Supporting Information

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Supporting Materials and Methods

Materials and Methods

Generation of MEF2C Mutant and Transgenic Mice. The *Mef2c*-null allele referred to as $Mef2c^{KO}$, and *Mef2c* conditional allele referred to as $Mef2c^{loxP}$, have been described previously (1, 2). To generate mice that lack MEF2C in the brain, mice heterozygous for the null allele of *Mef2c* were mated to mice transgenic for Cre recombinase under control of a human GFAP promoter (hGFAP) (3, 4). The resulting mice were mated to mice homozygous for the $Mef2c^{loxP}$ allele to produce conditional null mice.

The neuronal-specific transgene was constructed by subcloning an in-frame fusion of the MEF2C DNA binding-domain and the VP16 activation domain to a construct containing 1.8kb of 5′ flanking sequence from the rat NSE gene (5). The transgenic founders were produced by pronuclear injection of the linearized transgenic construct into fertilized oocytes. The founder mice were then intercrossed with wild type littermates to generate F1 and subsequently F2 mice that were used for behavioral and electrophysiological experiments. All animal and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center at Dallas.

Tissue Processing, Histology, TUNEL, Immunohistochemistry, and RNA *In Situ* Hybridization. Age P60 animals were anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde. The brains were dissected and postfixed in 4% paraformaldehyde for 12 h at 4°C. Samples were then embedded in paraffin, sectioned at 5 μm, and stained with Cresyl violet (Nissl staining). Perfused brains were fixed overnight in DEPC-treated 4% paraformaldehyde. Riboprobes were labeled with ^{35}S-UTP by using MAXIscript *in vitro* transcription kit (Ambion). *In situ* hybridization was performed as described (6).

For TUNEL assay, brains from P14 and P40 animals (two animals of each genotype) were dissected as described above. Free DNA ends were detected on coronal brain sections of animals using the *In situ* cell death detection kit (Roche) according to the manufacturer's protocol.

We performed all immunohistochemistry on triplicate sections per group. Based on anatomy, matched sections from control and mutant were used. Mice were perfused as described above. Brains were postfixed and then transferred to a 30% sucrose solution in PBS and cryoprotected at 4°C overnight. The brains were sectioned (30-μm sections) on a freezing microtome and mounted onto glass slides. MEF2C immunostaining using goat anti-MEF2C (Santa Cruz) or monoclonal antibody to the neuronal-specific nuclear protein NeuN (Chemicon) was performed as described (7)

For RT-PCR, frontal cortex, hippocampus and dentate gyrus were dissected and immediately frozen. From individual samples (six wild type and six $Mef2c^{BKO/KO}$ littermates), total RNA was isolated using TRIzol reagent and standard protocols. Total RNA (1 μg) was used as a template for first strand cDNA synthesis using the SuperScript first strand synthesis kit from Invitrogen. Quantitative analysis was performed by real-time PCR using TaqMan chemistry on an ABI PRISM 7000 sequence detection system (Applied Biosystems). Predesigned intron-spanning primers were purchased from Applied Biosystems. The relative quantities of mRNA were determined using standard curves.

Electron Microscopy. Age P30 $Mef2c^{BKO/KO}$ and wild-type littermate mice ($n = 2$ each genotype) were anesthetized and transcardially perfused with 0.1 M phosphate buffer, pH 7.3 at 37°C, followed by 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at 37°C. Brains were removed and postfixed overnight in the same fixative at 4°C. Square blocks, 500 μm, of the dentate gyrus were then cut using a Leica vibratome. These were then fixed with 1% osmium tetroxide and embedded. Sections, 60 nm thick, were cut on the Leica Ultracut UCT microtome. Sections were collected on 200 mesh copper grids and stained with uranyl acetate and lead citrate. Sections were imaged on the Jeol 1200 EX TEM at magnification of ×40,000–50,000. Images were collected at a distance of 100–200 μm from the dentate gyrus granule cell bodies.

Quantification of Dendritic Spine Density. Golgi-Cox silver impregnation of neurons was performed using commercially available reagents (FD Rapid Golgi Stain Kit, NeuroTechnologies). P21 Animals (two of each genotype) were perfused with cold PBS, and the whole brain placed in silver impregnation solution for 12 days, transferred to Solution C for 2 days, and cut into 100 μM sections on a cryostat.

