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Early deprivation induces competitive subordinance in C57BL/6 male mice

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ABSTRACT

Rodent models have been widely used to investigate the impact of early life stress on adult health and behavior. However, the social dimension has rarely been incorporated into the analysis due to methodological limitations. This study characterized the effects of neonatal social isolation (early deprivation, ED) on adult C57BL/6 mouse behavior in a social context using our recently developed behavioral test protocols for group-housed mice. During the first two postnatal weeks, half of the pups per dam were separated from their dam and littermates for 3 h per day (ED group). Post weaning, ED and control pups were electronically tagged and co-housed. At 12 weeks, the mixed cohorts were transferred to IntelliCages, equipped with computer-controlled operant chambers. Access to the chambers was used as an index to analyze novel object response, behavioral flexibility, and competitive dominance with minimal experimenter intervention. In general, ED had greater effects on males; ED males exhibited reduced body weight, increased novelty response, and were subordinate to control littermates when competing for reward access. Male ED mice also demonstrated mildly impaired reversal learning. Analyzing gene expression changes in brain regions controlling emotion, stress, spatial memory, and executive function revealed reduced *BDNF* and *c-Fos* in hippocampal CA1, enhanced *c-Fos* in the basolateral amygdala, reduced *Map2* while enhanced *HSD11β2* in prefrontal cortex of ED males. In male mice, it was suggested that neonatal social isolation results in sustained changes in social behavior with altered function of limbic and frontal cortices.

1. Introduction

Severe early life stress has been proposed as a major contributor to physical and psychosocial disadvantages in adulthood [1,2]. For instance, perinatal glucocorticoid exposure may lead to later physical and mental disorders by interfering with the development and responsiveness of the neuroendocrine system [3]. Rat and mouse models of early life stress, such as separation from the dam and nest for defined periods during infancy, are widely used to investigate the neuroendocrine basis for alterations in adulthood behavior. However, inconsistent behavioral phenotypes have been documented, presumably due to differences in duration of the separation protocol [4], animal strain [5–7], and subject sex [8,9]. In addition, it is important to consider the context in which the experiments were conducted because mouse behavioral responses are markedly sensitive to social context.

We speculated that maintaining social context during behavioral testing may reveal additional effects of early separation, as disruption of the infant–mother–litter relationship is in part a social contextual stressor and so should impact the development of sociality among other neurobehavioral abnormalities. Indeed, social context plays a critical role in both the etiology and expression of psychopathology in humans [10].

Social context-dependent traits, such as performance during competitive challenges, have not been characterized extensively in early life stress models due to the limitations in assessing rat and mouse behavior in undisturbed social contexts. For example, conventional methods for evaluating sociality in mice, such as the three chamber test and the resident-intruder test, involve no more than three individuals and are dependent on experimenter handling. Alternatively, the IntelliCage [11] has suitable features for studying the behavior of over a dozen group-housed mice in the home cage with minimal direct handling by the experimenter. The apparatus is equipped with programmable operant conditioning chambers that enable sensitive, automated

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behavioral monitoring optimal for robust phenotype characterization. In the present study, we examined the lasting effects of early life social isolation stress on adult cognitive behavior under group-housed environment using IntelliCage [12,14].

We adopted the neonatal social isolation paradigm (hereafter referred to as *early deprivation* or ED), according to the nomenclature defined in previous studies [4,5] as the early life stress mouse model. Individual pups were isolated for 3 h daily from their dam and littermates during the first 2 weeks of postnatal development. Because maternal behavior is a critical determinant of pup stress responsiveness [13], the present study was designed to minimize the variability of maternal care by dividing all litters equally between ED and control groups. IntelliCage-based behavioral experiments were conducted when the pups were fully mature. We further investigated whether an ED experience could induce molecular changes in brain regions involved in stress responses and social behaviors by quantifying expression of various immediate early genes, genes associated with synaptic plasticity, and genes of the glucocorticoid signaling pathway in the hippocampus, amygdala, and medial prefrontal cortex (mPFC).

2. Methods

2.1. Animals and early deprivation treatment

C57BL/6J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan), and 12- to 24-week-old females were time-mated over 4 days in our laboratory to yield sufficient numbers of male and female pups for experiments. Parturition was monitored once a day by 10:00, and the day litters were found was defined as postnatal day (PND) 1. Sex of the pups was determined by external genitalia on PND 1, and the litters were culled to 6 — 8 pups per dam. At the time of culling, the sex ratio was adjusted to as close to one to one as possible. ED was conducted according to the procedure reported previously [4,6]. To establish an ED group for each sex, half of the pups (3 or 4 pups/litter) were randomly selected on PND 2, and thereafter, the same pups were removed from the nest for 3 h per day during the late light phase (15:00 — 18:00) up to PND15. Tail coloring with permanent marker pens was used to distinguish pups in the control group from those in the ED group. During ED, the pups were kept at 37 °C in individual cages in a room separate from the home cage. Rest of the littermates (the control group) remained in the nest with their corresponding dams. After weaning, all animals were group-housed with 5–7 animals of the same sex. Therefore, the ED group underwent brief (3 h) separation during the first 2 weeks after birth only and were raised in a social context thereafter. The animal facility was maintained at 23 ± 1 °C with 50 ± 10% humidity on a 12 h light–dark cycle (lights on at 8:00). Rodent chow (Labo MR stock, Nosan, Yokohama, Japan) and purified water were provided *ad libitum* throughout the study, except during the behavioral examinations, at which time water access was permitted only between 22:00 and 1:00. All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine, The University of Tokyo.

