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CDKL5 knockout leads to altered inhibitory transmission in the cerebellum of adult mice.

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1 ABSTRACT

2
3 Mutations in the X-linked cyclin-dependent kinase-like 5 gene (CDKL5) are associated to
4 severe neurodevelopmental alterations including motor symptoms. In order to elucidate the
5 neurobiological substrate of motor symptoms in CDKL5 syndrome, we investigated the motor
6 function, GABA and glutamate pathways in the cerebellum of CDKL5 knock-out female
7 mice.

8 Behavioural data indicate that CDKL5-KO mice displayed impaired motor coordination on
9 the Rotarod test, and altered steps, as measured by the gait analysis using the CatWalk test. A
10 higher reduction in spontaneous GABA efflux, than that in glutamate, was observed in
11 CDKL5-KO mouse cerebellar synaptosomes, leading to a significant increase of spontaneous
12 glutamate/GABA efflux ratio in these animals. On the contrary, there were no differences
13 between groups in K⁺-evoked GABA and glutamate efflux. The anatomical analysis of
14 cerebellar excitatory and inhibitory pathways revealed a selective defect of the GABA-related
15 marker GAD67 in the molecular layer in CDKL5-KO mice, while the glutamatergic marker
16 VGLUT1 was unchanged in the same area. Fine cerebellar structural abnormalities such as a
17 reduction of the inhibitory basket “net” estimated volume and an increase of the pinceau
18 estimated volume were also observed in CDKL5-KO mice. Finally, the BDNF mRNA
19 expression level in the cerebellum, but not in the hippocampus, was reduced compared to WT
20 animals.

21 These data suggest that CDKL5 deletion during development more markedly impairs the
22 establishment of a correct GABAergic cerebellar network than that of glutamatergic one,
23 leading to the behavioural symptoms associated to CDKL5 mutation.

1 INTRODUCTION

2
3 Cyclin-dependent kinase-like 5 (CDKL5) is a gene located on the X chromosome also known
4 as serine/threonine kinase 9 (STK9) (Montini et al., 1998). Since early description of
5 chromosomal rearrangements involving this gene in two females (Kalscheuer et al., 2003),
6 around 150 individuals (with a large prevalence of females) with a CDKL5 mutation have
7 been described and diagnosed with the early-onset seizure variant of Rett syndrome (RTT),
8 autism, infantile spasms or West syndrome (Tao et al., 2004; Weaving et al., 2004). Thanks to
9 the International RTT syndrome phenotype database (InterRett), it has been established that
10 CDKL5 disorder can be considered as an autonomous clinical entity (Bebbington et al., 2008;
11 Fehr et al., 2013). The clinical features commonly associated with a CDKL5 mutation include
12 early-onset seizures, severe intellectual disability, and gross motor impairment, mainly
13 characterized by the delay in the ability to sit, independent walking, functional hand use,
14 motor dyspraxia leading in some cases to impaired ambulation, severe hypotonia (Bahi-
15 Buisson et al., 2008; Stalpers et al., 2012).

16 Little is known about the function and the mechanism of action of the protein. CDKL5
17 protein is involved in neuronal differentiation, dendritic arborization and synaptogenesis,
18 recognizing a number of substrates, and affecting several genes (Chen et al., 2010). CDKL5
19 expression is strongly induced in early postnatal stages, and in the adult brain CDKL5 is
20 present in mature neurons in the cerebral cortex, hippocampus, cerebellum and brain stem
21 (Rusconi et al., 2008). Loss-of-function studies using RNA interference revealed that CDKL5
22 is required for neurite growth and excitatory synapse stability through Rac signaling
23 remodelling the actin fibers of cytoskeleton (Chen et al., 2010; Ricciardi et al., 2012).
24 Mutations of CDKL5 resulted in the loss of kinase activity toward its substrates (Sekiguchi et
25 al., 2013), which includes the 120-kDa protein amphiphysin 1, a protein involved in clathrin-
26 mediated endocytosis; DNA methyltransferase 1 (DNMT1) (Kameshita et al., 2008), and
27 netrin-G1 ligand (NGL-1), a lipid-anchored protein that is structurally related to the netrin
28 family of axon guidance molecules (Ricciardi et al., 2012). CDKL5 also mediates the
29 phosphorylation of methyl-CpG-binding protein 2 (MeCP2) (Mari et al., 2005), the protein
30 encoded by MECP2 gene which is considered to be the primary cause of RTT (Amir et al.,
31 1999).

Two animal models lacking the CDKL5 gene have been generated up-to-now. The mouse generated by Wang et al. (2012) models a splice site mutation found in a CDKL5 patient by deleting the mouse CDKL5 exon 6 through homologous-mediated recombination in embryonic stem cells, thereby disrupting kinase activity. The mouse generated by Amendola et al. (2014) carries a constitutive knockout (KO) allele obtained by deletion of exon 4. The phenotypic characterization of these mice reveals autistic-like deficits in social interaction, impairments in fear memory, alterations in event-related potentials, disruption of multiple signal transduction pathways (Wang et al., 2012). Furthermore, limb clasping, hypoactivity, abnormal eye tracking, abnormal electroencephalograph responses to convulsant treatment, decreased visual evoked responses have been reported (Amendola et al., 2014). An impairment of hippocampus-dependent memory has been also described in this mouse (Fuchs et al., 2014). However, the neurobiological substrate of the complex behavioural phenotype produced by CDKL5 mutation in human and mice is still unclear. In the attempt to overcome this gap, in the present study we investigated the neurochemical organization of the cerebellum of CDKL5 KO female mice (Amendola et al., 2014), with particular attention to the balance between the excitatory glutamate and inhibitory GABA signalling. The cerebellum has been chosen in view of its role in movement planning and execution, and cognitive functions (Buckner et al., 2013). Furthermore, in both mice and human, CDKL5 is expressed in virtually all neurons, but not astrocytes, of the cerebellar granular (gl) and molecular (ml) layers, and Purkinje (Pj) cells (Rusconi et al., 2008).

