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SUPPLEMENTARY METHODS

CNF1 preparation

CNF1 was obtained from the 392 ISS strain kindly provided by V. Falbo (Rome, Italy) and purified as previously described (Falzano et al, 1993). Briefly, bacteria grown in LB medium were collected, lised, centrifuged and the supernatant was ammonium sulphate precipitated. Following extensive dialysis against 25 mM Tris buffer pH 6, the lysate was applied to a DEAE ion-exchange column (Pharmacia). Eluted CNF1 was ammonium sulphate precipitated and further applied to a series of gel filtrations columns and finally to hydrophobic column of butyl(C6) sepharose, and eluted by a linear decreasing gradient of ammonium sulphate. The fractions were dyalised, tested for CNF1 activity, pooled and applied to a monoQ FPLC column (Parmacia). CNF1-containing fractions were pooled and verified to SDS polyacrylamide gel. In all experiments, the recombinant protein CNF1 C866S, in which the enzymatic activity on RhoGTPases is abrogated by change of cystein with serine at position 866 (Schmidt *et al*, 1998), was used as a control. The plasmid coding for CNF1 C866S, purified as above described, was kindly provided by E. Lemichez (U627 INSERM, Nice, France).

Animals and treatment

Experimental mice were bred in our animal facility and obtained from crossings of MeCP2-308 mice [heterozygous (HZ) females and hz males; B6.129S-MeCP2tm1Hzo/J, stock number: 005439; backcrossed to C57BL/6J mice for at least 12 generations from the Jackson Laboratories (USA)]. Like most of the studies on RTT mouse models (Moretti *et al*, 2006; Stearns *et al*, 2007), the potential efficacy of CNF1 was evaluated on hz male mice. After general anesthesia (Equithesin, 3 ml/kg ip), mice were secured in a stereotaxic apparatus. Five minutes after icv injection, the needle was slowly removed and animals were sutured with tissue glue. After five minutes on a heating pad to regain normal body temperature, animals were returned to their home cages where they remained undisturbed for one week. The same methodology was adopted in an

additional experiment which was performed to exclude that the volume of the injection in the ventricles could damage brain tissue and/or elicit an astrocytic response. Three wt animals were icv injected, as above described, with 2 µl of TRIS and three received no infusion. After icv injections, mice were singly housed in polycarbonate transparent cages (33 × 13 × 14 cm) with sawdust bedding and provided with drinking water and a complete pellet diet (Rieper, BZ, Italy) *ad libitum*. Mice were kept on a 12-h light–dark schedule (lights off at 8:00) and the temperature was maintained at 21° ± 1°C, relative humidity 60 ± 10%.

Contextual and cued fear-conditioning tests

This cognitive test takes advantage of the natural tendency of mice to freeze in response to fearful stimuli. During the training session, mice were first left undisturbed in an apparatus with a grid floor connected to an electric shocker (Coulbourn Instruments, Allentown, USA) for 3 min (baseline). Afterwards they were exposed for 30 s to an acoustic stimulus (a 80 dB sound) which was associated, during the last 2 s, with a single footshock (0.6 mA) (conditioning phase). After an additional 30 s interval, mice were removed from the experimental apparatus and returned to their home cages. Each mouse was placed again in the same training context 24 h after the training session (context test). The context test lasted 4 min, after which the mouse was removed and returned to its home cage. The cued test was performed 1 hour later in a new context (a Plexiglas irregular box with a 50 cm-high wall made of grey plastic): after 3 min of acclimation, animals were exposed to the same sound (80 dB) they heard during the conditioning phase for 3 minutes without any footshock (cued test). Each session (training, context and cued) was videorecorded and subsequently scored by means of a specific software (THE OBSERVER, Noldus Information Technology, Wageningen, The Netherlands). The time mice spent inactive was used as an index of retention of the unconditioned stimulus.