2.2. Behavioral analysis in a group-housed environment

2.2.1. General scheme

At 12 weeks, mice were subcutaneously implanted with radiofrequency identification (RFID) microchips for identification (Datamars, SA, USA) and behaviorally phenotyped using the automated behavioral testing apparatus IntelliCage (TSE Systems, GmbH, Bad Homburg, Germany). Males and females were separately tested using different IntelliCage apparatuses. The control and ED groups for each sex comprised 7 individuals per cage. To minimize dam and litter effects, no more than one mouse of each group, per sex, was selected from any one litter.

IntelliCage consists of a large cage (55 × 37.5 × 20.5 cm ($w \times d \times h$)) equipped with four corner chambers (15 x × 15 × x 21 cm (w × x d × x h)). The chambers permit access to the drinking water through motorized doors that sense poking action (referred to as nose pokes hereafter) for opening or closing. Only a single mouse can enter each chamber at a time and is identified by the RFID reader located at the chamber entrance. The visiting and the nose poke patterns are recorded automatically for each mouse. The rules for opening the doors are flexibly programmable, and can be assigned uniquely for each mouse. Therefore, the corner chambers function as fully automated operational units. In the present study, we analyzed novelty-induced exploratory behavior, repetitive nose poking, behavioral flexibility, and competitive dominance, using the same protocol as the previous studies [12,14]. After the novelty response analysis which is described in the next section, mice were kept in the IntelliCage for a week (*Habituation*). For the behavioral flexibility test and the competition task, mice were deprived of water except for the 3 h session period between 22:00 and 1:00, during which water served as a reward. A blue LED light on the cage wall served as a cue to indicate the test period. Mice were trained for 1 week as per the water acquisition schedule before the series of behavioral examinations, when water drinking was permitted only during the LED-cued 3 h period (*Shaping*). Time spent occupying the corner chambers (visit duration) at the beginning of the LED-cued session was considered an index of competitive dominance for reward acquisition since the drinking time per visit was not restricted and re-entries were permitted during the *Shaping* period.

2.2.2. Novelty response test

Before introduction to the operant chamber-equipped IntelliCage apparatus, all mice were habituated for 1 week with their new cagemates in a cage used in the IntelliCage apparatus without four corner chambers. Therefore, the mice were familiarized to the social as well as the physical environments. Corner chambers were placed as the only novel objects to these mice when they were transferred to the fully equipped IntelliCage apparatus. On the day of transfer, the number of visits made per 20 min (visit frequency) to the corner chambers was quantified as an index of the novelty response. From the following day (day 2) to the end of the *Habituation* period (day 7), the visiting pattern was considered to reflect basal activity.

2.2.3. Behavioral flexibility test

Tests on behavioral flexibility, a reflection of executive function, were conducted during the LED-cued period. The test consisted of a behavioral sequencing task in the *Acquisition* phase, followed by its reversals, as described previously [12]. In brief, each mouse was assigned to a pair of diagonally positioned incorrect (never-rewarding) corner chambers and a pair of alternately active/inactive rewarding corner chambers controlled using the IntelliCage software. The spatial assignments were counterbalanced among groups. A visit to the incorrect corner chambers was counted as a discrimination error. Mice were permitted to drink water as a reward for 4 s upon visiting the "active" correct (rewarding) corner chamber. After obtaining the reward, the previously "active" corner chamber became "inactive" and the previously "inactive" corner chamber instead switched to the "active" rewarding (water-dispensing) state. It was thus necessary for mice to acquire the behavioral sequence of shuttling between the pair of diagonally positioned rewarding corner chambers in order to continuously drink water. The alternation of the active/inactive switches was automatically controlled for each mouse by the IntelliCage software program. After the *Acquisition* phase (Acq.) of the behavioral sequencing task, behavioral flexibility was evaluated in the following 7 reversal phases (Rev. 1 to Rev. 7). The previously assigned spatial patterns of the correct and incorrect corner chambers were maintained within each reversal phase, which was switched every 7 sessions in Rev. 1 and Rev. 2, and every 4 sessions from Rev. 3 to Rev. 7. The adaptation to the new rules was assessed based on discrimination errors. Discrimination error rate (%),

used as an index of learning performance, was determined for each session by the number of visits to the incorrect corner chambers within the first 100 visits. Nose poke frequency per visit at the "active" correct corner chamber was considered an index for compulsive repetitive behavior, since excessive nose-poking was behaviorally useless in obtaining additional reward (water).

2.2.4. Competition task

The competition task was conducted as described previously [12]. In brief, mice competed against each other for the reward in limited access sites, the four corner chambers with water. Since mice were deprived of water except for the session period (22:00 − 1:00), the competition was greatest at the beginning of the session. The session period was cued by the LED light on the wall of the cage, and mice were thoroughly trained to learn the association of the cue and the session period. Only one mouse can enter each chamber to access the water at a time, and the water drinking was limited to 4 s per visit for each mouse. Once rewarded at one corner chamber, a mouse had to enter a different corner chamber for another reward since the inactivation of chamber upon re-entry was programmed by the IntelliCage software. In this study, mice were subjected to two competitive conditions: high and low. For the high competitive condition, mice from both the ED and control groups (7 mice per group; total of 14 mice per cage) competed for access. For the low competitive condition, the two groups of mice ($n = 7$ each) were kept and tested in two separate cages. Time spent occupying the corner chamber (visit duration) at the beginning of the session was considered the primary index for competitive dominance. Competitive ranking was determined based on the sum of the visit durations during the first 5 min of the session under both of the high competitive conditions combined, in ascending order: the higher the competitive dominance, the smaller the ranking number.