MATERIAL AND METHODS

Animals and genotyping

Mice were produced by crossing CDKL5 heterozygous +/- female mice with CDKL5 Y/- male, and CDKL5 heterozygous +/- KO female mice with WT male mice (Amendola et al., 2014, the genetic background is C57BL/6J). The animals had access to water and food ad libitum and lived in a room with a 12:12 hour dark/light cycle. Adult homozygous female (-/-, N=17) and WT female (+/+, N=12) were used in this study, housed in groups of 3-4 animals *per* cage. Mice tails were used for genotyping analysis. The mice genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA MiniPrep Kit (Sigma-Aldrich, Milan, Italy)

according to the instructions of the manufacturer, and eluted in 100 µl of elution solution. DNA concentration was determined using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA USA). The presence of the DNA deletion was identified by standard PCR technique, using the enzyme GO TAQ Flexi DNA Polymerase (Promega, Milano, Italy) and specific primers. The sequences of the primers used for mice genotyping were the followings: 108F, F: 5'- ACGATAGAAATAGAGGATCAACCC -3'; 109R, F: 5'- CCCAAGTATACCCCTTTCCA -3'; 125R, F: 5'- CTGTGACTAGGGGCTAGAGA -3'. For each sample the reaction was performed in a mix containing Buffer 1x, MgCl₂ 1.5mM, dNTPs 0.2mM, primer 108F 1µM, primer 109R 0.5µM, primer 125R 0.5µM, Taq Polymerase 1.25U/µl and 100ng/ml of DNA. After 4 minutes at 95°C, 33 PCR cycles were performed (94°C 40 sec, 59°C 30 sec, 72°C 40 sec), followed by 7 minutes at 72°C. Resulting PCR products were resolved by electrophoresis in a 2.0% agarose gel stained with Gel Red™ (Biotium, Hayward, Canada), using a 100 bp DNA ladder (Fermenta, Milano, Italy) as DNA marker. The 249bp band represents the WT chromosome while the presence of a 344bp band is due to the deletion. Results were obtained crossing the mice gender with the resulting PCR bands, as follows: WT male = 240bp; CDKL5 Y/- male = 344bp; WT female= 240bp; CDKL5 heterozygous female = 344bp, 240bp; CDKL5 homozygous female = 344bp. All the animal protocols described here were carried out in compliance with the European Community Council Directives of 24 November 1986 (86/609/EEC) and approved by our intramural committee and Ministero Salute, in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals and ARRIVE guidelines.

Locomotion behavioural analysis

Cerebellar function was analysed by Rotarod and computerized gait analysis (Stroobants et al., 2013). Twelve animals were included in each group. Gait analysis was performed 7 days after RoraRod test. Tests were carried out in the light phase. To minimize stress, animals were individually handled daily for 10 min for 1 week before the behavioural experiments. Motor coordination ability was tested using the LE 8500 RotaRod (2Biological Instruments, Varese, Italy), which consists of a central drum (6cm diameter), gnarled to provide a suitable grip, elevated 20cm above the floor. Mice were placed gently on the drum for 3 consecutive training days, performing 4 trials each day, accelerating from 3.5 rpm to 35 rpm for a

maximum of 5 min with 1 h inter-trial interval. The latency to fall during the observation period was recorded (s) by the SEDACOM32 program (PanLab, Barcelona, Spain). The CatWalk apparatus (Noldus Information Technology, Wageningen, the Netherlands), comprising a long glass plate with a fluorescent light beamed into the glass walkway floor was used for computerized gait analysis, that was performed at least one week after the last RotaRod test (in animals 5.5month old). In a dim environment, the light is reflected downward and the footprints of the animal as it walks along the walkway are recorded by a camera mounted under the glass. Mice were trained to cross the walkway daily one week prior to the test. The mice performed uninterrupted runs for at least three times and the paw print footages were analysed by using the automated gait analysis system Cat Walk software, classified and analysed using the Catwalk XT module (Bozkurt et al., 2008). The following parameters were considered: “Print area” (the total floor area contacted by the paw during the stance phase) and “Base of support (cm)” (the distance between the two hindpaw or forepaw measured perpendicularly to the direction of walking) as spatial static parameters of each paw; “Run duration” (the duration of the run based on the selected steps in the data selection), “Stand” (the duration in seconds of contact of a paw with the glass plate), “Swing” (the duration in seconds of no contact of a paw with the glass plate), “Swing Speed” (the speed - distance unit/second- of the paw during swing) as kinetic parameters of each paw; “Stride Length” (the distance -in Distance Units- between successive placements of the same paw), “Step Cycle” (the time in seconds between two consecutive initial contacts of the same paw) as stride parameters; “Regularity index” (the number of normal step sequence patterns relative to the total number of paw placements) and “Cadence” (the number of steps) as gait coordination.

Spontaneous and K⁺-evoked endogenous GABA and glutamate efflux from cerebellum synaptosome preparation

Crude synaptosome (P2) fraction was prepared from the cerebellum of six animals from each groups under investigation. Briefly, on the day of the experiment a lightly anesthetized animal was sacrificed by decapitation and the cerebellum was dissected out from the brain. The tissue was then homogenized in ice-cold buffered (pH-7.4) sucrose solution (0.32 M). After the homogenate centrifugation (10 min; 2500 × g, 4 °C), the supernatant was collected and the

synaptosomes were isolated by centrifugation (20 min; $9500 \times g$, 4 °C). The P2 pellet fraction was resuspended in 5 ml of Krebs solution (mM: NaCl 118.5, KCl 4.7, CaCl₂ 1.2, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 10; gassed with 95% O₂/5% CO₂) (Chiodi et al., 2012). After their preparation, the synaptosomes were maintained in warmed (37°C) for 20 min. Thereafter, identical aliquots (0.5 ml) of synaptosomal suspension were distributed on microporous filters, placed at the bottom of a set of parallel superfusion chambers, maintained at 37 °C and continuously perfused with aerated (95% O₂/5% CO₂) Krebs solution (0.3 ml/min). After a 30 min wash-out period, 9 consecutives 5-min fractions were collected. In details, after the collection of three basal samples, synaptosomes were depolarized with 15 mM K⁺ for 90s.

In each sample, GABA and glutamate levels were simultaneously measured by HPLC coupled with fluorimetric detection. 30 µl/sample were transferred into glass microvials and placed in a temperature-controlled (4°C) Triathlon autosampler (Spark Holland, Emmen, The Netherlands). Before the injection, the system added 30 µl of o-phthaldialdehyde/mercaptoethanol reagent to each sample and, after 60 s of reaction, 40 µl of the mixture were injected onto a Chromsep analytical column (3 mm inner diameter, 10 cm length; Chrompack, Middelburg, The Netherlands). The column was eluted at a flow rate of 0.52 ml/min (Beckman 125 pump; Beckman Instruments) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). Glutamate and GABA were detected by means of a Jasco fluorescence spectrophotometer FP-2020 Plus (Jasco, Tokyo, Japan). The retention times of glutamate and GABA were ~3.5 and ~15.0 min, respectively.

