Supplementary Information for

Functional Recovery with Electro-Acupuncture Stimulation in an *Mecp2*-Knockout Rat Model of Rett Syndrome

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Materials and methods

1. Materials

5-Bromo-2′-deoxyuridine (BrdU; Cell Signaling Technology, 5292S, mouse monoclonal); anti-c-Fos (Abcam, 190289). doublecortin antibody (Cell Signaling Technology, 4604S). Alexa Fluor® 488(Abcam, 150077, rabbit polyclonal) and Alexa Fluor® 594 (Abcam, 150116). DAPI (Beyotime, C1002). oxytocin ELISA kit (Abcam, 133050); arginine-vasopressin ELISA kit (Abcam, 205928).

2. Animal

Four-to-five-week male *Mecp2*$^{-/y}$ and wild type (WT) Sprague Dawley (SD) rats were prepared according to previous study [1]. Typical symptoms of Rett syndrome in male rats appear relatively early, such as short survival time, decreased motor ability and social ability. Therefore, in this study the male *Mecp2*$^{-/y}$ rats were used as Rett, and littermate male WT rats were used as control. All rats were group-housed in the animal houses, with a 12 h light/dark cycle and a thermo-regulated environment. The use and care of animals complied with the guideline of the Biomedical Research Ethics Committee at the Shanghai Institutes for Biological Science, Chinese Academy of Sciences (CAS) [2].

3. Electro-acupuncture treatment

Rats were lightly immobilized in the rat holder (Fig. S1), their feet were straightened and fixed with medical tape. The electro-acupuncture stimulation was performed with a distant-dense wave stimulation for 20 min with an electrical potential difference of 9 V, an electric current of 10 mA, a pulse width of 20 μs and a frequency of 2:10 Hz (distant wave:dense wave) using the electrostimulator (Hwato SDZ-V nerve and muscle stimulator, Hwato Suzhou Medical Instruments Factory, China). The acupuncture needles (10 mm in length, 0.25 mm in diameter, Hwato Suzhou Medical Instruments Factory) were inserted to a depth of 3 mm at the HT 7 (located at the depression on the posterior lateral side of the wrist, between flexor carpi ulnaris tendon and flexor digitorum superficialis tendon), KI 1 (located at the depression in the middle of the metatarsal area, on the plantar side) and GV 20 (located at the vertex, on the dorsal midline) [3,4]. The bilateral HT 7 and KI 1 were stimulated by electroacupuncture. GV 20 was performed with needle retention. The *Mecp2*$^{-/y}$ rats and WT rats were treated by electroacupuncture six times per week for four continuous weeks. The WT and *Mecp2*$^{-/y}$ rats in control group were also lightly immobilized in the rat holder without electroacupuncture stimulation.

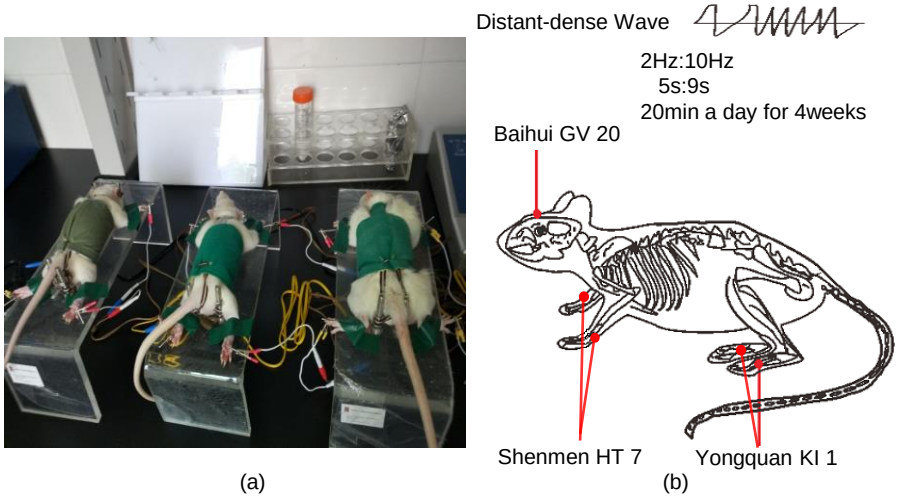

Fig. S1. Acupoints position and experimental parameter. (a) Rats fixation and electro-acupuncture; (b) acupoints and experimental parameter.

4. Behavior test

Test of open field, accelerating rotarod, three-chamber interaction were performed a week before fear conditioning. Before each test, the rats were transported to the behavioral room to habituate for at least 30 min.