Neuronal Cell Culture. Dissociated hippocampal neurons were fixed for 30 min with 4% paraformaldehyde, rinsed twice in PBS/Glycine, then blocked in 2% goat serum for 1 h. The cells were then incubated with primary antibodies, anti-postsynaptic density-95 (PSD-95, Affinity Bioreagents) and antisynapsin polyclonal (Synaptic Systems) overnight at 4°C. The next day the cells were washed, then incubated with fluorescent secondary antibodies. Coverslips were mounted with Vectashield (Vector Laboratories) and neurons were visualized on a Zeiss Confocal microscope.

Behavioral Studies. Behavioral tests were performed on adult male mice at least 2 months old, with littermates of both genotypes. As control littermate animals for tests using $Mef2c^{BKO/KO}$ mice ($n = 18$), we used mice carrying the floxed *Mef2c* allele but no cre transgene ($n = 17$). As control littermate animals ($n = 14$) for tests using MEF2-VP16 transgenic animals ($n = 17$), we used mice negative for the NSE-MEF2C-VP16 transgene. The experimenters were blinded to the genotype in all tests.

Locomotor Activity. Animals were placed in a fresh home cage and locomotor activity was measured for 2 h by photocell beams linked to computer data acquisition software (San Diego Instruments).

Rotarod. Each mouse was placed on the rotarod (IITC Life Science). The rotarod was activated and its speed ramped up from 0–45 revolutions per minute in 60 seconds. The time to fall off the rotarod or turn one full revolution was measured for three consecutive trials. The mouse was returned to its original cage for 2 hours. The test was repeated for a total of three runs/day for 4 consecutive days.

Elevated-Plus Maze. Mice were placed in the center of an elevated-plus maze (arms are 33 × 5 cm, with 25-cm tall walls on the closed arms) under dim lighting and their behavior was videotaped for 5 min. The tapes were analyzed by an observer blind to the genotype of the animals. The time spent in the closed and open area, as well as the number of explorations of open arms, was determined.

Open Field. Mice were tested for their activity during 6 min in a 72-cm-diameter open field under dim lighting. A video tracking system (Ethovision 3.0, Noldus) was used to measure the locomotor activity of the animal, as well as the time spent in the center and borders of the test. Anxiety-like behavior is measured as the total time in the center of the open field as well as the latency to enter the center of the field.

Fear Conditioning. Mice were placed in individual chambers (Med Associates) with four mice tested at once. A total of three tests were performed, the first being the training test. Mice were placed in the chambers for 2 min followed by a loud tone (90 dB) for 30 sec then immediately followed by a 0.8-mA footshock for 2 sec. Mice remained in the box for 1 min at which time they again received a loud tone (90 dB) for 30 sec and then an immediate 0.8-mA footshock for 2 sec. The mice were immediately removed and placed back into their home cages. Each chamber was cleaned. To test for context-dependent fear conditioning, 24 h later, the mice were placed back in the same boxes without a tone or shock, and their behavior videotaped for 5 min. The amount of time the animal spent freezing was assessed by the FreezeFrame program (Actimetrics). Freezing behavior was defined as no movement except for respiration. Four hours later, the cue test was performed. To test for cue-dependent fear conditioning, mice were placed in a novel environment scented with vanilla odor with no tone or shock for 3 minutes followed by 3 minutes of the tone. The amount of time the mice spent freezing was assessed as described above. Cue-dependent fear conditioning was determined by subtracting the three minute baseline freezing from the freezing during the tone.

Pain Sensitivity. Mice were placed in individual chambers (MedAssociates) for 2 minutes to habituate. The animals were then shocked (0.05 mA; 1 sec), and their behavior was scored as either no movement, flinching, or jumping by an observer blind to their genotype. Every 30 seconds, the shock was increased by 0.05 mA, with a maximum shock of 0.6 mA, until the animal flinched and jumped in response to the shock.