2.2.5. Gene expression analysis

Mice were sacrificed 1 h into the last (40th) session of the competition task (23:00), and brains were rapidly collected, frozen in powdered dry ice, and stored at −80 °C until analysis. The frozen brains were sectioned on a cryostat (CM3050, Leica Microsystems K.K., Tokyo, Japan) at a thickness of 20 μm and placed on steel-framed PPS membrane slides (Leica Cat. No.11505268). The following sub-regions within the corticolimbic system were chosen as the targets for sampling by laser microdissection (LMD) using the Leica Microsystems LMD7000: the prelimbic cortex (PrL), infralimbic cortex (IL), anterior cingulate cortex (ACC), basolateral nucleus of the amygdala (BLA), central nucleus of the amygdala (CeA), dentate gyrus (DG), and CA1 sub-region of the hippocampus (CA1). Regions of interest (ROI) collected with LMD ranged from 300,000 to 600,000 $\mu m^2 \times 20$ μm in volume. Samples for RNA quantification were prepared as described previously [15]. In brief, RNA containing solution was obtained by dissolving the laser-microdissected tissue samples in the CellAmp Direct RNA Prep Kit lysis buffer (Takara. Otsu, Japan) with proteinase K (0.3 U, Takara), incubated at 50 °C for 30 min, and sonicated for 1 min. Proteinase K was inactivated by incubation at 75 °C for 5 min, followed by DNase treatment (0.05 U, Takara) at 37 °C for 5 min, and DNase inactivation at 75 °C for 5 min in solution adjusted to an optimal concentration for the subsequent reverse transcription (PrimeScript, Takara). For gene expression analysis, 5 of 7 mice per group that underwent behavioral examinations were used. Gene expression levels were quantified by SYBR Green I-based real-time qPCR using Thunderbird qPCR mix (Toyobo, Osaka, Japan) and a Light Cycler instrument (Roche Molecular Biochemicals, Indianapolis, USA). The expression levels of the transcripts are presented as copy number normalized to 18S rRNA expression levels, as it has been previously demonstrated that the amount of 18S rRNA linearly correlates with the size of the ROI [15].

2.3. Statistical analysis and data presentation

Values are expressed as mean ± standard error of the mean (SEM). Student's *t*-test, Wilcoxon signed-rank test, one-way analysis of variance (ANOVA) followed by Tukey post hoc test, or two-way ANOVA with repeated measures followed by Bonferroni post hoc test, and Pearson's or Spearman's test of correlation were employed for statistical analysis using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) for Windows. The significance level between groups was defined as $p < 0.05$. Graphs were generated using the GraphPad Prism 5.0.

3. Results

3.1. Effects of ED on body weight and other gross health conditions

Pups subjected to daily ED tended to be lighter than the control pups, regardless of sex, throughout the 2 weeks of deprivation, although the difference was not significant (Fig. 1A). In males, body weight in the ED group was significantly lower than that in the control group at the time of behavioral experiments [$F(6, 6) = 2.98$; $p < 0.01$] (Fig. 1B). On the other hand, no weight difference was observed between control and ED females (Fig. 1B). No differences were observed between the control and ED groups in appearance of fur or whiskers, and neither group exhibited bald patches, lesions on the feet/tail, and scabs on the tail, rump, or back at the time of first behavioral examination. No difference was observed in the pattern of body weight fluctuations in response to changing housing conditions or during the behavioral examination periods between control and ED groups in either sex (Fig. S1A).

3.2. Effects of ED on novelty response behavior

Upon introduction of animals to the IntelliCage apparatus, the number of visits to any of the four corner chambers during the first day was considered an index of the novelty response. The number of visits during the second day was interpreted as an index of basal activity. ED mice showed sex-dependent alterations in the novelty response compared with controls (Fig. 1C–F), with male ED mice made more visits during the first day than controls, especially during the first few hours and during the dark (active) period (Fig. 1C). The effect of ED treatment was considered significant [$F(1, 1036) = 7.82$; $p < 0.05$], with no treatment × time interaction [$F(74, 1036) = 1.16$; $p = 0.18$]. This increased novelty response of ED males on the first day was not observed on the next day (Fig. 1D) or during the rest of the *Habituation* period (Fig. S1B). In females, the ED group exhibited a tendency for decreased visit frequency during the first 6 h [$F(1, 187) = 4.21$; $p = 0.06$] with no significant interaction with time [$F(17, 187) = 1.31$ and $p = 0.19$] (Fig. 1E and F). The hypoactivity of the ED females normalized within several hours of transfer to the IntelliCage apparatus; therefore, the difference in total visit frequency for the first 24 h between ED and control females did not reach statistical significance [$F(1, 814) = 2.99$; $p = 0.11$], while a significant interaction was observed between ED treatment and time [$F(74, 814) = 1.49$; $p < 0.01$] (Fig. 1F).

3.3. Effects of ED on executive function

Behavioral flexibility was examined by evaluating incorrect (never-rewarding) chamber visits (discrimination error rate) for each mouse using the implanted microchip (Fig. 2A–C). No apparent deficits in the acquisition of the behavioral sequencing task (Fig. 2B) were observed in ED males (Fig. 2D) or ED females (Fig. S3) during the *Acquisition* phase. Overall learning performance throughout the experiment was not significantly impaired in the ED males [$F(1, 480) = 0.47$; $p = 0.51$], with no interaction between session (days) and ED treatment [$F(40, 480) = 1.00$; $p = 0.44$] (Fig. 2D). However, a partial defect in the ability to adjust to the reversal was detected. On the second session of each reversal phase from Rev. 1 to Rev. 5, an analysis of variance yielded