GABA and glutamate efflux was expressed as pmol/min/g of protein while K⁺-evoked GABA and glutamate efflux was expressed as percent increase over the respective spontaneous efflux (calculated by the mean of the two fractions collected prior to the depolarising stimulus). Protein was determined according to Bradford (1976).

Immunohistochemistry, microscopy, confocal microscopy and image analysis.

Animals (six animals per group) were deeply anesthetized and perfused through the ascending aorta with physiological saline at 37°C (20 mL), followed by paraformaldehyde 4% (w/v) and 14% picric acid saturated aqueous solution in Sørensen buffer 0.1 M pH 7 at 37°C (20 mL)

1 and at ice-cold temperature (20 mL). The brains were then removed and immersed for 90 min
2 in the same ice-cold fixative, before being rinsed for at least 48 h in 5% sucrose in 0.1 M
3 phosphate buffer. Cerebella were frozen in CO₂ and 14 µm thick cryostat sagittal sections
4 (HM550 Microm, Bio-Optica, Milano, Italy) were collected on gelatin coated slides from
5 1.95 mm to 1.525 lateral level according to Paxinos and Franklin (2001).

6 Sections were firstly incubated in 0.1 M phosphate buffered saline (PBS) at room temperature
7 for 10–30 min, followed by overnight incubation at 4°C in a humid atmosphere with the
8 primary antibodies diluted in 0.3% PBS-Triton X-100, v/v. The followings antisera were
9 used: goat, anti-calbindinD-28K, Santa Cruz Biotechnology, (Dallas, USA), 1:75; rabbit anti-
10 Vesicular Glutamate Transporter 1 (VGLUT1), Abcam (Cambridge, United Kingdom),
11 1:1000; mouse anti-glutamic acid decarboxylase 67 (GAD-67), Chemicon (Merck-Millipore,
12 Darmstadt, Germany), 1:800; rabbit anti-post synaptic density protein 95 (PSD-95), Abcam,
13 1:1000; mouse anti-neurofilament 200 (NF-200), Sigma Aldrich, 1:1000. After rinsing in PBS
14 for 20 min (2 × 10 min), the sections were incubated at 37°C for 30 min in a humid
15 atmosphere with the secondary antisera conjugated with different fluorochromes:
16 DyLight488-conjugated affinity-pure Goat anti-Mouse IgG (ThermoScientific, Monza, Italy),
17 DyLight488-conjugated affinity-pure Donkey anti-Goat IgG (Jackson ImmunoResearch,
18 Suffolk, UK), Rhodamine Red™-X-conjugated-conjugated affinity-pure Donkey anti-Goat
19 IgG (Jackson ImmunoResearch) and Rhodamine Red™-X-conjugated affinity-pure Donkey
20 anti-Rabbit IgG (Jackson ImmunoResearch) diluted in 0.3% PBS-Triton X-100. Sections were
21 then rinsed in PBS (as above) and mounted in glycerol containing 1,4-phenyldiamine (0.1
22 g/l).

23 In order to minimize technical bias, the following technical features were taken: all sections to
24 be compared were processed in the same experimental session; 5 brains from different
25 experimental groups were included in each slide, according to a random distribution; the same
26 batch, dilution and preparation of primary such as secondary antibodies were used for all
27 slides to be compared; the same conditions were established for all sections. For all indicated
28 markers, three sections/animal were analyzed, and the mean value/animal was used for the
29 statistical analysis. All analyses were performed in blinded manner.

30 Immunofluorescence images were captured by a Nikon Eclipse E600 microscope (Nikon,
31 Shinjuku, Japan) equipped with digital CCD camera Q Imaging Retiga-2000RV (Q Imaging,
32 Surrey, BC, Canada). Measurements were performed using the NIS-Elements AR 3.2

software. The immunoreactive area was calculated as % area of the region of interest (ROI). The mean intensity was expressed as optical density (OD) of the immunoreactive signal, subtracted of the background.

For fine morphological analysis, sections were scanned with Nikon Ti-E fluorescence microscope coupled with A1R confocal system. Diode laser system with 405 wavelength output, air-cooled argon-Ion laser system with 488 wavelength output, yellow diode-pumped solid state laser system with 561 wavelength output and diode laser system with 638 wavelength output were used. Images were acquired with oil immersion 60x with an optical resolution of 0.18 micron, using NIS-Elements AR 3.2 software (Nikon). All the z-stacks were collected in compliance with optical section separation (z-interval) values suggested by NIS-Elements AR 3.2 software. All images to be compared were acquired using the same microscope configuration.

For the analysis of GAD-67 and VGLUT1 synaptic contacts, images were acquired with 4x scanner zoom and 1024x1024 pixels resolution as confocal stacks with optical section separation (z-interval) of 0.4 micron and a z-volume of 6 micron. For the analysis of NF-200 around Purkinje cell body, images were acquired with 512x512 pixels resolution as confocal stacks with optical section separation (z-interval) of 0.2 micron and a z-volume of 8 micron. For the analysis of PSD-95, images were acquired with 1024x1024 pixels resolution as confocal stacks with optical section separation (z-interval) of 0.3 micron and a z-volume of 8 micron.

The number of synaptic spots was measured by 3D voxel-based reconstruction followed by spot detection and count by Imaris BitPlane software 7.7.2 version (Bitplane AB, Saint Paul, MN, USA). The NF200- and PSD95-IR was detected by 3D voxel-based reconstruction on calibrated images, followed by filament and surface recognition, respectively, by Imaris BitPlane software, and finally referred as “estimated volume”. 3D rendering of surfaces, spots and filaments are based on the identification of voxels belonging to an object (detected by their gray values). To create detailed surfaces and spots detection, we used confocal z stacks as source for Imaris 3D reconstruction and analysis. See results for further details.

Total RNA isolation, reverse transcription and mRNA quantification

1 The cerebellum (six animals per group) was homogenized and total RNA isolation was
2 performed using RNAeasy Mini Kit[®] (Quagen, Milano, Italy). Total RNA was eluted in
3 RNase-free water and using a spectrophotometer (Nanodrop 2000, Thermo Scientific)
4 absorbance values at 260, 280 and 320 were measured.

