Supplementary Methods

Skull Morphometry

8-12 month old NFATc2/c4 DKO and control mice were analyzed by μCT. Skulls were scanned with a GE Medical Systems eXplore RS MicroCT System at 45μM resolution with white voxels representing bone. Measurement of reconstructed 3D images where analyzed with eXplorer software. Histological analysis was performed using standard methods.

Behavioural analysis of NFATc knockout mice

NFATc2/c4 DKO mice and *NFATc2/c4* double *c3* heterozygous knockout (NFATc2/c4 DKO c3 HKO) mice used were on a mixed background of 129/Sv x BALB/c, therefore we chose these two inbred strains as control mice. Mice (BALB/c mice, n=16; 129/SvEv mice, n=16; NFATc2/c4 DKO mice, n=15; NFATc2/c4 DKO c3 HKO mice, n=16) were subjected to a comprehensive behavioral test battery, including the grip strength test, the wire hang test, the elevated plus maze test, and the social interaction test in a novel environment, in a manner similar to those previously described[1]. Inbred BALB/c mice and 129/SvEv were obtained from Taconic (Germantown, NY). An average of 78.1% of markers that correspond to 129/SvEv was confirmed in four NFATc2/c4 DKO c3 HKO mice by Genetic Testing Services (Charles River Laboratory). Body weight was not significantly different among them, but rectal temperature was lower in BALB/c than other groups ($P=0.0016$ for NFATc2/c4 DKO versus BALB/c; $P<0.0001$ for NFATc2/c4 DKO c3 HKO versus BALB/C; $P<0.0001$ for 129/SvEv versus BALB/C). Age of mice at start of the behavioural tests was 9~13 weeks old for neuromuscular strength tests, 11~15 weeks old for elevated plus maze test, 12~16 weeks old for social interaction test in a novel

environment, 16~20 weeks old for eight-arm radial maze test and 22~26 weeks old for contextual and cued fear conditioning test, respectively. Mice were housed in a room with a 12-hr light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m.. After the tests, all apparatus were cleaned with super hypochlorous water to prevent a bias based on olfactory cues with the apparatus.

Neuromuscular Strength

Neuromuscular strength was assayed with the grip strength and wire hang tests. A grip strength meter (O'Hara & Co.) was used to assess forelimb grip strength. Mice were lifted and held by their tail so that their forepaws could grasp a wire grid. The mice were then gently pulled backward by the tail with their posture parallel to the surface of the table until they released the grid. The peak force applied by the forelimbs of the mouse was recorded in Newtons (N). Each mouse performed the test three times and the greatest value measured was used for statistical analysis. In the wire hang test, the mouse was placed on a wire mesh which was then inverted and waved gently, so that the subject gripped the wire. Latency to fall was recorded, with a 60 sec cut-off time.

Elevated Plus-Maze Test

The elevated plus-maze consisted of two open arms (25 x 5 cm) and two enclosed arms of the same size, with 15 cm high transparent walls. The arms and central square were made of white plastic plates and were elevated to a height of 55 cm above the floor. In order to minimize the likelihood of animals falling from the apparatus, 3-mm high plexiglas ledges were provided for the open arms. Arms of the same type were arranged at opposite sides to each other. Each mouse was placed in the central square of the maze (5 x 5 cm), facing one of the closed arms. Mouse behavior was recorded during a 10 min test period. The number of entries into, and the time spent on open and enclosed arms were recorded. For data analysis, we employed the following four measures: the

percentage of entries into open arms, the stay time on open arms (sec), the number of total entries, and total distance traveled (cm). Data acquisition and analysis were performed automatically, using Image EP software.

Social interaction test in a novel environment

Two mice of identical genotypes, which were previously housed in different cages, were placed into a box together (40 x 40 x 30 cm) and allowed to explore freely for 10 min. Social behavior was monitored by a CCD camera, which was connected to a Macintosh computer. Analysis was performed automatically using Image SI software. The software can discriminate a mouse with darker coat color from one with lighter coat color. The number of contacts and total distance traveled were measured.