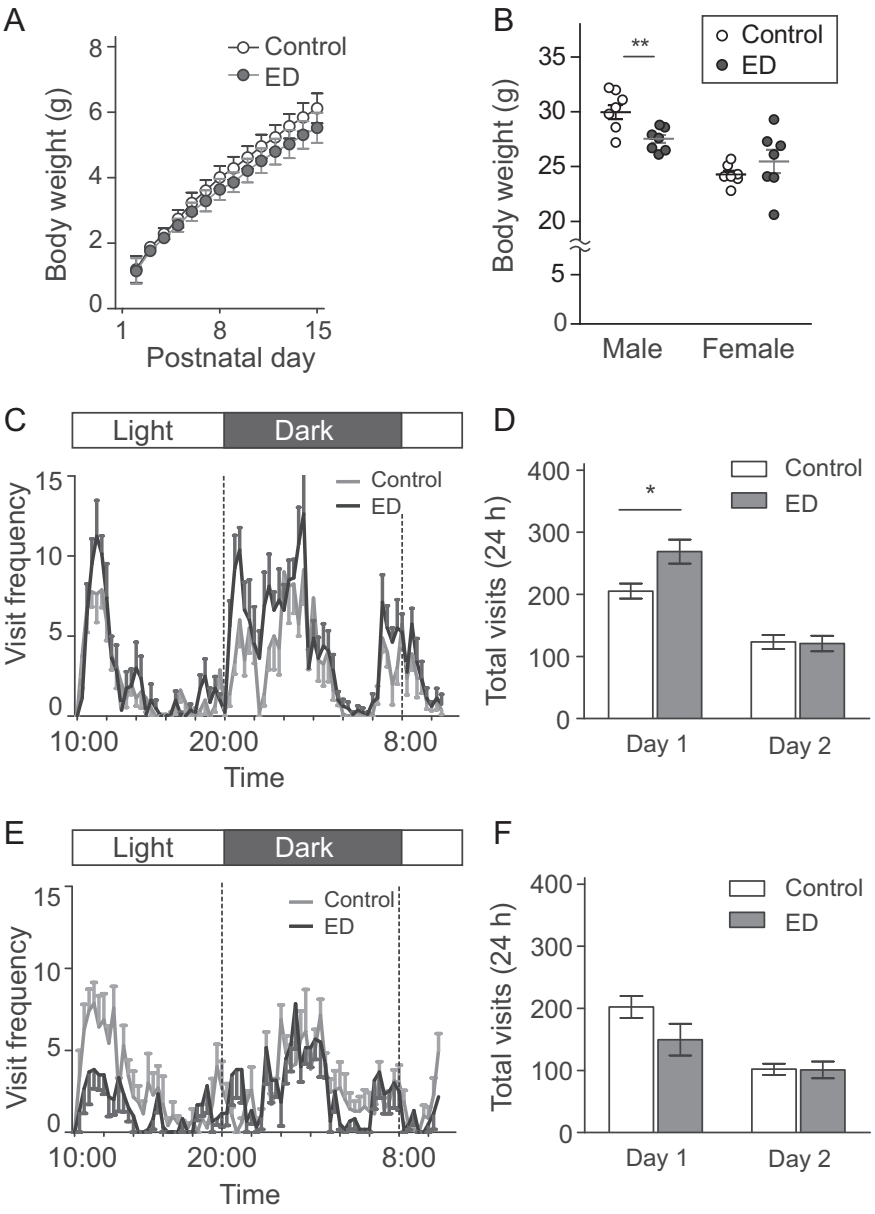

Fig. 1. Effects of early deprivation (ED) on body weight and novelty response. (A) Body weight of the pups during the first 14 neonatal days. The litter average per treatment group plotted against postnatal day regardless of sex since differences in sex were negligible during this period. (B) Body weight in adult mice. (C–F) Number of visits to the novel cage sites (corner chambers) during the first and second days in the IntelliCage apparatus by (C and E) male ED and control mice, and (D and F) female ED and control mice. (C and E) Visit frequency was plotted every 20 min since introducing mice to the novel apparatus at 10:00. (D and F) The total number of visits during the first 24 h (day 1), and the next 24 h (day 2) which indicates the habituation period. Values are presented as mean ± SEM. (A, C, and E) Two-way ANOVA with repeated measures and (B, D, and F) Student's *t*-test. Asterisk denotes statistical significance between the groups ($p < 0.05$). $n = 7$ per group.

significant variation among the ED males and the control males [$F(1, 48) = 5.04$; $p < 0.05$; Fig. 2E], with no significant interaction [$F(4, 48) = 1.00$; $p = 0.42$]. In addition, ED males exhibited a lower nose poke frequency at the rewarding corner chamber compared with controls throughout the behavioral flexibility test (Fig. 2F). There were no significant differences in the number of nose pokes at the incorrect (never-rewarding) corner chambers or at the "inactive" (no longer rewarding) corner chamber (Fig. S2). The suppression of the nose poke frequency by the ED group reached statistical significance throughout the behavioral flexibility test [$F(1, 480) = 5.16$; $p < 0.05$]. However, a significant interaction was observed between the effect of session (days) and the effect of ED [$F(40, 480) = 1.72$; $p < 0.01$]; therefore, the meaning of the difference in nose poke frequency, while significant, is difficult to interpret. In females, the learning performance on the reversal learning task was unaffected by ED (Fig. S3A), and there was no statistical difference in the nose poke frequency between ED females and controls during the behavioral flexibility test (Fig. S3B).

