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Supplemental Data

Removal of FKBP12 Enhances mTOR-Raptor Interactions, LTP, Memory, and Perseverative/Repetitive Behavior

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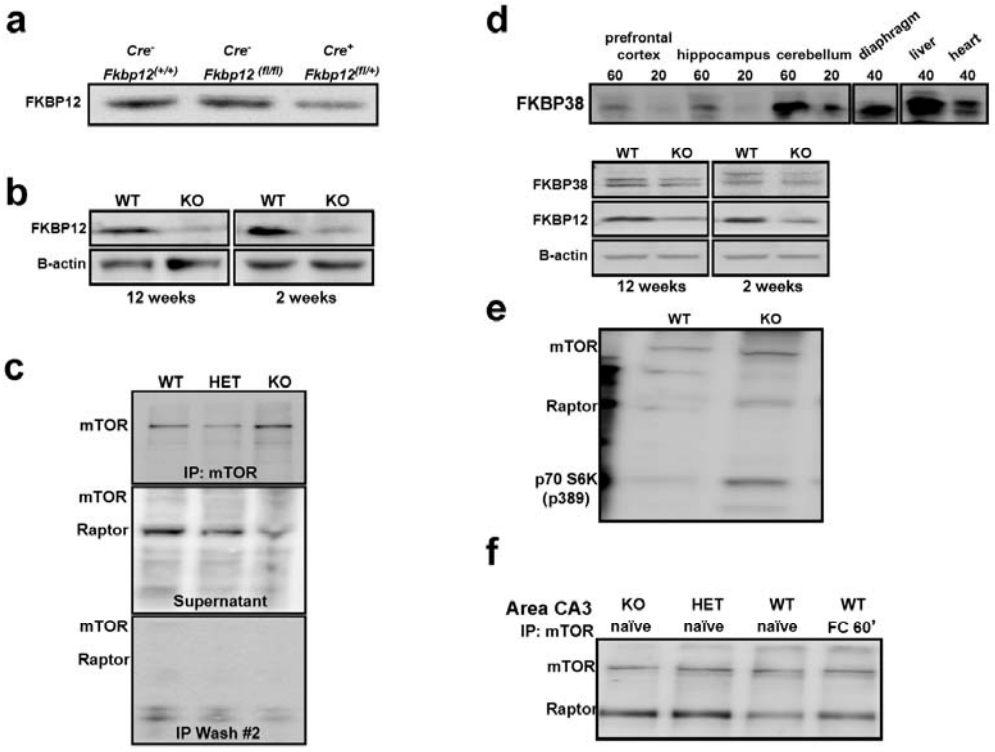
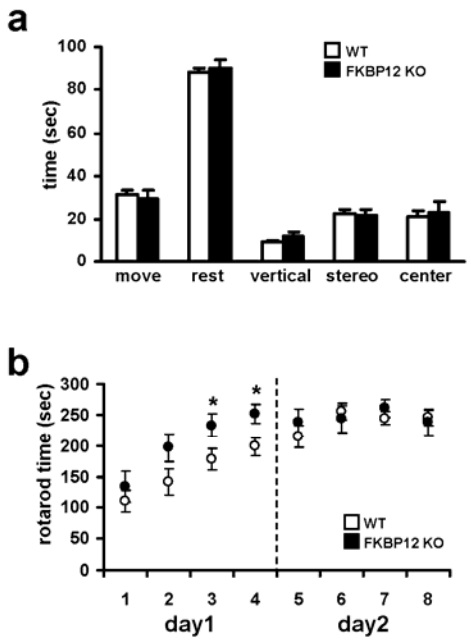
Supplemental Figures and Legends