Electrophysiology. Hippocampal slices were prepared from 12- to 21-day-old *Mef2c*$^{BKO/KO}$ and wild-type littermates or NSE-MEF2C-VP16 transgenic and wild-type littermate mice (six animals of each genotype). Synaptic activity was recorded from dentate gyrus granule cells ($n = 23$ for WT and $n = 20$ for *Mef2c*$^{BKO/KO}$; $n = 24$ for WT and $n = 26$ for NSE-MEF2C-VP16 transgenic mice) by a whole-cell voltage–clamp technique. Recordings were obtained with an Axopatch-200B patch-clamp amplifier (Molecular Devices). Patch electrodes were fabricated from borosilicate glass capillaries (Warner Instruments) with a Flaming/Brown model P-97 micropipette puller (Sutter Instruments). Pipettes had resistance of 3–6 MΩ when filled with the internal pipette solution that contained (in mM): 110 K-gluconate, 20 KCl, 10 NaCl, 10 Hepes, 0.6 EGTA, 4 Mg-ATP, 0.3 GTP, 10 Lidocaine *N*-ethyl bromide (QX-314), and buffered to pH 7.2–7.3 with CsOH (280–290 mOsm). Recordings were obtained with an Axopatch-200B patch–clamp amplifier (Molecular Devices). The membrane potential (Vm) was held at −70 mV. Fast and slow capacitances were neutralized. No series resistance (Rs) compensation was used during the experiments. In the whole-cell configuration, Rs ranged between 5 and 15 MΩ, and experiments in which Rs varied ≥20% within a recording session were discarded. In all experiments, voltage errors were less than 10 mV. Signals were low-pass filtered at 2 kHz, digitized at 10 kHz, and fed to a PC computer through a Digidata 1322A interface board (Molecular Devices). The Clampex 9.0 and Clampfit 9.0 software (Molecular Devices) were used for data display, acquisition, and storage. Constant current pulses (200 μs duration, 10–100 μA amplitude) were applied through a stimulus isolation unit (A365, WPI) driven by a dual channel stimulator (Grass S88; Grass Instruments). Spontaneuous events were recorded in the presence of 1 μM TTX. To record and isolate miniature and evoked EPSCs, GABA receptor antagonist PTX (50 μM; Sigma) was added to the bath solution.

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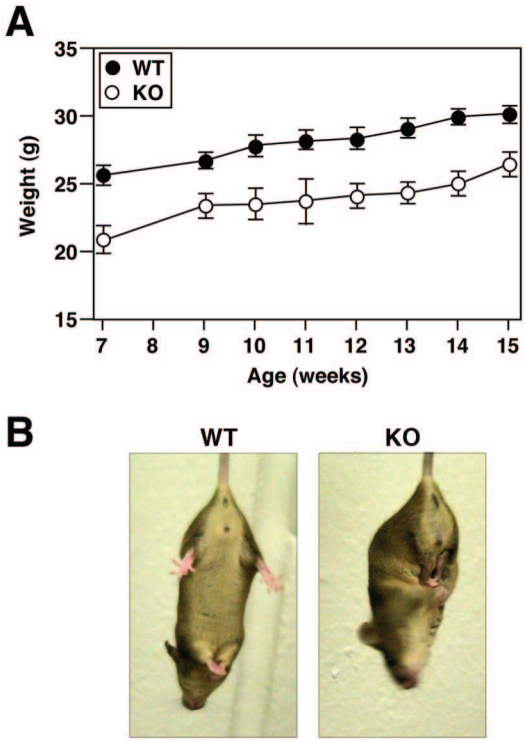

Fig. S1. MEF2C deficiency in brain leads to mice with neurological deficits despite normal brain anatomy. (*A*) *Mef2C*$^{BKO/KO}$ mice (open circle) are slightly reduced in size compared to littermates and significantly weigh less than wild type (WT) littermates (filled circle). (*B*) When suspended by the tail, wild type mice splayed their fore and hindlimbs while the *Mef2C*$^{BKO/KO}$ mice displayed an abnormal fore/hindlimb clasping reflex.