3.4. Effects of ED on competitive dominance in a group-housed environment

Due to the 21-h water deprivation, the beginning of each session during the behavioral flexibility test, when mice were at last permitted access to water, was highly competitive. Time-course evaluation revealed that visits to the corner chambers at the beginning of session had a trend of being dominated by the control group, whereas visits by the ED group peaked only after visits by the control group had declined and stabilized (Fig. 3A). A similar pattern was observed during the last 3 days of the *Shaping* period prior to the behavioral flexibility test. Again, corner chambers were dominated by the control group immediately after water access was permitted at 22:00 (Fig. 3B). A

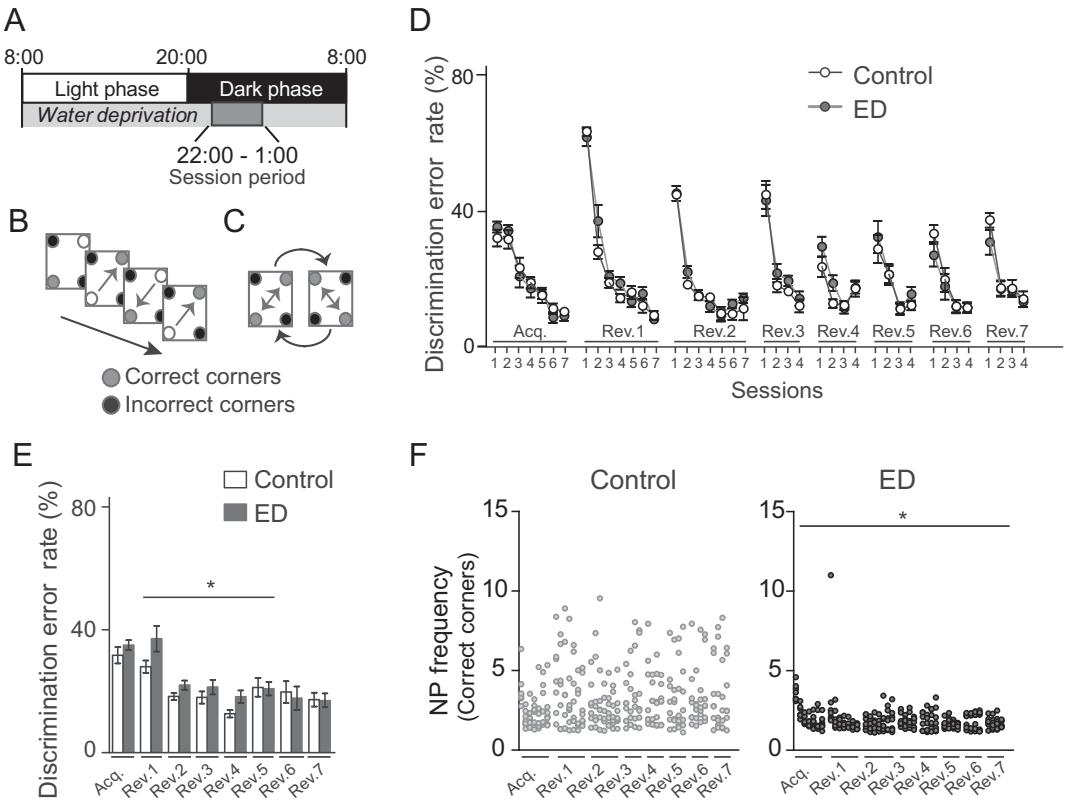

Fig. 2. Effects of early deprivation (ED) on executive function in male mice. (A) Scheme of the experimental schedule. The schematic illustrations of (B) behavioral sequencing task and (C) reversal. (D) Learning performance on the behavioral flexibility test. Each plot represents the session score based on the proportions of visits to the incorrect corner chambers out of the first 100 trials (discrimination error rate in %). (E) Discrimination error rate on the second session of the *Acquisition* phase (Acq.) and each reversal phases. (F) Nose poke (NP) frequency at the rewarding corner chambers during the initial 100 visits of the behavioral flexibility test. Values are presented as mean ± SEM. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test. Asterisk denotes statistical significance between the groups ($p < 0.05$). $n = 7$ per group. For readability, data of two groups are displayed in two panels.

significant effect of ED was observed between 22:00 and 22:10 [F (1, 130) =5.08; $p < 0.05$], with no significant interaction observed between the effects of ED and time during this period [F (10, 130) = 1.00; p = 0.45]. In females, however, no significant effect of ED on competitive dominance was detected during the behavioral flexibility test based on the time course of visits (Fig. S3C). This observation was confirmed by analysis of visit frequency during the first 5 min of all sessions (Fig. S3D). Therefore, we pursued further analysis in male mice only.

To further characterize the effect of ED on competitive dominance in males, we compared visits under high and low competitive conditions (Fig. 3C). A low competitive condition assesses the subordinate group's motivation for drinking in the absence of the dominant group [12]. Under a high competitive condition, in which the ED and control groups were housed in the same IntelliCage apparatus (i.e., $n = 14$ mice per cage), visit duration (Fig. 3D) and frequency (Fig. S4A) at the corner chambers during the first 5 min (22:00 − 22:05) were lower in the ED group, where significant group differences in visit duration were detected between sessions 4–9 [F (1, 60) = 4.75; $p < 0.05$] and sessions 30–34 [F (1, 48) = 5.29; $p < 0.05$] (Fig. 3D). The visit duration (Fig. 3E) and frequency (Fig. S4B) of the ED group increased during the subsequent 5 min period (22:05 − 22:10) as the visits by the controls decreased, and there were significant differences in the visit duration [F (1, 156) = 5.92; $p < 0.05$] (Fig. 3E) and frequency [F (1, 156) = 6.48; $p < 0.05$] (Fig. S4B) throughout the first high competitive condition. Differences in visit duration (Fig. 3F) and visit frequency (Fig. S4C) between the ED and control groups diminished 10 min into the session. Therefore, under a high competitive condition, the ED mice were subordinate to control mice (Fig. 3 and Fig. S4). In contrast, when the ED and control groups were separated in two IntelliCage apparatuses (a low competitive condition, $n = 7$ mice per cage), there were no significant differences in visit duration (Fig. 3D–F) or frequency (Fig. S4A–C) at the corner chambers during the first 5 min of the competitive task between groups. The difference reappeared when these groups of mice were again co-housed in the same IntelliCage apparatus (Fig. 3D–F and Fig. S4A–C).