5 RNAs were subjected to DNase treatment (1 U/μl, 1x DNase buffer, 2 U/μl ribonuclease
6 inhibitor, at 37°C for 30 min) (Fermentas) and were retrotranscribed using the enzyme M-
7 Moloney murine leukemia virus reverse transcriptase (M-MuLV-RT, 10U/μl) (Fermentas), in
8 the presence of 1x first strand buffer, 1mM d(NTP)s (Fermentas), 25 ng/μl Oligo (dT)₁₈
9 primers (Fermentas) by incubating at 42°C for 60 min. A sample with no reverse transcriptase
10 enzyme in the reaction mix was processed as NO-RT control.

11 Semi-quantitative real-time PCR was performed using the Mx3005P[™] real-time PCR system
12 (Stratagene, Santa Clara, CA, USA). The chemistry chosen to perform these PCR
13 experiments was based on SYBR Green I fluorescent detection. Each reaction mix consisted
14 of 10 ng of template cDNA, 1x Maxima[™] SYBR Green/ROX qPCR Master Mix (Fermentas)
15 and 0.4μM of both primers (sense and antisense). Specific primers for each gene of interest
16 were the followings: GAPDH, F: 5'- AAA GCC TCG TGC TGT CGG ACC-3', R: 5'- GCA
17 GGG TGG GTG TGC CGT C-3'; BDNF, F: 5'- GTG ACA GTA TTA GCG -3', R: 5'- GCC
18 TTC CTT CGT GTA ACC-3'; VEGF, F: 5'- AAG AGA AGG AAG AGG AGA G -3', R: 5'-
19 ACC CAA GAG AGC AGA AAG -3'; IGF1, F: 5'- TCA TGT CGT CTT CAC ACC TCT T -
20 3', R: 5'- ACA CAC GAA CTG AAG AGC AT -3'; among these, GAPDH was considered as
21 housekeeping gene. All primers were obtained from IDT (Coralville, IA, USA).

22 In order to check for possible contamination of genomic DNA, the NO-RT control was
23 processed. No template controls were also used for each reaction mix. Thermal profile of PCR
24 reactions was performed as follow: an activation step of master mix Taq polymerase (95°C,
25 10 min) and 40 cycles of denaturation (95°C, 15 sec) and annealing/extension (60°C for
26 30sec). At the end of the amplification cycles the dissociation curve was obtained by
27 following a procedure consisting of first incubating samples at 95°C for 1 min to denature the
28 PCR-amplified products, then ramping temperature down to 55°C and finally increasing
29 temperature from 55°C to 95°C at the rate of 0.2°C/s, continuously collecting fluorescence
30 intensity over the temperature ramp.

31 The specificity of the amplified product was verified by the presence of a single peak at the
32 expected melting temperature. Random amplified products were resolved by electrophoresis

1 in a 2.0% agarose gel stained with Gel Red™ (Biotium, Hayward, CA) in order to check the
2 specificity of the PCR reaction. This was confirmed by the presence of a single band of the
3 expected size. A 100 bp DNA ladder (Fermentas) was used as DNA marker.

4 No significant variations were found in the expression of GAPDH, confirming its suitability
5 as housekeeping gene. The semi-quantitative analysis of gene expression was performed on
6 the values of the threshold cycle (Ct) obtained for each sample, considering GAPDH as
7 housekeeping gene.

8 The efficiency of the primers used for real-time PCR reactions was calculated by amplifying
9 control cDNA serial dilution. Standard curve slope and primer efficiency were calculated with
10 the MxPro QPCR software 3.1 (Stratagene, La Jolla, California). Samples were always
11 processed in duplicate. The relative gene expression was calculated by the formula $2^{(-\Delta\Delta C_t)}$
12 using a defined group as reference ($2^{(-\Delta\Delta C_t)} = 1$).

14 **Western blot**

16 For this experiment, four animals were included in each group. The cerebellum was
17 homogenized twice using the Ultra Turrax homogenizer (IKA, Staufen
18 Germany) in ice-cold RIPA lysis buffer: 150 mM NaCl, 50 mM TRIS pH 8.0, 1% Triton X-
19 100, 0.5% Sodium deoxycholate and 0.1% Sodium Dodecyl Sulphate supplemented with
20 1mM phenylmethanesulphonylfluoride (PMSF), 10 mM Sodium fluoride, 1 mM Sodium
21 orthovanadate and 1% inhibitors cocktail (Sigma-Aldrich). Samples were then incubated in
22 ice for 30 minutes and centrifuged at 12 000 RPM for 20 minutes at 4°C.

23 Proteins in the supernatant fraction were quantified using the Lowry method. Equivalent
24 amounts of protein (15 µg for cerebellum and hippocampus, 25 µg for cortex) were subjected
25 to electrophoresis on 4-20% polyacrylamide Mini-protean TGXTMgels (Bio-Rad, Milano,
26 Italy). The gel was then blotted onto a nitrocellulose membrane (Protran BA85 Whatman,
27 Sigma-Aldrich), and equal loading of protein in each lane was assessed by brief staining of
28 the blot with 0.1% Ponceau S (Sigma-Aldrich). Membranes were blocked for 1 h in 2,5%
29 BSA/ 0,1% Tween-20 in PBS and incubated overnight in PBS/0,1% Tween-20 at 4°C with
30 primary antibodies: anti-goat polyclonal, rabbit monoclonal anti PSD-95 (Millipore,
31 Darmstadt, Germany; dilution 1:1000), and rabbit polyclonal anti-β-Actin (Sigma Aldrich;
32 dilution 1:10000). Membranes were washed, incubated with HRP-conjugated anti-rabbit, anti-

1 goat or anti-mouse secondary antibodies (1:2000, Dako, Milano, Italy) and specific reactions
2 revealed with the enhanced chemiluminescent (ECL) Clarity™ ECL Substrate (Biorad).
3 Densitometry analysis on digitized images was performed with Fluor-STM Multimager using
4 the Quantity One Software (Bio-Rad) (cerebellum) and Chemi DOCTM MP Imaging
5 systems, using the software ImageLAB 5.2.1. (cerebral cortex and hippocampus). Intensity for
6 each band was normalized to the intensity of the corresponding β -Actin band. The relative
7 protein content was expressed as arbitrary units (AU).