Eight-arm radial maze test

The floor of the maze was made of white plexiglas, and the wall (25 cm high) consisted of transparent plexiglas. Each arm (9 x 40 cm) radiated from an octagonal central starting platform (perimeter 12 x 8 cm) like the spokes of a wheel. Identical food wells (1.4 cm deep and 1.4 cm in diameter) with pellet sensors were placed at the distal end of each arm. The pellet sensors were able to record pellet intake by the mice automatically. The maze was elevated 75 cm above the floor and placed in a dimly-lit room (25lux) with several extra-maze cues. During the experiment, the maze was maintained in a constant orientation. One week before pretraining, animals were deprived of food until their body weight was reduced to 80-85% of the initial level. Pretraining started on the 9th day. Each mouse was placed in the central starting platform and allowed to explore and to consume food pellets scattered on the whole maze for a 5 min period (one session per mouse). After completion of the initial pretraining, mice received another pretraining to take a pellet from each food well after being placed at the distal

end of each arm. A trial was finished after the subject consumed the pellet. This was repeated 8 times, using 8 different arms, for each mouse. After these pretraining trials, actual maze acquisition trials were carried out. In spatial working memory task of the eight-arm radial maze, all 8 arms were baited with food pellets. Mice were placed on the central platform and allowed to get all 8 pellets within 25 min. A trial was terminated immediately after all 8 pellets were consumed or 25 min had elapsed. An 'arm visit' was defined as traveling for more than 5 cm from the central platform. The mice were confined in the center platform for 5 sec after each arm choice. The animals went through one trial per day (10 trials total). For each trial, choices of arms, latency to get all pellets, distance traveled, number of different arms chosen within the first 8 choices, and the number of revisiting and omission errors were automatically recorded. Data acquisition, control of guillotine doors, and data analysis were performed by Image RM software. Seven 129/SvEv mice couldn't finish the task within 25 min at the last trial because they didn't move readily, and three NFATc2/c4 DKO c3 HKO mice died during the test battery. Data from those mice were excluded from the statistical analysis.

Contextual and cued fear conditioning

Each mouse was placed in a test chamber (26 x 34 x 29 cm) inside a sound-attenuated chamber and allowed to explore freely for 2 min. A 60dB white noise, which served as the conditioned stimulus (CS), was presented for 30 sec, followed by a mild (2 sec, 0.5 mA) footshock, which served as the unconditioned stimulus (US). Two more CS-US pairings were presented with 2 min inter-stimulus interval. Context testing was conducted 7 days after conditioning in the same chamber. Cued testing with altered context was conducted 7 days after conditioning using a triangular box (35 x 35 x 40 cm) made of white opaque plexiglas, which was located in a different room. Data acquisition, control of stimuli (i.e. tones and shocks), and data analysis were performed automatically, using Image FZ software. Images were captured at 1 frame per second. For each pair of

successive frames, the amount of area (pixels) by which the mouse moved was measured. When this area was below a certain threshold (i.e. 20 pixels), the behavior was judged as 'freezing'. When the amount of area equaled or exceeded the threshold, the behavior was considered as 'non-freezing'. The optimal threshold (amount of pixels) to judge freezing was determined by adjusting it to the amount of freezing measured by human observation. 'Freezing' that lasted less than the defined time threshold (i.e. 2 sec) was not included in the analysis.

Image analysis

The applications used for the behavioral studies (Image LD, Image EP, Image RM, Image FZ, and Image SI) were based on the public domain National Institutes of Health's Image program (developed by Wayne Rasband at the National Institute of Mental Health and available at http://rsb.info.nih.gov/nih-image/) and was modified for each test by Tsuyoshi Miyakawa (available through O'Hara & Co., Tokyo, Japan).

Statistical analysis

Statistical analysis was conducted using StatView (SAS institute). Data were analyzed by ANOVA Bonferroni - Dunn test. Values in graphs were expressed as mean ± SEM.

Cell culture and transfections

E15.5 mouse neurons were cultured and transfected as previously described[2]. For luciferase assays the neurons were treated 16 hrs after transfection with 100ng/ml recombinant FGF8 (R&D). For studies with FK506 (200ng/ml) /CsA (2 μg/ml) the cells were preincubated for 10 min with the inhibitors before stimulation and the inhibitors were present during the stimulation. 293T cells were transfected by calcium phosphate precipitation in triplicate in 12-well plates with the indicated amount of pCEP4-Flag-

DYRK1a or pTargeT-DSCR1 with 50 ng each of NFAT-luc and Renilla-luc plasmids. Empty pCEP4-Flag plasmid was used to maintain total transfected material at 500 μg/well. Dual luciferase assays were performed according to standard protocols.