The time-course of corner chamber occupancy at the beginning of the session (22:00 − 22:15) was analyzed based on the sum of visit durations (SUM duration) across all sessions. Under a high competitive condition, control group occupancy peaked within the first 5 min, whereas that of the ED group peaked after 22:05 (Fig. 3G and J). Under a low competitive condition, SUM duration of the ED mice peaked earlier, at the same time as the control mice (Fig. 3H and J). When the competitive condition was switched back to high, the peak SUM duration of the ED mice again followed that of the control group (Fig. 3I and J). Detailed peak analysis confirmed that the latency to reach the peak was significantly shorter during the low competitive condition compared with the high competitive conditions in ED mice ($p < 0.05$), while latency was unaffected by the competitive conditions in control mice (Fig. 3J). Ranks in competitiveness were assessed by the total number of visits during the first 5 min of the session under high and low competitive conditions (Fig. S4D). Competitive ranking was also determined based on the sum of visit durations, where lower the SUM duration, lower the ranking. It was revealed that ranks of the ED mice were significantly lower ($p < 0.05$) than control mice under the first high competitive condition, becoming indistinguishable from controls under the low competitive condition, and tended to be lower under the second high competitive condition ($p < 0.1$). Motivation for drinking water was also assessed by analyzing the total duration and frequency of visits in longer temporal windows of 15 min (22:00 − 22:15: Fig. S5A–D) and 30 min (22:0022:30: Fig. S5E and F). Data from the