4.1. Open-field test

WT and *Mecp2*$^{-/y}$ rats were subjected to a 10 min open-field test and their movement traces were recorded by infrared camera, the open field (100 cm × 100 cm × 100 cm) consisting of central and surrounding zones were divided into ninths to examine the locomotor activity. The trace and videos were collected and analyzed by super-maze soft to assess the motion activity of *Mecp2*$^{-/y}$ and WT rats.

4.2. Accelerating rotarod

The assay was performed as previous procedure [3,5]. WT and *Mecp2*$^{-/y}$ rats were placed on the rotating cylinder of an accelerating rotarod apparatus (Shanghai Xinruan Information Technology. Co., Ltd.) and allowed to move freely as the rotation increased from 5 to 40 r·min^{c} for 5 min period. Before experiment, we first trained rats for 5 min every day for 3 days on the instrument. The testing conditions are the same as the training. The latency to fall was recorded when the rat fell from the rod. Data was shown as mean ± standard error of the mean (SEM).

4.3. Social interaction behavior

The test was performed as previous study [6]. At the first stage, the rat was placed in the middle chamber of the three-chamber apparatus (Shanghai Xinruan Information Technology. Co., Ltd.) equipped with two empty, barred cages in the corners of the left and right. Rats were explored freely and habituated for 10 min. At the sociability test stage, an unfamiliar SD rat (stranger 1) was placed inside a wire cage on right side chamber, an empty wire cage was placed on the left chamber. The test rat was placed in the middle chamber and allowed to move freely. We recorded and analyzed the time spent in each chamber and the sniffing time of test rat with stranger 1 to assess the social ability. At the social novelty test stage, the original stranger rat (stranger 1) remained in its wire cage on the right-side chamber. A new unfamiliar rat (stranger 2) was placed in the wire cage on the left-side chamber. We recorded and analyzed the time spent in each chamber and the sniffing time of the test rat with stranger 1 and stranger 2 to assess the social novelty.

4.4. Fear conditioning

The fear conditioning test was performed according to previous study to evaluate l fear memory [7,8]. Before the experiment, the rats were trained in a rat fear conditioning chamber (Shanghai Xinruan Information Technology. Co., Ltd.). Each rat was placed in the chamber and left undisturbed for 2 min, then a tone (30 s, 4 kHz, 80 dB) coincided with a foot shock (2 s, 0.7 mA). The tone and foot-shock stimuli were recurred after 1 min. After an additional 1 min, the rat was placed to its home cage. Fear memory was assessed at different times of 3 h, 1 day, 3 days, and 7 days after training. The percentage of freezing time was served as an index of fear memory. Data are shown as mean ± SEM.

5. Tissue preparation and immunofluorescence

Rats were transcardially perfused with 0.1 mol·L^{-1} cold phosphate buffer (PB) followed by 4% paraformaldehyde (PFA) in 0.1 mol·L^{-1} PB fixation. The removed brains were post-fixed in 4% PFA in 0.1 mol·L^{-1} PB for 12 h and dehydrated in 10%, 20% and 30% sugar solution at 4 °C respectively. Sections with 40 μm thickness were prepared by the freezing microtome (LEICA CM1950) and stored in poly(butylene succinate) PBS [9]. The brain sections were incubated with 0.25% Triton X-100 in 5% BSA for 1 h at room temperature[7]. Next, the sections were co-incubated with anti-c-Fos polyclonal (Abcam, 190289, rabbit, 1:5000) or double cortin (Cell Signaling Technology, 4604S, rabbit polyclonal, 1:400) and BrdU (Cell Signaling Technology, mouse monoclonal, 5292S, 1:140) for 24 h at 4 °C. Then, the sections were co-incubated with Alexa 488 and 594 secondary antibodies (ab150077 & ab150116, 1: 500) for 2 h and counterstained the cell nucleus by 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, 1:1000) for 15 min at room temperature. After washed three times with 0.1 mol·L^{-1} PBST. The sections were covered with anti-fade reagent (Invitrogen). The image was obtained from the confocal microscope (Leica), the positive cells were counted by Image J software.

6. Statistical analysis

The Graph Pad Prism software (v6.0) was employed for statistical analysis. Survival time curves was analyzed using Log-rank (Mantel–Cox) test. Other statistics among each experimental group was analyzed using two-tailed *t*-test or one-way analysis of variance (ANOVA) (Bonferroni's *post hoc*) test. All data were presented as mean ± SEM. In all analyses, statistical significance was considered at $p < 0.05$.