8

9 **Statistical analysis**

10
11 Data from single animals were the unity of analysis in all experiments. Statistical testing was
12 performed with two-ways ANOVA followed by post-hoc multiple comparisons test
13 (accelerating Rotarod), and Student's t test (overall Rotarod, and all other experiments). A
14 probability level of $p < 0.05$ was considered to be statistically significant.

15

16 **Data Accessibility**

17
18 The Standard Operating Procedures (SOPs) used to plan the study and conduct all
19 experiments, all raw data (including CatWalk movies) generating the complete data-set used
20 for manuscript, figures preparation and statistical analysis are available upon editorial request.

21

22 **RESULTS**

23

24 **Animals and locomotion analysis.**

25
26 Breeding, nursing and weaning were comparable in all genotypes, and no spontaneous
27 mortality was observed over the experimental time.

28 The motor performance in accelerating Rotarod test was evaluated in 2.5month- and re-tested
29 in the same animal when 5.5month-old. Results are presented in Fig. 1, where graph A refers
30 to the test performed in 2.5month- and graph B to the re-test in 5.5month-old mice. The
31 overall evaluation of performance (from trial 1 to 12) indicates that CDKL5 mutated animals
32 fell down earlier compared to the respective WT (two ways ANOVA, genotype effect:

F(1,26)=7.933, P=0.0091). Moreover, a trial effect is also observed, showing an impairment in Rotarod learning in CDKL5 mutated animals (two ways ANOVA, trial effect: F(11,286)=2.289, P=0.0108; genotype x trial interaction: F(11,286)=3.074, P=0.0006). We re-tested animal at the time of sacrifice (graph B), confirming that the motor coordination defect in -/- mice was still present (two ways ANOVA, genotype effect: F(1,25)=11.91, P=0.0020). Results from gait and CatWalk computerized analysis are reported in Table 1 and analysed by Student's t test. A significant alteration in the spatial single paw support is observed in CDKL5 -/- mice, which was characterized by a decreased print area (fore paw: P=0.0016; hind paw: P=0.0156) and base of support (P=0.0074). Moreover, the duration of contact of hind paws with the glass plate was shorter in CDKL5 -/- mice than in WT +/+ mice (P=0.0314).

GABA and glutamate efflux from cerebellar and hippocampal synaptosomes

Spontaneous and K⁺-evoked endogenous GABA and glutamate efflux from synaptosomes obtained from CDKL5 -/- compared to the WT mice have been evaluated. A nearly 4-folds and 2-folds reduction in spontaneous GABA and glutamate efflux, respectively, was observed in cerebellum synaptosomes from CDKL5 -/- mice (Fig. 2A, P=0.0009; Fig. 2B, P=0.0324). There were no significant changes in spontaneous GABA and glutamate efflux in cerebellar synaptosomes (Fig. 2B). However, a trend to a reduction was observed in synaptosomes from CDKL5 -/- mice. Based on the above results, the glutamate/GABA efflux ratio in cerebellar synaptosomes from each animal was calculated. Interestingly a significant increase of this ratio was observed in CDKL5 -/- mice when compared to WT animals (Fig. 2C).

As shown in Fig. 2D and 2E, there were no differences between groups in K⁺-evoked endogenous GABA and glutamate efflux from cerebellar synaptosomes, calculated as percent increase over the respective spontaneous efflux. By comparing spontaneous and K⁺-evoked endogenous GABA and glutamate efflux from hippocampal synaptosomes obtained from CDKL5 -/- with those measured in WT mice, only a significant reduction in spontaneous GABA efflux was observed. However, the reduction in spontaneous GABA efflux obtained in hippocampal synaptosomes from CDKL5 -/- mice was lower than that observed in the cerebellum (CDKL5 -/- = 22.67 ± 5.38 pmol/min/g of protein; WT +/+ = 45.27 ± 6.15 pmol/min/g of protein; P<0.05) (*data not shown*).

GABAergic and glutamatergic pathways in the cerebellum

In view of the above results, possible alterations of cerebellar excitatory and inhibitory pathways have been evaluated in CDKL5 $-/-$ mice. Glutamic acid decarboxylase (GAD67) and vesicular glutamate transporter 1 (VGLUT1) were used as markers for inhibitory and excitatory synaptic contacts, respectively (Kaneko et al., 2002).

Fig. 3 show representative images of GAD67-IR (A, B) and VGLUT-IR (C, D) in the cerebellar cortex of WT and CDKL5 $-/-$ female mice. The analysis of immunoreactivity (IR) has been carried out in the granular and molecular layer. We evaluated both IR (immunoreactive area -IR % area- or immunoreactive mean intensity -OD-) and the number of “puncta” in the layers of interest, thus estimating both the size/number of the structural elements (IR % area) and the expression level/staining intensity (proportional to the antigen content) in each element (OD). The number of synaptic contacts was calculated using the Imaris “spot” program, which allows to semi-automatically identify point-like (“puncta”) structures in 3D images (FIG. 3E-G). We first estimated the mean spot diameter (around 0.3 μ m) using the “slice visualization tool” in both WT and CDKL5 $-/-$ mice. Positive spots in the area of interest were then automatically selected by applying the “quality filter”, which classifies the spots based on the intensity at the center.

Results of quantifications are reported in Fig 3 H-J (GAD67-IR) and K-M (VGLUT-IR). A general decrease of GAD67-IR in the molecular (IR % area: $P=0.0217$; n° of spots: $P<0.0001$) and granular ($P=0.0017$) layers was observed in CDKL5 $-/-$ mice when compared to WT $+/+$ mice. No differences between the genotypes were observed by evaluating the VGLUT-IR.

