Postnatal Loss of *Mef2c* Results in Dissociation of Effects on Synapse Number and Learning and Memory

Supplemental Information

Generation of Conditional *Mef2C* KO Mice in Postnatal Brain

The CaMKII-Cre93 line expresses Cre recombinase postnatally at days 10-14 in broad forebrain regions (1). For our breeding, homozygous floxed *Mef2c* females were mated with CaMKII-Cre93 male mice, and then the resultant heterozygous f*Mef2c* males expressing the Cre transgene were mated with homozygous f*Mef2c* females to produce the conditional KO mice. The genotypes were identified by PCR using genomic DNA extracted from tail snippets as described previously (2). Conditional KO and littermate control male mice were 4 to 6 months of age in the present study and group housed, 3-4 per cage. Mice were kept on a 12 hr light/dark cycle with ad libitum access to food and water with all testing conducted during the light cycle. All experiments were conducted in a manner blind to genotype. All animal procedures were approved by the institutional animal care and use committee at UT Southwestern Medical Center in compliance with US Public Health Service guidelines.

Quantitative PCR

Briefly, mice were rapidly decapitated and the whole brain removed from the skull and placed in a brain block. Four brain regions (frontal cortex, striatum, hippocampus, and cerebellum) were dissected from coronal slices (1 mm thick) and total RNA from each brain region was extracted using Trizol (Invitrogen). Complementary DNA was constructed using SuperScriptase III and random hexamers. The primers for *Mef2c* are 5'- AGGATCACCGGAACGAATTCCA-3' and

5’- GCATGCGCTTGACTGAA GGACTTT-3’ while the primers for *Gapdh* have been previously described (3).

Quantification of Dendritic Spine Density

Animals were anesthetized with chloral hydrate and transcardially perfused with phosphate buffered saline. Whole brain was removed and processed for Golgi impregnation. Brain sections were cut coronally at a 100 mm thickness on a cryostat and mounted on gelatin coated slides. Four neurons per section were analyzed for spine counting using Neurolucida software. In each animal, we examined 4 sections (100 µm apart between the section) encompassing dorsal (or anterior) hippocampus and obtained the average value for the number of spines.

Behavioral Overview

Three cohorts of animals were used for the behavioral analyses. The first cohort of mice was tested in novel object recognition and cue-dependent fear conditioning paradigms. The second cohort was examined in locomotor activity boxes, the rotarod to assess motor performance, and hind limb clasping. The third cohort was assessed for grooming behavior, nest building, social interaction with adult mice, social interaction with juvenile mice, context-dependent fear conditioning, foot shock, and performance in olfaction tests.

Locomotor Activity

Mice were placed in fresh home cages and their activity was recorded for 2 hours under red light by photocell beam breaks linked to a computer using PAS software (San Diego Instrument).

Rotarod

Mice were placed on an accelerating rotarod (IITC Life Sciences) and examined as described previously (3). Each mouse was placed on the accelerating rotarod for a maximum of 5 minutes and the time to the first fall off the rotarod was determined by Series 8 software (IITC Life Science). The mouse was then returned to its home cage. The test was repeated for a total of 8 trials over 2 days (4 trials/day).

Fear Conditioning

For context-dependent fear conditioning, each mouse was placed in a fear conditioning chamber and habituated for 2 min. The mouse was then presented with a train of foot shocks consisting of three 0.5 mA shocks that were a 2 sec duration separated by a 30 sec interval. One minute after the last foot shock, the mouse was removed from the chamber and returned to the home cage. To assess short-term memory, the mouse was returned to the chamber 90 min after the training for 5 min in the absence of any foot shocks and freezing behavior assessed. Twenty-four hours after the training the mouse was returned to the same chamber for 5 min without a foot shock and freezing behavior was monitored. Freezing behavior is defined as the absence of movement except respiration and is scored every 10 seconds during the testing by an observer blind to the genotype.

For cue-dependent fear conditioning, a separate cohort of mice was tested. During training, each mouse was placed in the chamber for 2 minutes to habituate, then presented with a tone (90 dB) for 30 sec, which was terminated with a 2 sec foot shock at 0.5 mA. The mouse remained in the chamber for 1 min and received another tone paired with the foot shock. One minute after presentation of the second tone/foot shock, the mouse was removed from the

chamber and returned to the home cage. Twenty-four hours after training, mice were placed in a novel environment to habituate for 3 min followed by the presentation of the tone for 3 min and freezing behavior was measured. Cue-dependent fear conditioning is determined by subtracting baseline freezing from the freezing observed during the 3 minute period presented with tone.

Pain Sensitivity Test

A mouse was placed in the fear conditioning chamber and allowed to habituate for 2 min, followed by a train of foot shocks starting from 0.05 mA and increasing in intensity by 0.05 mA every 20 sec until a maximum of 0.75 mA or the mouse presented pain by vocalization. The shock intensity required to flinch, jump, and vocalize was scored by an observer.