Reference

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Supplementary figures

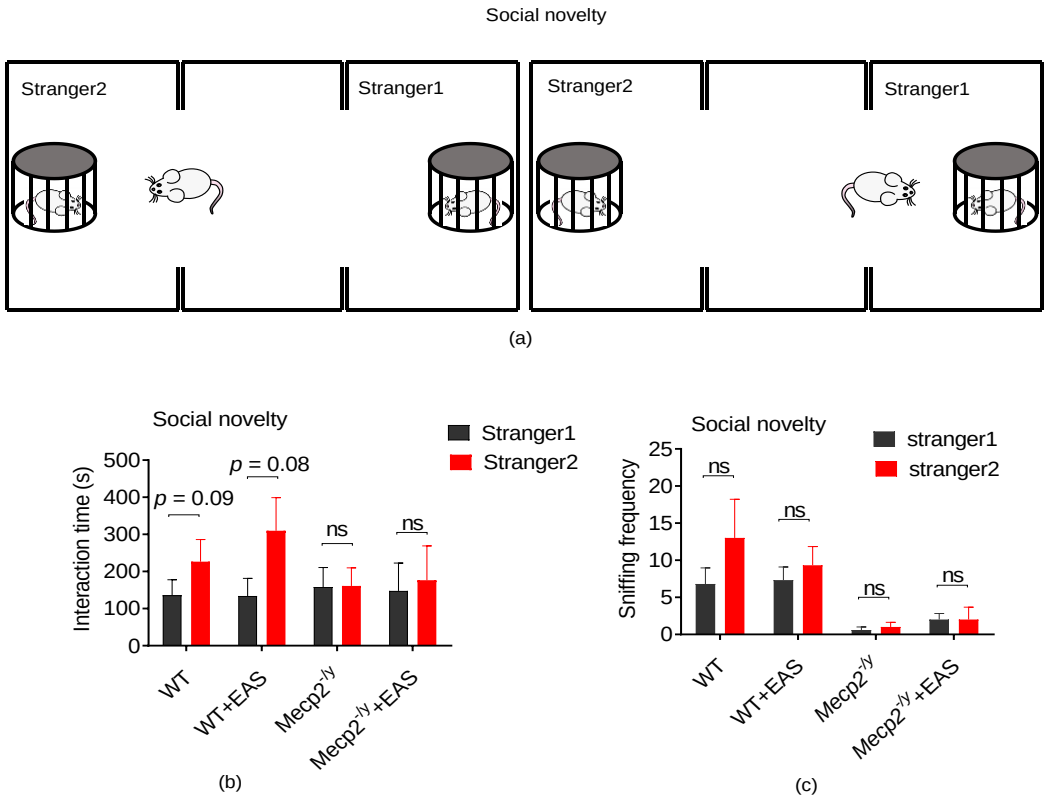

Fig. S2. The effect of EAS on the social novelty behavior of *Mecp2*$^{-/y}$ rats. (a) Schematic diagram of three-chamber apparatus test; (b) interaction time with stranger 1 rat and strange 2 rat side; (c) sniffing frequency with strange rat. All data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01; ns: no significant; one-way ANOVA (Bonferroni's *post hoc*) test.

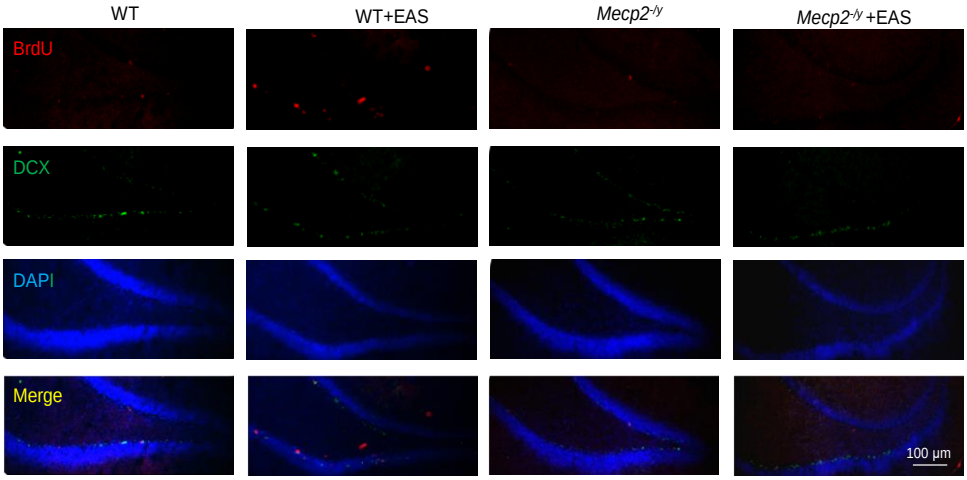

Fig. S3. The immunofluorescence images of BrdU and DCX cells in hippocampus showing BrdU^{+} cells (red), DCX^{+} cells (green) and the merge (yellow) from different groups.