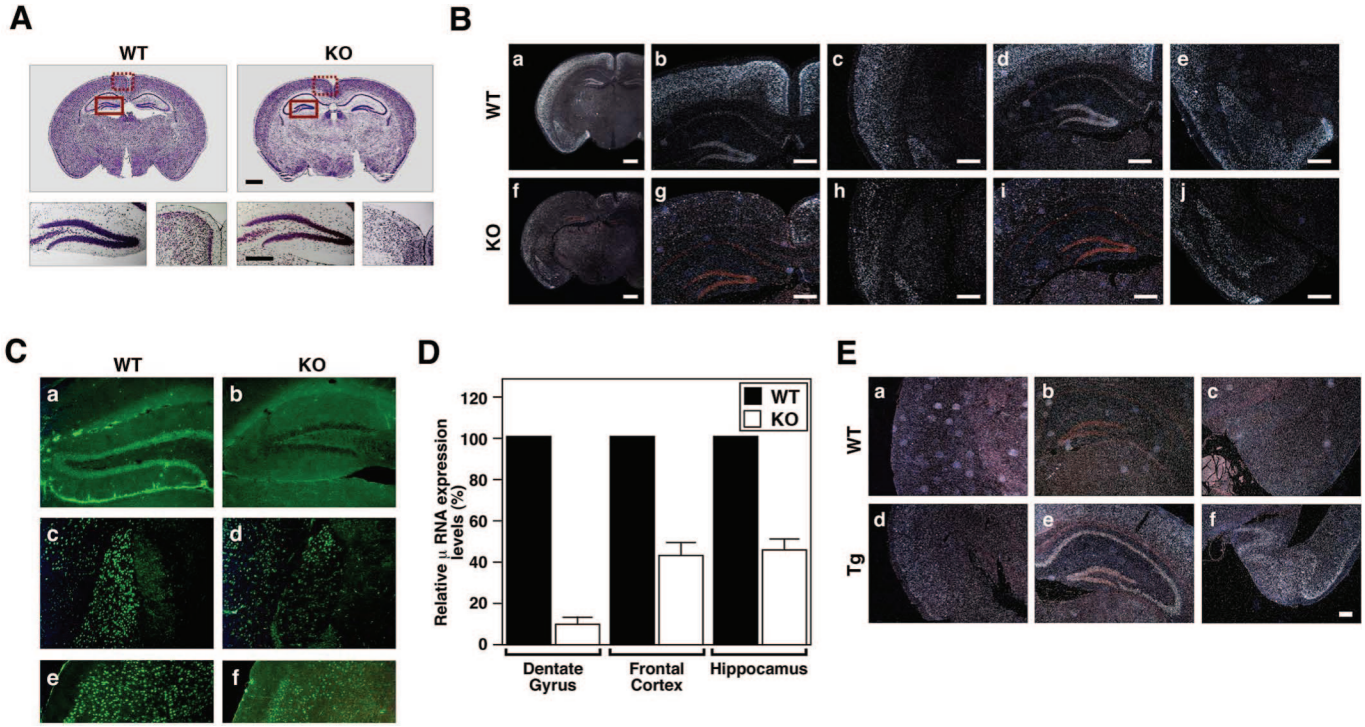

Fig. S2. GFAP-specific Cre-mediated recombination of *Mef2c*. (*A*) Cresyl violet-stained coronal sections of wild type and *Mef2C*$^{BKO/KO}$ mouse brains. For comparison, a region of the cerebral cortex (indicated with dashed rectangle) and the dentate gyrus (indicated with a rectangle) are shown at higher magnification. (Scale bar, 300 μm.) (*Lower*). (*B*) Expression of *Mef2c* was examined by *in situ* hybridization of coronal sections of frontal cortex (b and g), entorhinal cortex (c and h), dentate gyrus (d and i) and amygdala (e and j) of wild type (a-e) and *Mef2c*$^{BKO/KO}$ (f-j) in P60 mice. *Mef2c* mRNA expression was almost undetectable in frontal cortex and dentate gyrus of *Mef2c*$^{BKO/KO}$ mice. (Scale bar, 1 mm.) (a and f); (Scale bar, 400 μm.) (b-e, g-j) (*C*) Immunofluorescence studies of coronal hippocampal sections of P60 mice immunostained with antibody against MEF2C showed MEF2C expression abolished in the *Mef2c*$^{BKO/KO}$ dentate gyrus (b) compared to wild type littermate (a). MEF2C expression in the amygdala (c and d) and lateral entorhinal cortex (e and f) were diminished in the *Mef2c*$^{BKO/KO}$ mice, with only a few neurons still expressing MEF2C. (Scale bar, 200 μm.) (*D*) Detection of *Mef2c* mRNA by real-time RT-PCR using RNA extracted from dentate gyrus, hippocampus and frontal cortex of wild type and *Mef2c*$^{BKO/KO}$ P60 mice. Values are normalized to 18S expression. *Mef2c* expression was almost undetectable in dentate gyrus of *Mef2C*$^{BKO/KO}$ mice and greatly reduced in hippocampus and frontal cortex of the mutant mice. Bars are means $\pm$ SD. (*E*) *In situ* hybridization with an anti-sense *Mef2c-VP16* probe was used to examine the expression of the transgene in coronal sections of entorhinal cortex (a and d), dentate gyrus (b and e) and amygdala (c and f) of wild type (a to c) and MEF2C-VP16 (d to f) P60 littermate mice. Hybridization using anti-sense *Mef2c-VP16* probe did not generate signal in the wild type tissues. *Mef2c-VP16* mRNA was highly expressed in the dentate gyrus of MEF2C-VP16 transgenic mice and weakly expressed in the amygdala of transgenic mice. Expression of *Mef2c-VP16* mRNA was not detected in entorhinal cortex. (Scale bar = 400 μm.)