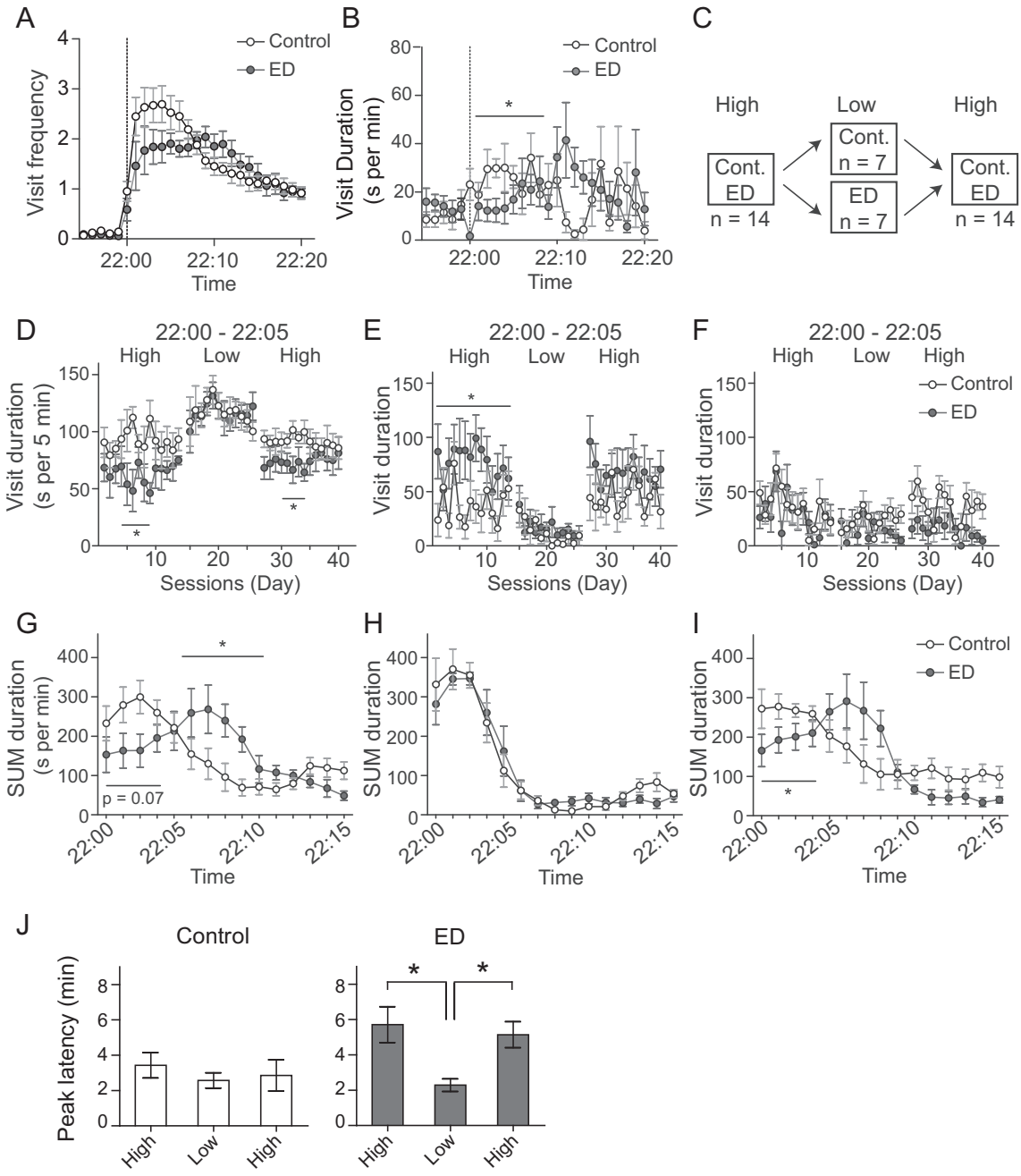

Fig. 3. Effects of early deprivation (ED) on competitive dominance of male mice. (A) Visit frequency during the behavioral flexibility test, illustrating the averaged reward acquisition pattern of control male vs. ED male mice at the beginning of all sessions. (B) Visit duration at the beginning of the test period during the *Shaping* period. The value for an individual animal was determined by taking the average value of the last 3 days of the *Shaping* period when mice were shaped to the water drinking schedule. (C) Scheme of the competition task for male mice. The competitive level was adjusted to high or low by inclusion or exclusion of the control mice in the ED cage (reducing the number of mice per cage competing for the water reward). A total of 14 mice, composed of both groups, were subjected to the competition task for 14 days (High), then the mice were divided according to the treatment group, reducing the competitive condition for the following 12 days (Low), followed by reunion of the groups under the high competitive condition (High) for another 2 weeks. Visit duration at the corner chambers during (D) the first 5 min (22:00–22:05), (E) the next 5 min (22:05–22:10), and (F) the following 5 min (22:10–22:15). Visit duration is plotted against session (days). (G–I) Time-course of corner chamber occupancy based on the sum of visit durations (SUM duration) during (G) the first high, (H) the low, and (I) the second high competitive conditions plotted against time elapsed from the beginning of the session (22:00) in 1 min intervals. (J) Peak latency determined from the sum of visit durations during the High and Low competitive conditions. Values are presented as mean ± SEM. Two-way ANOVA with repeated measures followed by Bonferroni post hoc test (A, B, and D–I) and one-way ANOVA followed by Tukey post hoc test (J), where asterisk indicates $p < 0.05$. $n = 7$ per group.

habituation period (6 days, omitting the day of introduction to IntelliCage) as well as the beginning of the shaping period (first 3 days) confirmed that there was no difference in the visiting pattern (visit frequency) between the groups prior to the competition task (Fig. S5G–J).

Halfway through the competition task, bald patches appeared on the backs of all control males, while only a single ED male had a bald patch. On the day of sampling, bald patches were still present on the backs of all control males as well as on those of 3 of 7 ED males. No other physical changes, such as in the appearance of whiskers or the presence of lesions and scabs, were observed in either group throughout the behavioral tests.

3.5. Transcript levels in the brains of ED male mice

Brains of male mice were removed during the last session of the competition task. We quantified the transcript levels of immediate

early genes, neurotrophic factor gene, and genes involved in glucocorticoid signaling in the mPFC, hippocampus, and the amygdala. Compared with the control group, ED mice exhibited a significantly enhanced level of *cFos* transcript expression in the BLA ($p < 0.05$) and a significant reduction in the CA1 region of the hippocampus ($p < 0.05$), but not in the adjacent DG (Fig. 4A). *BDNF* was unaltered in all regions analyzed in this study, except the CA1 region, where it was significantly downregulated ($p < 0.05$; Fig. 4C). *TrkB* transcript level was not altered in the CA1 (data not shown). *Arc* transcript level in the PrL region of the mPFC was slightly reduced, although the effect was not statistically significant (Fig. 4B). In the PrL region, downregulation of *Map2* ($p < 0.05$; Fig. 4D) and *MR* ($p = 0.06$; Fig. 4F) transcript levels were also observed. No alterations in *GR* transcript level were detected in any of the examined regions (Fig. 4E). However, *HSD11β2* was found to be slightly upregulated in the IL ($p = 0.05$; Fig. 4G).

3.6. Correlation between competitive ranking and transcript levels

To identify genes that may regulate competitive behavior, transcription levels of differentially expressed genes in various brain regions of interest (presented as fold-change relative to controls) were plotted against competitive rank. Indeed, significant correlations with competitive rank were observed for several genes exhibiting differential expression between groups. There was a positive correlation between competitive rank and the *cFos* transcript level in CA1 ($r = 0.74$; $p < 0.05$; Fig. 5A) and a tendency for an inverse correlation between rank and the *cFos* expression level in BLA ($r = -0.63$; $p = 0.05$; Fig. 5B). In addition, a significant inverse correlation was observed between competitive rank and the *cFos* transcript level in IL ($r = -0.65$; $p < 0.05$; Fig. 5C). A tendency for a positive correlation was observed between competitive rank and the *BDNF* transcript level in CA1 ($r = 0.61$; $p = 0.06$; Fig. 5D). In the PrL region, a significant correlation was observed between competitive rank and *Map2* expression ($r = 0.72$; $p < 0.05$; Fig. 5E) but not between competitive rank and *MR* expression ($r = 0.53$; $p = 0.12$; Fig. 5F).

4. Discussion

The early postnatal environment is critical for optimal cognitive development, but the effects of early social deprivation on behavior in