Nuclear export assays of NFATc4 were performed as previously described[3]. Confocal images were acquired using a two-photon microscope (Zeiss LSM510 with a Coherent MIRA laser, Cell Sciences Imaging Facility) and Volocity (Improvision) software was used to measure fluorescence intensity of nuclear and cytoplasmic EGFP-NFATc4.

Antibodies and constructs

Rabbit polyclonal antibodies to DYRK1a were generated against an N-terminal fusion of maltose binding protein (MBP) to the first 499 residues of rat DYRK1a (MBP-DYRK1aΔC). Serum was affinity purified on immobilized DYRK1aΔC fused to glutathione S-transferase (GST-DYRK1aΔC). Rabbit polyclonal antibodies to DSCR1 were a gift from E. Olson.

DYRK1a in EGFP-C1 and DYRK1aΔC in pGEX-2T were gifts from W. Becker. Full-length DYRK1a was cloned into pCEP4-Flag and DYRK1a-KI was generated by site directed mutagenesis (K188R), DYRK1aΔC was cloned into pMAL-c2 (New England Biolabs), MCIP1 (DSCR1-HA) in pTargeT was a gift from R. S. Williams.

NFAT-Luciferase, the EGFP-NFATc4 and GST-NFATc4 plasmids have been described elsewhere[3]. Serine to alanine mutations in NFATc4 to generate NFATc4-SRR-A and NFATc4-SRR/SP-A were introduced using PCR-directed mutagenesis. A 2.5 kb genomic BamH1/BamH1 fragment 5' of the NFATc4 start ATG in exon 1 was isolated from a 129 Sc/Ev genomic library and subcloned into the PGL3-Pro vector (Promega).

Renilla luciferase in the pRR6 vector was used as an internal standard in the transfections.

Transient Transgenic Mice

Full-length DYRK1a and DSCR1 were cloned into pCIG[4], which drives expression of the inserted gene under control of a β-actin promoter. The vector backbone was excised and DNA was injected into fertilized oocytes, which were implanted into the uteruses of pseudopregnant females. Transgenic embryos were harvested at E13.5 and analysed as indicated.

In vitro kinase assays

H19-7 neuronal progenitor cells (gift from B. Wainer) were stimulated for 30 minutes with 5 ng/ml bFGF (Invitrogen) and nuclear extracts were prepared.For priming reactions, 2.5 μg of extract was added to 1 μg of GST-NFATc4 (Supplementary Fig. 4a) immobilized on glutathione-sepharose beads in 20 μl Kinase buffer (KB)[5] + 1 mM ATP and 1 mM DTT for 20 minutes at 30°C. The beads were washed 2x with KB + 500 mM NaCl and 1x with KB, then 2.5 U rGSK-3 (NEB) was added in 20 μl KB + 200 μM ATP + 0.5 μCi γ-^{32}P ATP and 1 mM DTT for 20 minutes at 30°C. The reaction was stopped by addition of Laemmli SDS gel-loading buffer and the samples were subjected to SDS-PAGE, transferred to PVDF membranes, and exposed to film. DYRK1a constructs in the pCEP4-Flag vector were transfected into 293T cells and immunoprecipitated with anti-Flag M2 sepharose beads (Sigma) as the kinase source. GST-NFATc4 was added with 400 μM unlabelled ATP during the priming kinase reaction, and the supernatant was transferred to a new tube with γ-^{32}P ATP and rGSK-3 for the secondary phosphorylation.

Mathematical modelling

The model was constructed by means of a coupled set of 5 first order non-linear ordinary differential equations with gain and loss terms. The dynamic variables were the concentrations of Cn (Ca^{2+} activated and not), of NFAT (nuclear and cytoplasmic) and of DSCR1. The differential equations were solved numerically with the standard 4^{th}-order Runge-Kutta method, implemented with a C++ code of about 500 lines run on a Linux workstation.

1. Miyakawa, T. et al. Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia. Proc Natl Acad Sci U S A 100, 8987-92 (2003).
2. Graef, I. A. et al. Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. Cell 113, 657-70 (2003).
3. Graef, I. A. et al. L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. Nature 401, 703-8 (1999).
4. Megason, S. G. & McMahon, A. P. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development 129, 2087-98 (2002).
5. Beals, C. R., Sheridan, C. M., Turck, C. W., Gardner, P. & Crabtree, G. R. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science 275, 1930-4 (1997).