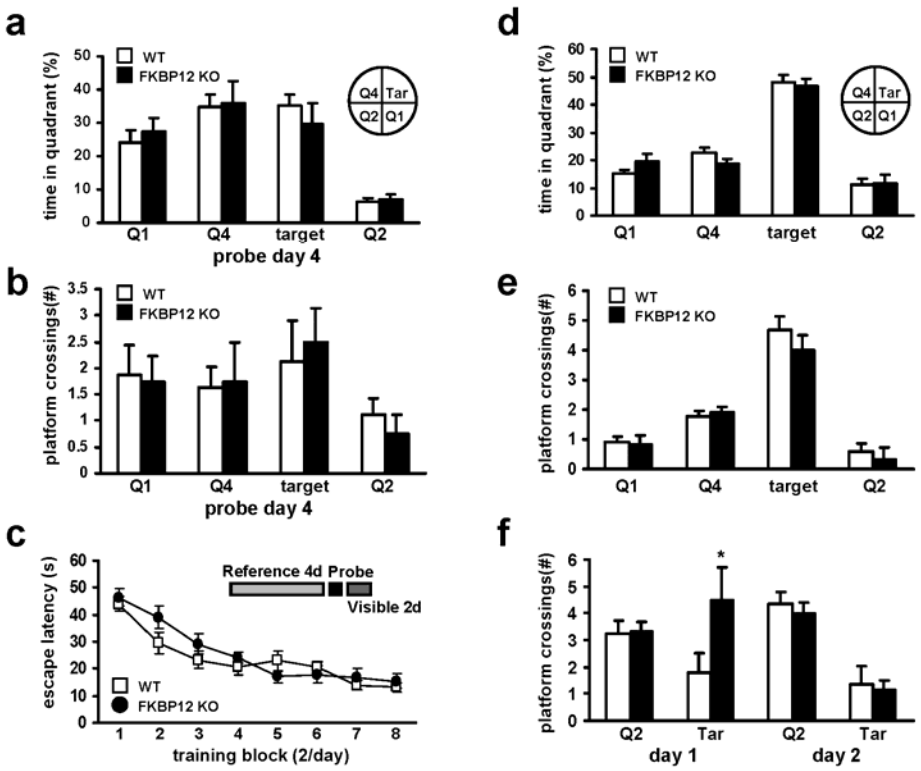


Supplemental Figure 1. FKBP12 is normally expressed by floxed alleles of FKBP12 in the hippocampi of T29-1 *CAMKIIα* driven conditional knockout mice. (a) FKBP12 protein levels are expressed in mice homozygous for floxed alleles of the FKBP12 gene compared to levels expressed in mice with only wild-type (WT) FKBP12 alleles. Lysates prepared from hippocampi extracted from appropriate mouse genotypes, 20 µg loaded/ lane, β-actin controls not shown.

(b) T29-1 driven removal of floxed FKBP12 from the hippocampus occurs as early as two weeks of age. FKBP12 levels in tissues isolated from the hippocampus show nearly identical expression at P14 compared to 12 week old adult animals **(c)** Immunoprecipitation (IP) with mTOR antibody efficiently extracts mTOR complexes from prepared lysates. mTOR is highly enriched in the IP fraction but is absent in the supernatant and the wash fractions. Raptor is present at greater levels in the WT supernatant compared to the KO supernatant. **(d)** FKBP38 is present at very low levels in the mouse brain but is present at high levels in the periphery. Lysates prepared from brain region or peripheral tissue extracted from appropriate genotype of mice, either 60 or 20 µg loaded/ lane, β-actin controls not shown. FKBP38 is nearly undetectable in the brain compared to FKBP12 when loaded at similar concentrations. FKBP38 levels are not upregulated in the hippocampi of adults or pups of FKBP12 conditional knockout mice (2 and 12 weeks old) in response to FKBP12 removal. **(e)** Phosphorylated S6K (at Ser 389) is enriched in mTOR IPs prepared from the hippocampus of FKBP12 mutant mice. **(f)** mTORC1 levels increase in Area CA3 response to fear conditioning.



Supplemental Figure 2. *FKBP12* conditional knockout mice demonstrate normal anxiety levels in OFA but enhanced performance on during rotarod learning task (a) FKBP12 conditional knockout mice (KO) display no difference when compared to wild-type (WT) littermates in OFA. No differences were observed between either genotype for total time in (s) spent moving (move), spent at rest (rest), spent rearing (vert), spent moving in stereotypic (repetitive) fashion (stereo), or spent in the center quadrants of the testing arena (center). WT, n=21; KO, n=22. **(b)** FKBP12-deficient mice demonstrate enhanced learning on the rotarod task. FKBP12 KO mice reach maximal latency on day three compared to day six for wild-type animals (* $p<.05$). WT, n=16; KO, n=14. Mean weight in (g) of animals tested, WT, 22.5 ± 0.9; KO, 23.1 ± 1.3 ($p>.05$).