Home-cage spontaneous activity and its circadian variation

The sensors (20 Hz) detected any movement of mice with a frequency of 20 events per second. Data were recorded by an IBM computer with dedicated software. No movements were detected by the sensors when mice were sleeping, inactive, or performed moderate selfgrooming. Scores were obtained during 1-h intervals and expressed as counts per minute (cpm). The 24-h profile of activity was obtained by averaging seven days of continuous registration. The position of cages in the rack was such that mice of each group were equally distributed in rows and columns. The access of the authorized personnel to the animal room was not restricted and followed the routine schedule.

Open field test

Eight days after icv inoculation mice underwent a 1 hour open field session. The testing apparatus consisted of a squared arena (40 × 40 cm) with a 50 cm-high wall made of black plastic. The sessions started placing the animal in one corner of the arena. The floor of the apparatus was cleaned with 50% ethanol after each session. Testing occurred under dim lights. The testing sessions were videotaped and the behavioral profile was subsequently scored by a trained observer, blind to genotype and treatment of mice, using a computer and a specific software (THE OBSERVER, Noldus Information Technology, Wageningen, The Netherlands). The detailed analysis of several behavioral parameters included 6 intervals of 5 min for a total of 30 min (Supplementary Fig. 1). The floor of the apparatus was subdivided into 12 square sections of the same dimension (10 cm x 10 cm) by lines placed on the video screen at the time of videotape analysis. The following items were scored: *Crossing* (number of line crossings with both forepaws), *Rearing* (body in vertical position), *Wall rearing* (body in vertical position with forepaws placed on the walls of the cage) *Grooming* (mouth or paws on body), *Inactivity* (time spent inactive), *Time in the centre* (time spent in the centre of the arena).

Novel object recognition test

Two weeks after the icv injection, Mecp2-308 and wt mice were evaluated in the novel object recognition test as described in (Papaleo *et al*, 2008). The testing apparatus consisted of a squared arena (40 × 40 cm) with a 50 cm-high wall made of black plastic. The sessions started placing the animal in one corner of the arena. The floor of the apparatus was cleaned with 50% ethanol after each session. Three experimental phases were carried out, each of them under dim lights. The day before the test, experimental mice were individually habituated for 1 h in the empty open field arena. The next day, during the acquisition session, each mouse was introduced for 10 min into the open field, which contained two identical copies of the same object (two rectangular white boxes, 4 × 1 × 3 cm). The objects were placed 8 cm from side walls. The 5 min retention test took place 1 h after the acquisition trial. The mice were placed back into the same arena with a duplicate of the old object and a new object (a conical black item, 4 × 3 cm). The testing sessions were videotaped and subsequently scored. The time spent exploring the objects by each animal during the acquisition and the retention trial were subsequently scored by a trained observer blind to genotype of mice, using a computer and specific software (THE OBSERVER, Noldus Information Technology, Wageningen, The Netherlands). To evaluate locomotor activity, the floor of the apparatus was subdivided into eight square sections of the same dimension by lines placed on the video screen at the time of videotape analysis and number of line crossings with both forepaws scored.

Dowel test

One month after CNF1 administration, experimental animals were also evaluated in the Dowel test as in (De Filippis *et al*, 2010). The hardwood round dowel we used, was 9.0 mm in diameter, mounted horizontally 50 cm above the floor level, and 35 cm long. At the beginning of the testing, each mouse was placed in the middle of the dowel so that the length of its body was parallel with the dowel for a two-minute criterion test. Latency to fall from the dowel into a cage of bedding was recorded.