The fibre net around the Purkinje cell soma visualized by neurofilament-heavy chain (NF-200) reflecting the inhibitory synaptic inputs from the basket cells, was then evaluated. Fig. 4A shows a confocal image of NF200-IR net (red) around the calbindin-positive soma (green) of Purkinje cells. The net was calculated cell-by-cell, selecting a ROI for each cell body by means of Imaris “filament” program on confocal images (Fig. 4B and 4C). Being threshold based, the filament program uses the AutoPath tracking function to trace filaments. This algorithm is based on local intensity contrast and connects large start- and small end-points, producing a tree-like filament. We firstly defined the ROI, the source channel along with the thinnest (filament ending) and the largest diameter (filament beginning). Using the absolute intensity histogram we then selected starting and seed points along with the threshold, on the

basis of the “shortest distance from distance map” algorithm, to calculate the filament estimated volume. As shown in Fig. 4D, the estimated volume of the NF200-IR GABA-ergic basket cell innervation was lower in CDKL5 $-/-$ mice than in WT $+/+$ animals ($P=0.0212$). Finally, the ramified axons of basket cells forming the “pinceau” (Cajal, 1911) that embraces the initial segment of cerebellar Purkinje cell axons has been evaluated. This structure was visualized by PSD-95-IR (Iwakura et al., 2012) (Fig. 4 E-G). The PSD-95 IR estimated volume and the staining intensity of GAD-67-IR in the pinceau (Fig. 4 I-K) were measured. The pinceau estimated volume was calculated cell-by-cell, building a surface for each cell, and using the absolute intensity histogram to select the PSD95-positive voxels, only, and excluding the background. The pinceau estimated volume was higher in CDKL5 $-/-$ than in WT $+/+$ mice (Fig. 4H; $P=0.0492$), while the GAD-67-IR intensity as measured in the 3D-rendering used to calculate the pinceau estimated volume, was similar in the two genotypes (Fig. 4L).

In order to establish whether the enlargement of the pinceau corresponded to an increase in PSD-95 protein, PSD-95 content was also assessed by Western Blot. The protein level was higher in CDKL5 $-/-$ than in WT $+/+$ mice (Fig. 4M; $P=0.0414$) in the cerebellum, but not in the cerebral cortex and in the hippocampus.

Neurotrophins and growth factors mRNA expression levels in the cerebellum

Due to the unbalance between excitatory and inhibitory neurotransmission in the cerebellum observed in CDKL5 $-/-$ compared to WT $+/+$ mice, and since the expression level of several neurotrophins is regulated by neural activity (Kuczewski et al., 2010), the expression levels of mRNA encoding for some neurotrophic and growth factors, were assessed. The BDNF mRNA expression level was strongly reduced in the cerebellum ($P=0.0129$), but not in the hippocampus, of CDKL5 $-/-$ compared to WT mice (Fig. 5). No differences between the two genotypes in the mRNA expression levels of the BDNF high-affinity receptor trkB, VEGF and IGF1 were observed (*data not shown*).

DISCUSSION

1 The impact of the deletion of CDKL5 protein on neurodevelopment has been mainly
2 investigated in the hippocampus and cerebral cortex of CDKL5-KO mice. An alteration of
3 postnatal neurogenesis and hippocampal-dependent behaviours (Fuchs et al., 2014), a reduced
4 dendritic arborization of cortical neurons (Amendola et al., 2014), and altered auditory-
5 evoked event-related potential (Wang et al., 2012) have been described. In this study we
6 indicate that cerebellum is also affected by CDKL5 deletion. In particular, our results indicate
7 severe alteration in the excitatory and inhibitory transmitters in the cerebellum of CDKL5 KO
8 compared to WT, and this is associated to an altered locomotor behaviour involving sensory-
9 motor integration and gait.

11 **CDKL5 mutated animals have a severe motor imbalance.**

13 CDKL5 mutations in humans are associated with severe motor symptoms, including gait
14 dysfunction, spasticity and movement disorders (Bahi-Buisson et al., 2008; Stalpers et al.,
15 2012). CDKL5 KO mice show a significant hypolocomotion in home cage (Amendola et al.
16 2014), while an impairment in motor coordination has been described in the Wang's animal
17 model (Wang et al., 2012). In the present study we demonstrate that the impairment in motor
18 coordination is also present in the CDKL5 KO mouse model generated by Amendola et al.
19 (2014). In addition, we also performed, for the first time, the gait analysis of CDKL5 mutated
20 female mice. Thus, we demonstrate the presence of an alteration in gait in CDKL5 $-/-$ mice,
21 characterized by a decreased print area, possibly due to a "tiptoed" locomotion or a "closed
22 finger walking", correlated to a decreased "stand" duration. The "base of support" is also
23 decreased for the fore, but not hindpaw CDKL5 $-/-$ mice, suggesting that mutated animals
24 need to have a wider base of support in posterior paws during locomotion than WT, possibly
25 to compensate balance defects. Notably, a similar gait abnormality has been described in the
26 mouse model for Refsum disease characterized by ataxia (Ferdinandusse et al., 2008).

27 The role of the cerebellum in achieving either movement skills in humans (Timmann et al.,
28 2001; Obayashi et al., 2004) and motor coordination in rodents (Kayakabe et al., 2013; Zhang
29 et al., 2014), led us to hypothesized that alterations in cerebellar neurotransmission and/or
30 neuroanatomy might underlie the motor coordination impairment observed in CDKL5 KO
31 mice.

1 However, we should also consider that the observed behavioural phenotype could be a
2 consequence of a GABA or glutamate defect during development, being both aminoacids
3 morphogens during development (Huang et al., 2007; Manent and Represa, 2007; Takayama,
4 2005).

6 **CDKL5 deletion induces a GABA/glutamate unbalance in the cerebellum.**

8 Considering that GABA and glutamate are the main neurotransmitters in the cerebellum
9 (Nakanishi, 2005), we firstly evaluated spontaneous and K⁺-evoked endogenous GABA and
10 glutamate efflux from synaptosomes (e.g. isolated synaptic terminals) obtained from WT and
11 CDKL5 KO mice. A higher reduction in spontaneous GABA efflux, compared to that in
12 spontaneous glutamate efflux, was observed in CDKL5 ^{-/-} mouse isolated nerve terminals.
13 The present results suggest that GABA neurons are more markedly affected by the loss of
14 CDKL5 than the glutamate ones. In accordance, we found an unbalance between these
15 inhibitory and excitatory transmitters in the cerebellum of CDKL5 ^{-/-} mice, as demonstrated
16 by the increase of glutamate/GABA ratio, in these mutated animals. Whether motor
17 coordination impairment in mutated mice depends on this unbalance cannot be determined at
18 this point. In this context, it is worth noting that the motor coordination impairment, observed
19 in a transgenic animal model of progressive ataxia (Massella et al., 2009), was associated with
20 a decrease in cerebellar GABA content. Thus, the reduced cerebellar GABA signalling in
21 CDKL5 ^{-/-} mice, and the consequent increase in glutamate/GABA ratio, might contribute, at
22 least partially, to their motor coordination dysfunctions. Finally, the evidence that there were
23 no differences between groups in K⁺-evoked endogenous GABA and glutamate efflux
24 suggests that CDKL5 ^{-/-} mouse cerebellar GABA and glutamate nerve terminals maintain
25 their stimulus-dependent exocytotic activity.