Supplemental Figure 3. FKBP12 conditional knockout mice have normal spatial learning and memory following eight day MWM reference testing but enhanced platform perseveration on the first day of reversal testing during eight day MWM training. (a) Escape latency times during eight day MWM training. **(b)** The mean of time spent in each maze quadrant during the probe test. **(c)** The mean number of platform crossings (equivalent location) for each quadrant during probe test. **(d)** The mean number of platform crossings (equivalent location) for each quadrant during probe test (four day training). **(e)** Perseverance for Q4 platform location (original training location) during reversal testing (eight day training) (*, p<.05). **(f)** Escape latency times during place learning and working memory phases of repeat acquisition paradigm (* p<0.05). For eight day MWM, WT, n=11; KO, n=12. For RAP, WT, n=11; KO, n=9 (* p<0.05).

Supplemental Experimental Procedures

FKBP12 cKO mice

Because we detected low level expression of Cre in the testes of male *CamKIIα-Cre*$^{+}$ mice, we used the following breeding strategy: males referred, to as *Cre*$^{-}$ *Fkbp12*$^{fl/-}$, were crossed to *CamKII-Cre*$^{+}$ *Fkbp12*$^{fl/-}$ females.

Western blots

The homogenizing buffer used for non-immunopreciptated tissue samples was (in mM): 50 Tris-HCl, pH 7.5; 150 KCl; 1 DTT; 1 EDTA; 1X complete protease inhibitor cocktail III; 1X phosphatase inhibitor cocktail I; 1X phosphatase inhibitor cocktail II (Sigma, St. Louis, MO). The antibodies and the dilutions for the Western blots used in used in these studies are as follows. Phospho-mTOR (Ser-2884) and total mTOR antibodies were diluted 1:1000. Phospho-S6K (Thr-389), Phospho-S6K (Thr-421/424), total S6K1, phospho-4E-BP1 (Thr-37/46), and total 4E-BP were diluted 1:1000 (Cell Signaling Technology, Beverly, MA). β-actin antibody was diluted 1:5000 and FKBP12 antibodies were diluted 1:200 (Santa Cruz, Santa Cruz, CA). Anti-rabbit and anti-goat HRP-tagged antibodies (Promega, Madison, WI) were diluted 1:5000. All blots were developed using enhanced chemiluminescence detection (GE Healthcare, Fairfield, CT). The bands of each Western blot were quantified from film exposures (KODAK) or were imaged using the KODAK 4000MM imaging system. All signals were obtained in the linear range for each antibody, normalized by total protein, and quantified via densitometry. Data represent mean ± SEM. A Student's *t*-test was used for Western blot analysis with $p < 0.05$ as significance criteria.

Immunoprecipitation

Transverse hippocampal slices (400 μm) were prepared from age-matched mice (12-24 weeks of age) using conventional techniques. Slices were maintained at 30˚C in an interface chamber perfused with oxygenated artificial cerebrospinal fluid (ACSF) containing in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose, 2 $CaCl_2$, and 1 $MgCl_2$ (2 ml/min). Slices were allowed to recover in ACSF for 60 min at 32ºC. Slices either were harvested for protein purification or were stimulated using HFS (identical stimulation to L-LTP induction). Area CA1 was extracted from individual mice on dry ice and was pooled to obtain 75-100 μg of protein. Tissue was homogenized in ice cold lysis buffer containing (in mM): 40 HEPES (pH 7.5), 150 NaCl, 10 pyrophosphate, 10 glycerophosphate, 1 EDTA and 0.3% CHAPS, Protease Inhibitor II, Phosphatase Inhibitor Cocktail I, II (Sigma, St. Louis, MO). Cleared homogenate was incubated with either anti-mTOR (1:50) (Cell Signaling Technology, MA) or anti-Raptor (1:100) antibody (Bethyl labs, Montgomery, TX) and gently shaken overnight at 4ºC. 10 μL/ 100 μL supernatant/antibody were added to slurry of IgG bound to agarose-beads (Pierce). The bead/sample slurry was incubated while rocking at 25ºC for two hrs. Immunoprecipitates were washed three times in lysis buffer, and once in wash buffer in mM (50 HEPES pH 7.5, 40 NaCl, 2 EDTA). SDS-PAGE buffer was added to the washed IPs, which were then resolved on SDS-PAGE gels. The specificity of the IP was tested using antibodies against dynamin, Ras, and β-tubulin (data not shown).