Novelty Object Recognition Test

On day 1, mice were habituated in an open-field arena for 10 min. On day 2, animals were habituated in the open-field arena again for 10 min after which the animals were removed and two identical objects placed in two corners of the arena (5 cm away from the walls), the mice were then returned and allowed to explore for 10 min. On day 3, the animals were placed in the open field arena with two objects in the same location; one a familiar object and the other a novel object. The animals were allowed to explore for 10 min and the time spent interacting with the objects was assessed. The discrimination index was calculated as (time spent with a novel object – time spent with a familiar object) / total time spent with objects x 100.

Social Interaction Tests

Briefly, an experimental animal was placed in an open-field arena containing an empty wire mesh cage located against one of the walls, and allowed to explore for 5 min. Immediately after, the animal was removed from the arena and a target animal (age matched C57BL/6 male) was placed behind the wire mesh cage. The experimental animal was then reintroduced to the arena and allowed to explore for another 5 min. The movement of the experimental animal was tracked using Ethovision software and the time spent in the interaction zone of the wire mesh cage in the absence and presence of the target animal was determined.

The juvenile social interaction test was performed across 4 days and sociability and social recognition behaviors were assessed as previously described (3). Briefly, an experimental animal was placed in a fresh cage and allowed to habituate for 10 min under red light. A juvenile (male 3-4 weeks old) was then introduced into the cage and an observer scored the time of interaction the experimental mouse made direct social interaction with a juvenile mouse during a 2 min time period. The experimental animal stayed in the cage with the juvenile for another 8 min to ensure the social memory. On the following three days, the experimental animal was placed into a fresh cage for 10 minutes after which the same juvenile was placed into the cage and the amount of time of direct social interaction during a 2 minute time period was measured.

Olfaction Test

The olfaction test was composed of three consecutive sessions and performed in red light. Each mouse was habituated in an empty cage with a cotton swab hanging from the lid for 10 min. In the first session, each mouse was presented with cotton swabs absorbed with water for 2 min for 3 consecutive times. Immediately after the first session, the cotton swab was scented with 10%

vanilla essence (nonsocial odor) and presented for 2 min for 3 consecutive times. The third session used the cotton swabs that were scented with soiled cage bedding from a different mouse cage (social odor). The amount of time a mouse spent sniffing the cotton swabs during each presentation was measured.

Nest Building Test

Nest quality scales are; 0 = no shredding, 1 = flat nest with partial shredding, 2 = shallow nest formed with mostly shredded materials but lacking fully formed wall, 3 = nest with well developed wall, 4 = nest with partial or complete roof (4).

Grooming Test

Each animal was placed in a fresh cage under red light between 10 am and 1 pm to minimize potential circadian-related behavioral changes and their behavior videotaped for 30 min. An observer blind to the genotype scored the time spent grooming during this time period (4).

Field EPSP Recording

Mice that were 8-12 weeks of age were anesthetized with isoflurane and decapitated. The brain was removed and immersed in ice-cold dissection buffer containing the following (in mM): 2.6 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 5 $MgCl_2$, 212 sucrose and 10 glucose. The hippocampi were dissected out and sliced with a vibratome into 400 μm-thick sagittal sections in the ice-cold dissection buffer bubbled with 95% O_2 and 5% CO_2 continuously. The CA3 subregion was surgically removed from each section.AC The hippocampal slices were recovered and incubated for 2-3 hrs at 30°C in oxygenated artificial cerebrospinal fluid (ACSF) containing

the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$ and 10 glucose, pH 7.4. The slices were transferred to the recording chamber and continuously perfused with ACSF at a rate of 2-3 ml/min at 30°C. Field EPSPs (fEPSPs) were evoked by inserting a concentric bipolar stimulating electrode to Schaffer collateral/commissural afferents. The extracellular recording electrodes were filled with ACSF (resistance, 1-2 MΩ) and placed into the CA1 stratum radiatum. Electrical signals were delivered using an Axoclamp 900A amplifier and Clampex 10.0 software. Input/output (I/O) relationship was determined by providing an ascending series of stimulus input intensities. Stable baseline responses were collected every 30 sec using an input stimulus intensity that induced 40-60% of the maximum response. Paired-pulse facilitation (PPF) was elicited by giving paired-pulse stimulations at decreasing interstimulus intervals of 500, 400, 200, 100, 50, 30 and 20 ms and analyzed by dividing the fEPSP slope of pulse 2 by pulse 1. After 20 min of stable baseline, long-term potentiation (LTP) was induced by theta burst stimulation (TBS) composed of 3 trains at 10 s intervals. Each train consists of five 100 Hz bursts with 200 ms inter-burst intervals. The initial slopes of the fEPSPs are expressed as percentages of the preconditioning baseline average, and the time-matched, normalized data were averaged across experiments. Data are presented as mean ± SEM. Two-way ANOVA was used to analyze the data from TBS-induced LTP. The data from I/O relationship and PPF were analyzed by Student's *t* test and significance was set with a *p* value of < 0.05.

Supplemental References

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