26 The altered neurotransmitter profile in CDKL5 ^{-/-} mouse could also impact on cerebellum
27 development. In particular, several lines of evidence indicates that a reduction of GABAergic
28 signalling during development leads to a severe unbalance between excitatory and inhibitory
29 transmitters in neuronal circuits during adulthood, possibly leading to behavioural deficit also
30 in RTT phenotype (Hirao et al., 2002; Wulff et al., 2009; Chao et al., 2010).

32 **CDKL5 deletion induces an alteration in excitatory/inhibitory circuits in the cerebellum.**

The possible mechanism linking CDKL5 mutation to the described neurochemical unbalance in spontaneous glutamate and GABA efflux from cerebellar synaptosomes is not clear. CDKL5 expression is highly regulated during development, being involved in neuronal maturation and maintenance, neuronal cytoskeleton rearrangement, dendrite growth and synaptic activity (Chen et al., 2010; Kilstrup-Nielsen et al., 2012; Della Sala and Pizzorusso, 2012). In the cerebellum, CDKL5 appears at P5 and reaches the peak in the adult brain, where it is localized in almost all neurons (Rusconi et al., 2008). Moreover, CDKL5 is present in both glutamatergic and GABAergic primary cultured neurons, the expression levels being higher in the latter cell population (Rusconi et al., 2008). Thus, the lack of CDKL5 during the development might affect the cerebellar connectivity development and related neurochemistry. These findings, together with the above reported neurochemical results, prompted us to perform an anatomical analysis of cerebellar excitatory and inhibitory pathways in CDKL5 $-/-$ mice. In particular, we analysed synaptic contacts and organization in the cerebellar cortex focusing on GABA and glutamate markers. Punctate positivity for GAD-67 is related to putative GABAergic nerve terminals (Benagiano et al., 2000) arising from a number of inhibitory interneurons, including Golgi-, basket-, stellate-cells (Hashimoto and Hibi, 2012). When analysed in the molecular layer, VGLUT1-IR reflects parallel fibres (thus, axons from granule cells), while in the granular layer it reflects mossy fibres (thus, cerebellar afferences from pontine nuclei, etc.). The neuroanatomical analysis reveals a defect of GAD-67 in the granular and molecular layer of CDKL5 $-/-$ mice, where GABAergic basket and stellate interneurons are located. Notably, the glutamatergic marker VGLUT1 is unchanged in the same area, thus supporting the existence of an unbalance between glutamatergic and GABAergic inputs, as observed in neurochemical experiment. Notably, also MeCP2 deletion in RTT seems to produce alterations of structural synaptic plasticity in inhibitory, but not excitatory, circuits in the cerebellum (Lonetti et al., 2010). Moreover, we found that the estimated volume of the basket “net” carrying inhibitory synaptic inputs from the basket cells to the Purkinje cells, is decreased in CDKL5 $-/-$ mice compared to WT $+/+$ animals, reflecting a structural abnormality or a decreased antigen content. The same fibres extend to wrap around the initial segment of the Purkinje cell axon in a structure called the pinceau (Cajal, 1911). This structure can be visualized by PSD-95 (Buttermore et al., 2012). In fact, although PSD-95 is mainly associated to the postsynaptic density of excitatory synapses through the

1 brain (El-Husseini et al., 2000), in the adult cerebellum is localized to pre-synaptic terminals
2 of GABA-ergic synapses, specifically to the specialized basket cell terminal pinceau
3 (Castejon et al., 2004). This structure possibly mediates inhibition via the electrical field
4 surrounding the Purkinje cell axon initial segment (Blot and Barbour, 2014). Strikingly, we
5 observed an increase in the pinceau estimated volume in CDKL5 $-/-$ mice compared to WT
6 $+/+$ animals. Moreover, we also found an increase in the content of PSD-95 in the cerebellum,
7 but not in the cerebral cortex and hippocampus, of CDKL5 $-/-$ mice compared to WT $+/+$
8 animals. Being PSD-95 in the adult cerebellum exclusively localized in the pinceau, this
9 finding supports the existence of an alteration of this structure in CDKL5 $-/-$ mice. It is worth
10 noting that the regular pinceau organization is required for normal motor behaviors including
11 gait (Bobik et al., 2004; Suarez et al, 2008). Thus, it might be speculated that the alteration of
12 this structure could participate to the behavioural impairments observed in CDKL5 $-/-$ adult
13 mice.

15 **CDKL5 deletion induces an impairment in BDNF mRNA expression in the cerebellum**

17 BDNF is highly expressed in the cerebellum through adulthood (Lindholm et al., 1994),
18 where it plays a key role in regulating the structure and phenotype of mature neurons
19 (Lindholm et al., 1997). In particular, the activity-dependent regulation of BDNF expression
20 is a critical event in synaptic plasticity (Kohara et al., 2001), and an altered glutamate-
21 receptor mediated BDNF mRNA expression has been described in *stargazer* mice,
22 characterized by ataxia, motor dyskinesia and epilepsy (Meng et al., 2007). In the present
23 study, we observed a reduction of BDNF mRNA expression level in the cerebellum of
24 CDKL5 $-/-$ mice. Other growth factors (NGF, IGF and VEGF) are not modified, and BDNF
25 itself is not altered in other brain areas, like hippocampus. While specific experiments are
26 necessary to evaluate the developmental profile and the regulation of BDNF synthesis by
27 excitatory stimuli, we can argue that the defect of basal synthesis of BDNF in adult CDKL5 $-$
28 $-/-$ mice might also be part of the complex neurochemical dysregulation observed in these
29 animals.

31 **CONCLUSIONS**

1 Collectively, the present study suggests that CDKL5 deletion during development severely
2 affects the establishment of a correct GABAergic network in the cerebellar cortex, as
3 indicated by the 3.9-fold decrease of GABA efflux in respect to the 1.51 decrease of
4 glutamate efflux, along with a general decrease in the inhibitory puncta density. CDKL5
5 deletion itself, or the GABA decline eventually derived from CDKL5 deletion during
6 development, could then lead to alterations in cerebellum neurochemistry and fine anatomy
7 supporting the motor symptoms associated to CDKL5 mutation.