Electrophysiology

Hippocampal slices were prepared in a fashion identical to those utilized for the immunoprecipation experiments. When indicated, ASCF was incubated with 20

nM rapamycin (Cell signaling Technology, Beverly, MA) or 40 μM anisomycin (Sigma-Aldrich, St. Louis, MO). Extracellular fEPSPs were evoked by stimulation of Schaeffer collateral pathway afferents and were measured by recording in stratum radiatum of area CA1. Stable baseline synaptic transmission was established for 20-30 min with a stimulus intensity of 50% of the maximum fEPSP prior to LTP-inducing high-frequency stimulation (HFS) or theta-burst stimulation (TBS). Stimulus intensity of the HFS was matched to the intensity used in the baseline recordings. LTP was induced by either one or four trains (5 min intertrain interval) of 100 Hz for one sec. TBS was delivered as either one or three patterns of stimulation consisting of five trains of four pulses at 100 Hz (200 ms interpulse interval) with an interburst interval of one min. Data were collected and presented as the average slope of the fEPSP from six individual traces collected over two min and then normalized to baseline recordings of fEPSPs. Two-way ANOVA were used for electrophysiological data analysis with $p < 0.05$ as significance criteria.

Associative Conditioned Fear

Mice were placed in a clean Plexiglas cage and habituated to the testing area for 30 min prior to training. Following the habituation session, the training sessions for contextual and cued fear conditioning consisted of a 150 sec exploration period followed by two conditioned stimulus-unconditioned stimulus (CS-US) pairings separated by one min (foot-shock intensity 0.9 mA, two s duration; tone 85 db white noise, 30 sec duration). Context tests were performed in the training chamber after one and 24 hrs. Cued tests were performed in an environmentally altered testing chamber (adding different flooring and vanilla odorant) either two or 24 hrs following training. Baseline freezing was monitored (three min) prior to

presentation of the tone (85 db white noise, three min duration). Data represent mean + SEM. A Student's *t*-test was used for statistical analysis with $p < 0.05$ as significance criteria.

Marble Burying

Mice were placed individually in clean Plexiglas cages containing fresh five cm deep bedding, along with 20 small black marbles arranged in five evenly spaced rows of four marbles. Testing was conducted for a 30-min period under normal room lighting and white noise conditions. After the test period, mice were removed and the unburied marbles were counted. The tester was blind to genotyping during testing. Marbles were considered buried if they were at least one-half covered with bedding. A one-way ANOVA was used for statistical analysis with $p < 0.05$ as significance criteria.

Novel Object Recognition

Mice from each genotype were tested for preference to the objects (Legos) to be used during the familiar and novel phases of the experiment. Objects first were tested for neutral preference strength using a naïve cohort of test mice. Objects that did not generate a preference difference were used for the object presentation on a new set of mice. Male mice from each genotype were habituated in a square testing arena (30 cm x 30 cm) for 30 min on day one. On day two the mice were habituated again to the arena, except that several equally spaced objects were contained within it. Mice then were presented with two objects (familiar) within specific areas (counter-balanced locations for presentation of objects) of the arena on days three and four. The mice were allowed to freely explore the arena and objects for 15 min then returned to their

home cages. On day five, one of the objects (used days three and four) was replaced with a third object (the novel object) and the mice were allowed to explore the environment for 15 min. Time (in sec during the first five min) spent exploring each object was recorded. Exploration was defined as contact with the object (tail only excluded) or facing the object (distance <2 cm). The amount of time spent exploring the novel object was divided by the amount of time exploring both the novel and familiar objects using a video tracking system. The resulting value was divided by total time to generate a preference index (PI). A PI value of 0.5 indicates preference for neither object. A PI>0.5 indicates a preference for the novel object, conversely a PI<0.5 indicates a preference for the familiar object. A one-way ANOVA was employed to assess the effect of genotype on the PI.