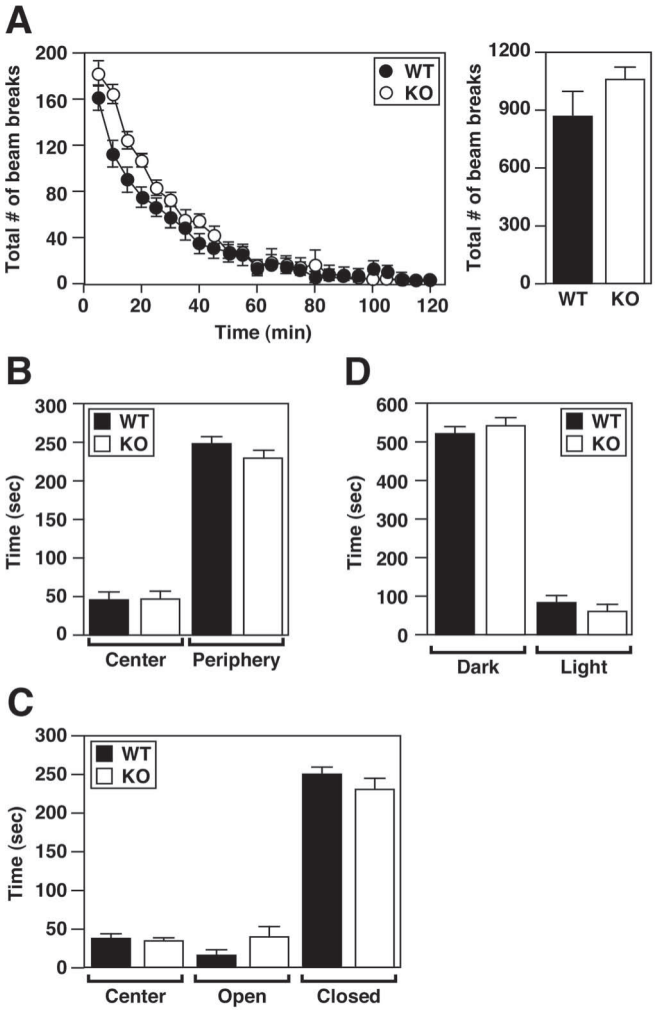

Fig. S3. *Mef2c*$^{BKO/KO}$ mice exhibited normal locomotor activity and anxiety-related behavior. (*A*) Locomotor activity was assessed by placing animals (18 *Mef2c*$^{BKO/KO}$ and 17 wild type littermates) in a new home cage. Locomotor activity was measured for 2 h by photobeams linked to computer data acquisition software. *Mef2c*$^{BKO/KO}$ mice exhibited similar locomotor activity to WT mice. To investigate anxiety-related behaviors, the *Mef2c*$^{BKO/KO}$ mice were examined in the open-field test, elevated plus-maze, and dark-light test. These paradigms rely on the fact that mice avoid open, brightly lit spaces; increased time spent in such spaces is interpreted as decreased anxiety-related behavior. *Mef2c*$^{BKO/KO}$ mice displayed similar levels of anxiety to their wild type littermates as determined by the (*B*) time in the center of the open field, (*C*) time spent in the open arms of the elevated maze and (*D*) time spent in the light side of the dark light test.

