	0,535
MaoB	0,84 ± 0,09	1,10 ± 0,08	0,152
Igf1	1,04 ± 0,09	1,19 ± 0,04	0,252
Mapk1/Erk2	0,82 ± 0,08	0,91 ± 0,04	0,918
Mapk3/Erk1	0,88 ± 0,11	1,05 ± 0,16	0,837

Neurotransmitters study

Tissue levels of the neurotransmitters DA, 5-HT, NA as well as their metabolites DOPAC, HVA, 5-HIAA, and MHPG (ng/g wet tissue weight) in prefrontal cortex, motor cortex, amygdala, striatum, hypothalamus, hippocampus, raphe nuclei and cerebellum of WT and KO mice. Data are presented as arithmetic means ± SEM. Pair-wise comparisons were performed applying the Mann-Whitney U- test. #: p-value ≤0.1 and >0.05; *: p-value ≤0.05 (indicated by arrows). DA (Dopamine); 5HT (Serotonin5-hydroxytryptamine), NA (Noradrenalin/Norepinephrine) DOPAC (3,4-Dihydroxyphenylacetic acid) 5HIAA (5-Hydroxyindoleacetic acid), HVA (Homovanillic acid) and MHPG (3-Methoxy-4-hydroxyphenylglycol). WT=Wildtype, KO=Knock-out.

Prefrontal cortex			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DA	43,65 ± 6,994	52,86 ± 8,445	0,4487
DOPAC	34,70 ± 5,367	72,37 ± 10,51	0,0424↑
5HT	68,00 ± 17,48	74,37 ± 19,53	1,0000
5HIAA	166,9 ± 30,75	266,5 ± 45,52	0,0727↑
NA	104,4 ± 16,36	108,9 ± 28,31	0,7879
MHPG	61,95 ± 7,989	51,83 ± 5,998	0,6485

Motor cortex			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DOPAC	36,00 ± 6,893	46,23 ± 11,62	0,9538
HVA	72,78 ± 11,42	113,1 ± 16,14	0,0721↑
5HT	114,8 ± 18,81	96,03 ± 16,82	0,6943
5HIAA	223,7 ± 32,67	225,3 ± 49,36	1,0000
NA	97,13 ± 28,28	115,7 ± 30,42	0,2319
MHPG	68,78 ± 8,271	51,49 ± 16,76	0,1520

Amygdala			
Neurotransmitter / Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DA	445,2 ± 88,26	374,4 ± 73,58	0,6058
DOPAC	174,9 ± 23,37	199,9 ± 31,14	0,3704
HVA	185,1 ± 30,24	192,8 ± 33,12	0,9626
5HT	202,6 ± 40,38	235,9 ± 47,12	0,6730
5HIAA	307,2 ± 44,15	383,1 ± 54,68	0,2766
NA	87,28 ± 27,04	122,2 ± 27,24	0,3704

Striatum			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DA	8202 ± 678,0	7608 ±1080	0,4499
DOPAC	2225 ± 274,6	2180 ± 116,8	0,6247
HVA	1076 ± 127,4	1083 ± 153,4	0,8968
5HT	210,8 ± 24,00	307,1 ± 44,13	0,1011

5HIAA	330,7 ± 40,13	425,5 ± 34,65	0,0831↑
NA	71,45 ± 18,99	59,56 ± 12,73	0,5936
MHPG	55,53 ± 9,976	89,36 ± 17,30	0,2370

Hypothalamus			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DA	160,4 ± 35,63	135,7 ± 31,53	0,6431
DOPAC	104,3 ± 14,27	117,4 ± 23,19	0,7282
HVA	183,7 ± 22,04	159,5 ± 31,24	0,6126
5HT	152,3 ± 37,31	197,5 ± 53,88	0,3357
5HIAA	425,5 ± 65,10	485,5 ± 82,40	0,6126
NA	294,1 ± 88,16	309,6 ± 72,03	0,8621
MHPG	65,60 ± 16,57	41,66 ± 7,740	0,3850