Open Field Analysis (OFA)

Mice were placed into the center of a clear Plexiglas (40x40x30) cm open-field arena and allowed to explore for 30 min on one day. Bright, overhead lighting provided ~800 lux of illumination inside the arenas. White noise was present at 55 dB inside the arena room. Data were collected in 2 min intervals by a computer-operated Digiscan optical animal activity system (RXYZCM, Accuscan Electronics). Average speed was calculated by dividing the total distance by time spent moving. The center distance was divided by the total distance to obtain a center distance: total distance ratio, which is used as a measure of anxiety-like behavior. Open-field activity data were pooled into three 10 min intervals and analyzed using a two-way (genotype by interval) analysis of variance (ANOVA) with repeated measures. The data is presented as summary of the intervals.

Rotarod test

Rotating rod learning was tested one day after OFA using an accelerating rotarod (UGO Basile, Varese, Italy). Mice were placed on a rotating drum, accelerated from 4 to 40 r.p.m. over a 300 sec period. Time spent walking on top of the rod before falling off the rod or hanging on and circling without moving independently around the rod was recorded. Mice were given four trials on two consecutive days with a maximum time of 300 sec and a 30 min intertrial rest interval. Rotarod data were analyzed using a two-way (genotype by trial) ANOVA with repeated measures (*$p<.05$).

Morris Water Maze and Repeated Acquisition Paradigm

For all water maze experiments, FKBP12 cKO mice and their wild-type littermates (2–4 months old) were used. Mice of both sexes were used, but were separately analyzed for sex specific differences, No sex differences were found, so the data were combined. Training for the hidden platform version of the Morris water maze consisted of four trials (60 sec maximum, intertrial interval (ITI) 30 min) each day for eight consecutive days. The probe trial was administered one hr following the completion of training on day eight. The visible platform task consisted of four trials (ITI 20 min) each day for two consecutive days with the escape platform marked by a visible cue and moved randomly between two locations.

The repeat acquisition paradigm was adapted from previous studies (Gimenez-Llort et al., 2005). Briefly, the paradigm consisted of a 24-day training period broken into several phases. On days one through five, mice were trained to locate a submerged hidden platform, using four trials (60 sec maximum, inter trial interval (ITI) 30 min). On days six and seven, reversal training occurred

(submerged platform is moved to opposite quadrant) using four trials (60 sec maximum, intertrial interval (ITI) 30 min). On days eight and nine, the mice were tested using a visible cue (as above). On days 14-17, the mice were tested for place learning and working memory with a submerged platform that was randomly placed in the maze. The platform location was different for each day of the four day testing phase. Mice that failed to locate the platform (60 sec trial) were placed on it for 15 sec before being retested. Mice were tested using another four random locations on days 21-24 in an identical format to days 14-17. The trajectories of the mice were recorded with a video tracking system (HVS Image Analyzing VP-200). Data represent mean + SEM. ANOVA and χ^2 tests were used for statistical analysis with $p < 0.05$ as significance criteria.

Arm Reversal in Y-Maze

Age matched male mice were habituated to the maze for 15 min on day one and then returned to their home cage. On day two the mice were trained to locate a submerged escape platform (in a pool of obscured water) in one arm or another of a Y-shaped maze (simple always right or always left arm pattern) for 20 trials. The mice were returned to their home cages after day two training. On day three, the mice were tested to see if they achieve an escape success criterion of 4/5 correct. For mice that achieved this criterion, the escape arm was reversed and the mice were tested to determine the latency to find the new escape location. Mice were allowed only 20 sec (maximum) to make an arm choice. Mice were not directed to the correct arm if they made an error. If mice made an error in arm choice, they were trapped in the incorrect arm for 20 sec. Mice then were returned to their home cage. Mice were assigned randomly to either left or right arms at the beginning of training and the researcher was blind to genotype during

training/testing. Repeated measures ANOVA tests were used for statistical analysis with $p < 0.05$ as significance criteria.