Immunohistochemistry and morphometric analysis of brain sections

Five months after CNF1 administration, experimental animals (5-6 per experimental group) were anesthetized and transcardially perfused with 100 ml of physiological saline (0.9% NaCl), followed by 200 ml of 4% paraformaldehyde in PBS, 0.12 M in sucrose. Brains were removed and postfixed for additiomal 24 h at 4°C. After fixation, the samples were rinsed for three times in PBS, 5% in sucrose and left overnight in PBS, 30% in sucrose. Samples were frozen at -30 °C in isopentane and stored at -80 °C. Serial 20 μm-thick coronal sections were cut at a Reichert–Jung Frigocut cryostat and collected in Petri dishes containing PBS. After treatment for 1 h in blocking normal horse serum, free-floating sections were incubated at +4°C overnight with the following primary antibodies: monoclonal anti-synatoptophysin (Millipore, MA, USA), SMI-32 (Covance, NJ, USA), polyclonal anti-GFAP (Dako, Denmark). After extensive washes, the following secondary antibodies were used for 1 h at room temperature: anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (Life Technologies Corporation, CA, USA). In some sections, nuclei were countestained with Hoechst 33258. Samples were observed and photographed using an Eclipse 80i Nikon Fluorescence Microscope, equipped with a VideoConfocal (ViCo) system. Morphometric analysis was conducted with the Optilab software (Graftek, Austin, TX). Astrocytic cross-sectional area was measured in 20 μm-thick coronal sections immunostained for GFAP. In dentate gyrus, three fields of 0.035 mm^2 were randomly chosen from both left and right hemispheres. For analysis of corpus callosum, two fields of the same area were recorded for each hemisphere in forebrain sections. After background subtraction, the GFAP-positive area was measured as percentage of the total field area. Measures obtained for each field were averaged to produce a single mean value for each animal. In dentate gyrus, the number of astrocytes was also counted in each field. Values obtained for each field were pooled to obtain a single mean value for each animal.

Western Blot analysis

Twenty-five micrograms of total protein protein extracts from hippocampus (lysis buffer: 50 mM HEPES, pH 7.4, 0.1 M NaCl, 10 mM MgCl2, 5% glycerol, 1% NP-40, and 10 mM NaF, protease inhibitors) were resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with TBS-T (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.02% Tween 20) containing 5% skimmed milk (Bio-Rad) for 30 min at room temperature, and then incubated overnight at 4°C with the primary antibodies diluted in 2% milk TBS-T . The following primary antibodies were used: rabbit polyclonal anti-GFAP (1:5000; Chemicon International, Inc.), rabbit polyclonal anti-erk (1:500; Santa Cruz Biotechnology, CA). After washing in TBS-T, immunocomplexes were detected with HRP-conjugated species-specific secondary antibodies (Jackson Laboratory, Bar Harbor, ME) followed by enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL).

MRS and MRI evaluations

Approximately 1 month after CNF1 icv injection, animals underwent MRI- MRS analyses. The animals were anaesthetised with 3.5% sevoflurane in oxygen 2 l/min (Sevoflo, Abbott SpA, Latina, Italy) within an induction chamber. This anaesthetic was preferred to isoflurane in order to avoid respiratory distress (apnea) in mutant mice. Once unresponsive to paw pinch, each mouse was transferred to a stereotaxic head frame, in prone position, under the continuous supply of anesthetic gases through a facemask, and fixed by using tooth bar, earplugs and adhesive tape to reduce head movement. During the MR analyses, anaesthesia was maintained at 3.5-3.0 % sevoflurane in oxygen (1 l/min). An integrated heating system allowed maintaining the animal body temperature at 37.0 ± 0.1 °C. Mice were left to spontaneous breathing, with no mechanical ventilation, during the whole experiment.

All MRI and MRS experiments were conducted on a 4.7 T Varian Inova animal system (Varian Inc. Palo Alto, CA, USA), equipped with actively shielded gradient system (max 200

mT/m, 12 cm bore size). A 6-cm diameter volume coil was used for transmission in combination with an electronically decoupled receive-only surface coil (Rapid Biomedical, Rimpar, Germany). The shape of this receiver coil (1.5 cm long, 1.5 cm wide and 0.6 cm high) was designed to optimally fit the dorsal surface of the mice' head, centered over forebrain regions.