Hippocampus			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DA	38,04 ± 4,715	41,58 ± 6,386	0,7197
DOPAC	19,82 ± 2,554	36,72 ± 6,081	0,0435↑
HVA	77,58 ± 10,71	59,64 ± 8,165	0,3562
5HT	214,5 ± 32,40	198,2 ± 34,60	0,7197
5HIAA	457,5 ± 66,25	428,1 ± 62,67	0,8421
NA	143,4 ± 46,39	169,2 ± 36,39	0,4967
MHPG	90,80 ± 13,77	50,20 ± 9,814	0,0220↓

Cerebellum			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DOPAC	15,44 ± 2,549	17,90 ± 2,817	0,8382
5HT	54,76 ± 6,947	53,74 ± 8,011	0,7750
5HIAA	153,2 ± 31,59	144,3 ± 30,80	0,9682
NA	114,7 ± 44,63	116,6 ± 29,64	0,5955
MHPG	53,73 ± 10,21	61,74 ± 10,96	0,4002

Raphe nuclei			
Neurotransmitter/ Metabolite	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P-value
DA	70,40 ± 14,34	63,73 ± 13,05	0,9497
DOPAC	55,07 ± 7,466	52,53 ± 7,825	0,9497
HVA	95,27 ± 13,58	96,95 ± 15,94	0,9497
5HT	246,0 ± 49,14	267,4 ± 47,04	0,9485
5HIAA	616,9 ± 140,8	853,9 ± 89,76	0,0813↑
NA	242,8 ± 74,70	313,7 ± 36,88	0,4136
MHPG	64,90 ± 13,90	51,35 ± 8,405	0,4908

Adult neurogenesis study

Data are presented as arithmetic means ± SEM. Pair-wise comparisons were performed applying the Mann-Whitney U- test. Total hippocampus: sum of SGZ and GCL. Nº of the estimated population: parameter given by Stereoinvestigator software, used to calculate the density.

Minichromosome maintenance complex component 2 (MCM2)-positive cells			
	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P. Value
Density:			
Total hippocampus	31430 ± 5358	31383 ± 4570	1,0000
Anterior hippocampus	14127 ± 2439	13352 ± 2223	0,9626
Posterior hippocampus	17303 ± 3491	18030 ± 2467	0,6730
Nº of estimated population:			
Total hippocampus	10615 ± 2177	12445 ± 2062	0,5414
Anterior hippocampus	4169 ± 1017	4484 ± 886,0	0,8884
Posterior hippocampus	6446 ± 1585	7960 ± 1313	0,2359

Doublecortin (DCX)-positive cells			
	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P. Value
Density:			
Total hippocampus	13151 ± 1039	13758 ± 711,8	0,5457
Anterior hippocampus	15557 ± 1060	16369 ± 684,9	0,6665
Posterior hippocampus	10885 ± 481,9	12295 ± 807,4	0,2581
Nº of estimated population:			
Total hippocampus	18250 ± 1326	19312 ± 909,2	0,1903
Anterior hippocampus	8140 ± 541,8	8404 ± 398,9	0,9314
Posterior hippocampus	10110 ± 986,3	10908 ± 639,3	0,2973

5-bromo-2`-deoxyuridine (BrdU)-positive cells			
	WT	KO	Mann-Whitney test
	Mean ± SEM	Mean ± SEM	P. Value
Density:			
Total hippocampus	559,0 ± 61,69	499,8 ± 66,75	0,4174
Anterior hippocampus	668,3 ± 81,01	604,5 ± 107,5	0,7430
Posterior hippocampus	449,7 ± 79,58	395,0 ± 68,04	0,5414
Nº of estimated population:			
Total hippocampus	165,5 ± 20,54	169,2 ± 27,13	0,8495
Anterior hippocampus	183,7 ± 28,64	201,8 ± 43,21	0,9626
Posterior hippocampus	147,3 ± 29,90	136,6 ± 31,54	0,6058

Acknowledgments

Affidavit

I hereby confirm that my thesis entitled “From behavior to neurobiological characterization of *Rsk2*-knockout mice as an animal model for Coffin-Lowry syndrome” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Santiago, January 2018.

Place, Date Signature

Curriculum Vitae