8 9 **ACKNOWLEDGMENT**

10
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12

Table 1

CatWalk analysis of individual paw and gait parameters. Twelve animals were included in each group. Statistical analysis: Student's t test, *P<0.05; **P<0.01

parameter			WT	-/-
(a) spatial paw	print area	<i>fore</i>	0.283±0.012	0.209±0.015 **
		<i>hind</i>	0.228±0.014	0.171±0.016*
	base of support (cm)	<i>fore</i>	1.545±0.048	1.348±0.048**
		<i>hind</i>	2.688±0.093	2.782±0.124
(b) kinetic paw	Run duration (s)		1.974±0.176	1.873±0.117
	Stand (s)	<i>fore</i>	0.148±0.009	0.130±0.005
		<i>hind</i>	0.143±0.007	0.121±0.007*
	Swing (s)	<i>fore</i>	0.117±0.005	0.119±0.003
		<i>hind</i>	0.109±0.005	0.115±0.005
	Swing speed (cm/s)	<i>fore</i>	65.24±3.820	63.70±2.04
		<i>hind</i>	64.31±4.087	61.09±2.36
(c) comparative paw	Stride length (cm)	<i>fore</i>	6.892±0.222	7.270±0.118
		<i>hind</i>	6.514±0.262	6.879±0.156
	Step cycle (s)	<i>fore</i>	0.269±0.013	0.248±0.006
		<i>hind</i>	0.257±0.011	0.235±0.006
(d) coordination	Regularity index (%)		94.21±2.021	93.80±1.512
	Cadence		16.49±1.026	16.48±0.629

Legend to the Figures

Figure 1

Somatosensory integration tested by accelerating RotaRod in CDKL5 ^{-/-} (N=17) and WT (N=12) female mice. Data are expressed as mean \pm SEM. In A, each point represents the latency to fall down in the fourth session during the 3 consecutive days of the test. Graph B shows performance of the same animal at 5.5 month of age, analysed by pooling the trials of each day to limit motivation and motor learning bias. Values represent the average mean latency to fall expressed in sec \pm SEM. Statistical analysis: two-ways ANOVA and post-hoc Bonferroni's test, **P<0.005; ***P<0.001.

Figure 2

GABA and glutamate efflux from cerebellar synaptosomes. Panels A, B, D, E: basal and K⁺-evoked GABA (A, D) and glutamate (B, E) efflux from synaptosomes isolated from the cerebellum. GABA and glutamate efflux is expressed as pmol/min/g of protein while K⁺-evoked GABA and glutamate efflux is expressed as percent increase over the respective spontaneous efflux (calculated by the mean of the two fractions collected prior to the depolarising stimulus). Eight-nine animals were included in each group. Data are expressed as mean \pm SEM. Statistical analysis: Student's t test, *p<0.05; **p<0.001.

Panel C: basal glutamate/GABA efflux ratio in synaptosomes isolated from the cerebellum. Data are expressed as mean \pm SEM. Statistical analysis: Student's t test, *p<0.05.

Figure 3

Analysis of GABA-ergic and glutamatergic markers in the cerebellar cortex of CDKL5 ^{-/-} and WT animals. Panels A, B: representative images of GAD-67-IR in WT (A) and CDKL5 ^{-/-} (B) mice. Panels C,D: representative images of VGLUT1-IR in WT (C) and CDKL5 ^{-/-} (D) mice; E-G: step by step scheme of three-dimensional analysis of VGLUT1 (red channel) and GAD67 (green channel) positive spots in the molecular layer of cerebellum; each channel is built separately and the number of VGLUT1 and GAD67-positive spots was calculated. H-J:

semiquantitative analysis of GAD-IR in the granular (H) and molecular layers (I,J), expressed as immunoreactive % area (H,I) and number of positive puncta in a standard area (J, area $26.5\mu\text{m}^2$). K-M: semiquantitative analysis of VGLUT1-IR in the granular (K) and molecular layers (L,M), expressed as optical density (OD) (K,L) and number of positive puncta in a standard area (M, area $26.5\mu\text{m}^2$). The numbers in A,B,C,D refer to the animal code. Six animals were included in each group. Data are expressed as mean \pm SEM. Statistical analysis: Student's t test, * $p<0.05$; ** $p<0.001$; *** $p<0.0001$. Scale bars: $50\mu\text{m}$

Abbreviations: gl, granular layer; ml, molecular layer; Pj, Purkinje cells.

Figure 4.

Fine neurochemical analysis of inhibitory inputs to the Purkinje cells in the cerebellar cortex of female CDKL5 $-/-$ and WT animals. Panels A-C: step by step three-dimensional analysis of the NF200-IR-positive “net” (red channel) surrounding Purkinje cell body (as identified by calbindin D28K-IR, green channel) and net volume quantification (panel D). Panels E-G: step by step three-dimensional analysis of the PSD-95-IR “pinneau” (red channel), surrounding the Purkinje cells hillock (as identified by calbindinD28K-IR, green channel). The surface was built in order to calculate the PSD95-IR volume (panel H). Panels I-K: GAD-67 (J, red channel, and K, 3D identification), PSD95 (I and K, blue channel) and merged image (I) in Purkinje cells, as identified by calbindinD28K-IR (green channel), and quantification of GAD-67 staining intensity (panel L). Six animals were included in each group. Results are expressed as mean \pm SEM. Statistical analysis: Student's t-test, * $p<0.05$.

M. Representative images of PSD95 Western blot in the cerebral cortex, hippocampus and cerebellum of CDKL5 $-/-$ and WT animals and quantification (N). Data are the % change in CDKL5 $-/-$ respect to WT animals and expressed as mean \pm SEM. Statistical analysis: Student's t test, * $p<0.05$.

Figure 5.

BDNF mRNA expression levels in the cerebellum and hippocampus of female CDKL5 $-/-$ and WT animals. The graphs represent the relative mRNA expression of BDNF normalized vs

the WT animals. (A), trkB (B) and p75 (C) genes. Six animals were included in each group. Data are expressed as mean \pm SEM. Statistical analysis: Student's t-test, *p<0.05.

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