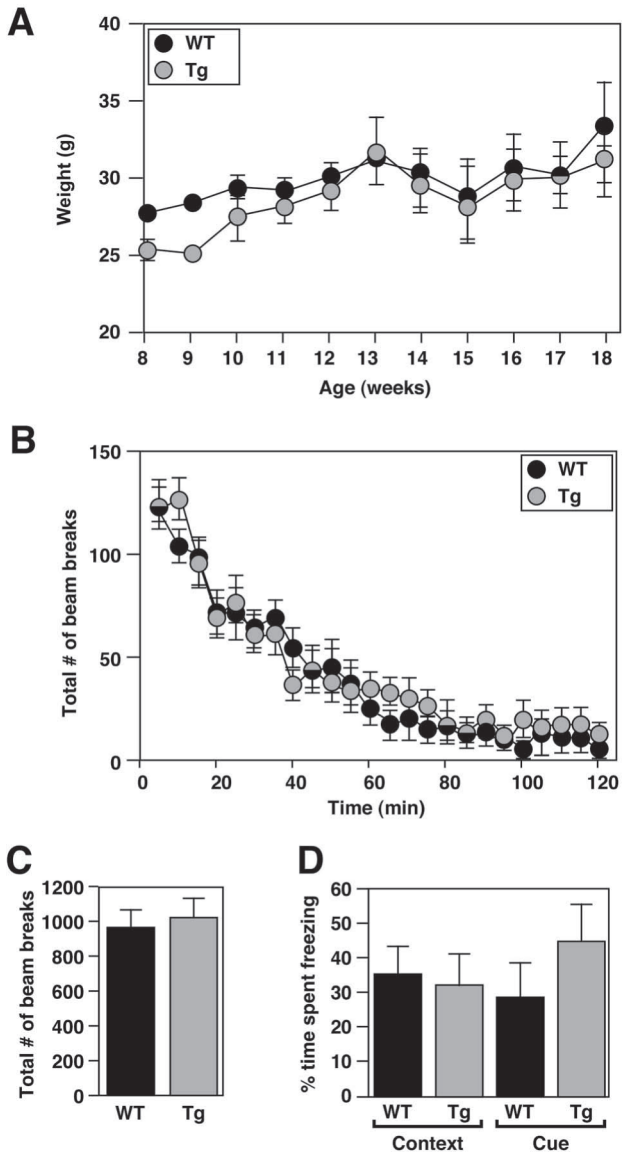

Fig. S4. Behavioral effects of MEF2C gain of function. (*A*) MEF2C-VP16 transgenic mice did not differ from the wild type littermates in body size or body weight. (*B* and *C*) Locomotor activity in the transgenics was indistinguishable from the wild type littermates. (*D*) No difference was observed in context and cue-dependent fear conditioning between MEF2C-VP16 transgenic and wild type littermate mice.

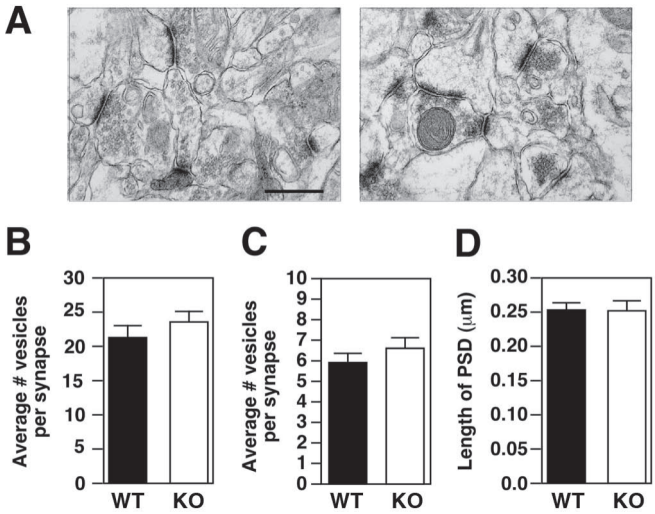

Fig. S5. Hippocampal synaptic ultrastructure is not altered in *Mef2c*$^{BKO/KO}$ mice. (*A*) Representative EM sections from wild type and *Mef2c*$^{BKO/KO}$ dentate gyrus (Scale bar: 500 nm). The total number of vesicles (*B*) and the number of docked vesicles (*C*) per synapse are similar in wild type and *Mef2c*$^{BKO/KO}$ synapses. Data represent average of mean vesicle number per synaptic plane for two animals of each genotype ± SEM. A total of 38 wild type and 52 synapses were counted. (Total number of vesicles, wild type, 21.06 ± 1.99; knockout, 23.68 ± 1.33; $P = 0.26$) (Docked vesicles, wild type, 5.94 ± 0.43; knockout, 6.66 ± 0.45; $P = 0.27$) (*D*) Postsynaptic densities (PSD) length did not differ between wild type and *Mef2c*$^{BKO/KO}$ (in μm) (wild type, 0.254 ± 0.011; knockout, 0.252 ± 0.014; $P = 0.95$).