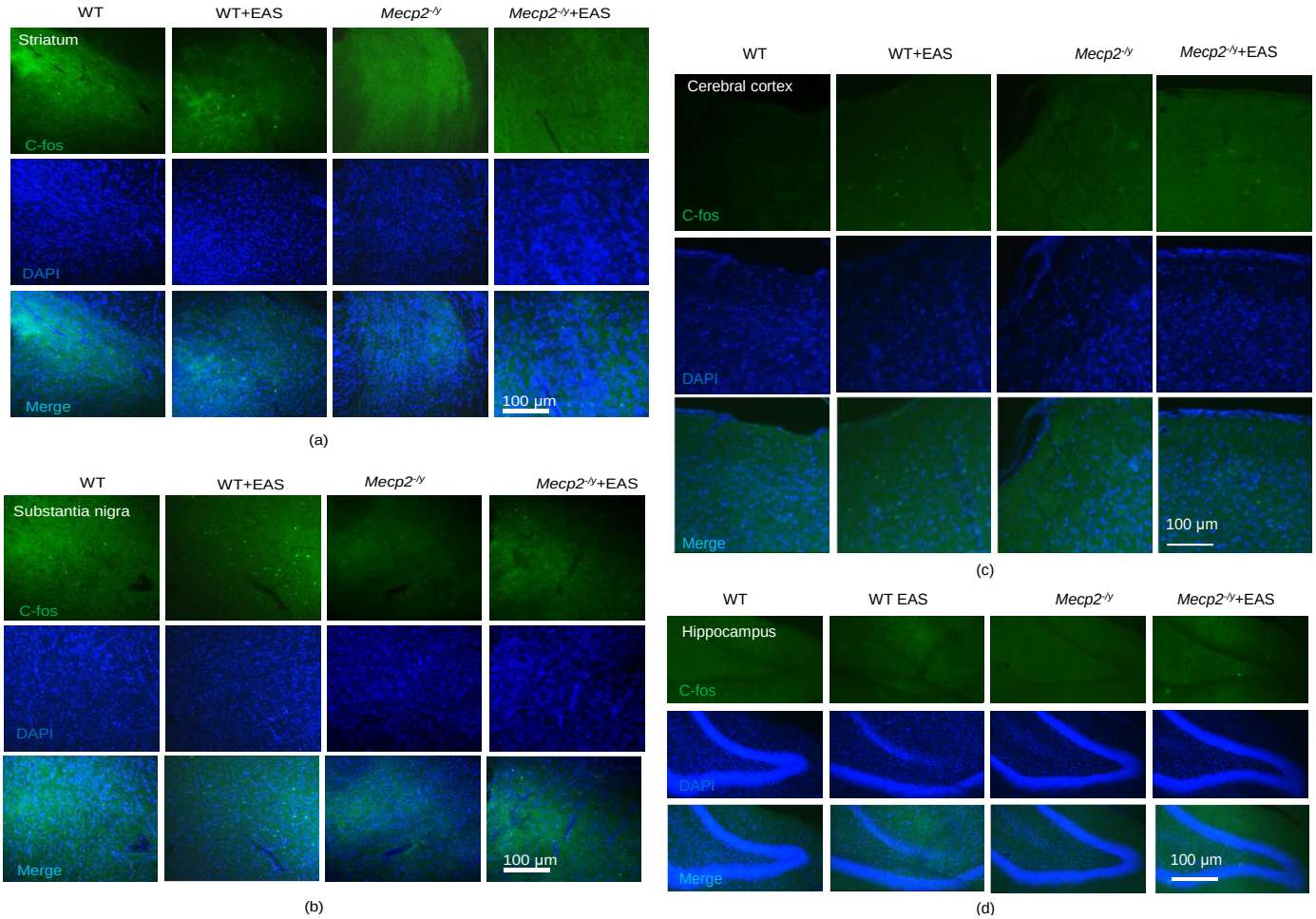

Fig. S4. c-Fos expression of (a) striatum, (b) substantia nigra, (c) cerebral cortex, and (d) hippocampus. c-Fos^{+} cells are shown green.