Gradient echo scout images were detected for accurate positioning of the head of the animal inside the magnet and fast spin echo sagittal anatomical images (TR/TEeff=3000/60 ms, 17 consecutive slices of 0.6 mm thickness, FOV=25 x 25 mm^2, matrix of 256 x 256, 4 averages, voxel resolution = 98 x 98 x 600 μm^3, scan time 6 min) were acquired for positioning the voxel for the MRS study.

Single voxel localised ^1H MR spectra (PRESS, TR/TE = 4000/23 ms, ns = 256) were collected from dorsal striatum, 16 ml, and hippocampus, 11.7 ml (Fig. 2d). Quantitative MRS protocol, including water T2 measurements, was applied (Canese *et al*, 2011). Localised shimming was performed up to water linewidths smaller or equal to < 12 Hz. The TR of 4 s was chosen in order to make negligible the limit effects due to the T1 relaxation time of water and metabolites (Mlynarik *et al*, 2001) The TE could not be reduced below 23 ms, because of spectral distortion due to the eddy currents. With this TE also T2 losses can be considered negligible. Nevertheless, T2 measurements were performed on water signal in order to identify any change due to illness progression or therapy (a set of water localised spectra was acquired from the same voxel with the same parameters but TE ranging from 23 to 300 ms, 15 values). The water signal was suppressed by using the VAPOR pre-sequence composed by seven CHESS pulses with optimized flip angles and timing in order to have a reduced sensitivity to B1 variation and thus it is highly efficient also for surface coil; 256 averages were sufficient to acquire metabolite spectra from all voxels with a S/N (referred to the highest signal) higher than 4. Unsuppressed water signal was acquired from the same voxel with the same parameters except for a reduced number of transients (4 instead of 256) and was used for metabolite quantification (assuming 79.9% for gray matter water content) (Kato *et al*, 1986; Schwarcz *et al*, 2001).

Spectra were analysed using LCModel (Provencher, 2001) that calculates the best fit to the experimental spectrum as a linear combination of model spectra (spectra of metabolite solutions). Seventeen metabolites were included in the basis set (Tkac *et al*, 2007; Tkac *et al*, 2009): alanine (Ala), aspartate (Asp), creatine (Cr), γ-aminobutyric acid (GABA), glucose (Glc), glutamate (Glu), glutamine (Gln), glycerophosphorylcholine (GPC), guanidoacetate (Gua), phosphorylcholine (PCho), myo-inositol (Ins), lactate (Lac), Nacetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphocreatine (PCr), scyllo-inositol, and taurine (Tau). Spectra of lipids and macromolecules were also included in the basis set (Pfeuffer *et al*, 1999). Only those metabolites that were estimated to have Cramer-Rao lower bounds (CRLB) less than 20%, which corresponded to an estimated concentration error <0.2 μmol/g (Tkac *et al*, 2003), were included into the quantitative analysis. In some cases, metabolites that have resonance overlapped or very close are also given as their sum.

The signals due to Ins, Glu and Gln underwent J-coupling modulation with increasing TEs. However, the decreases in these signals due to J-coupling at TE=23 ms were automatically accounted for in the LCModel basis sets.

Multislice fast spin echo axial images (TR/TEeff = 3200/60 ms, ns = 4, slice thickness 0.6 mm, 24 slices, matrix 256 x 256, FOV =25 x 25 mm^2) were acquired for volumetric analyses. Motor cortex (MC) and corpus callosum (CC) thickness and area were measured at +1.32 from bregma (Fig. 2a-c). Volumetric analyses of the whole brain have been performed from olfactory bulb to cerebellum included.

Data analysis

Parametric analyses of variance (ANOVA) were performed on all data. Specifically, ANOVA models included genotype and CNF1 treatment as between-subject factor. One or two repeated measures factors were also taken into account as for behavioral data. In particular, we considered as within-subject factors: baseline and conditioning sessions for the fear-conditioning tests; repeated measures (1, 48 and 72 h) for nest building behavior, phases (Light and Dark) for home cage

circadian activity and 10 minutes intervals for the open field test. Mann-Whitney U test was applied on latency data in the Dowel test, which had no normal distribution.

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