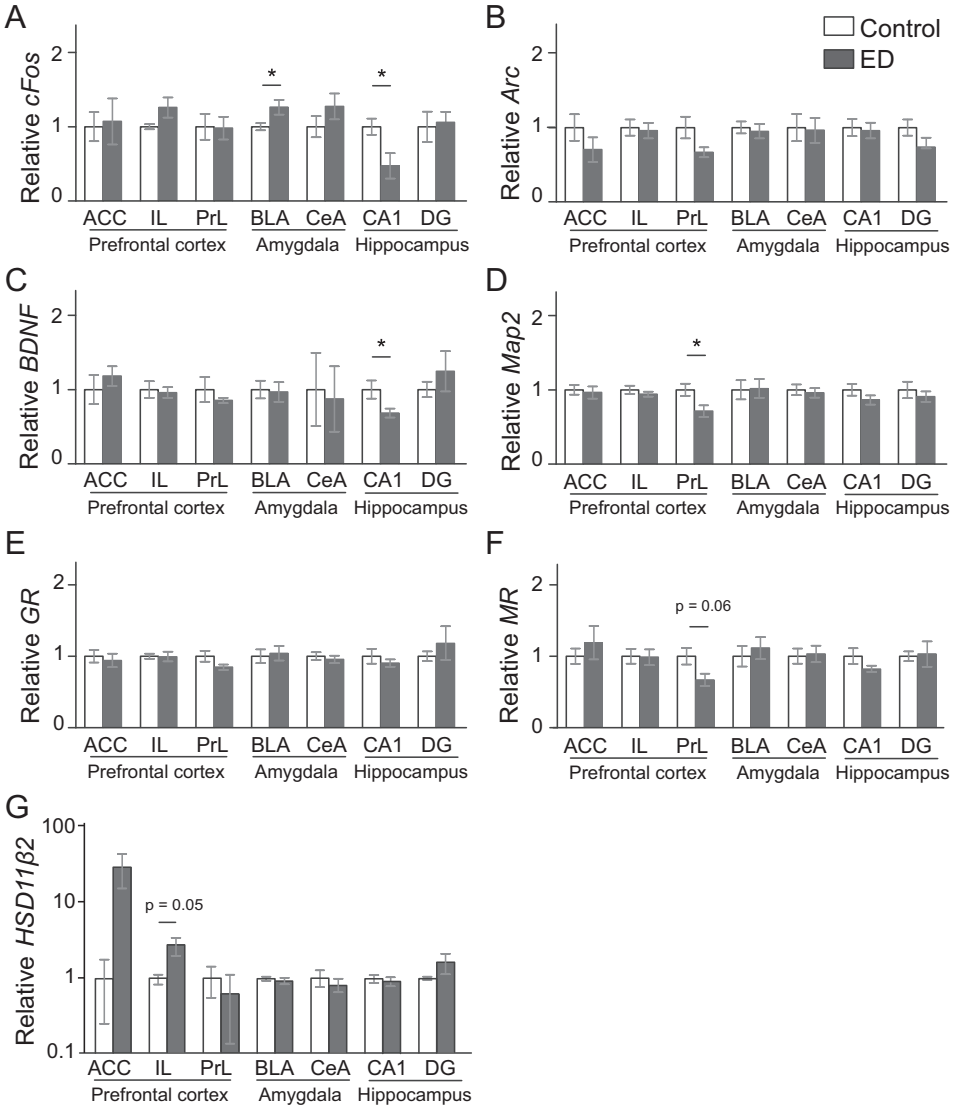

Fig. 4. Transcript expression levels in the medial prefrontal cortex (mPFC), hippocampus, and amygdala. Relative transcript levels of (A) *cFos*, (B) *Arc*, (C) *BDNF*, (D) *Map2*, (E) *GR*, (F) *MR*, and (G) *HSD11β2* in the mPFC, hippocampus, and amygdala of ED male mice compared with control male mice. Quantified copy numbers were normalized to *18S* rRNA expression levels and presented as the relative expression against the controls. Values in mean ± SEM. Asterisks denote statistical significance from the control group by Student's *t*-test at $p < 0.05$. $n = 5$ per group.

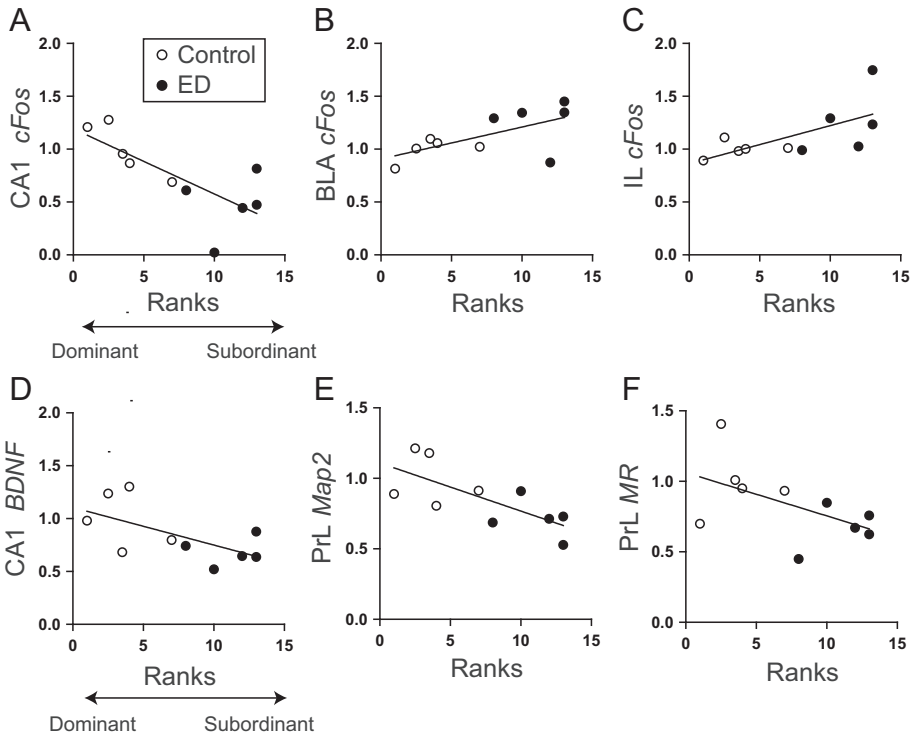

Fig. 5. Correlation between competitive rank and transcript expression levels. Competitive rank determined by the sum of the visit durations during the initial 5 min (22:00–22:05) of all the sessions under the high competitive condition, presented in ascending order from the 1st (the most dominant) to the 14th (the least dominant). The competitive ranks of the animals used for transcript expression level correlation analysis are as follows: Control, 1st, 2.5th, 3.5th, 4th, and 7th; ED, 8th, 10th, 12th, 13th, 13th. Correlation between competitive rank and (A) the *cFos* transcript level in CA1 ($r = -0.78$; $p < 0.05$), (B) the *cFos* transcript level in BLA ($r = 0.69$; $p < 0.05$), (C) the *cFos* transcript level in IL ($r = 0.72$; $p < 0.05$), (D) the *BDNF* transcript level in CA1 ($r = -0.58$; $p = 0.08$), (E) the *Map2* transcript level in PrL ($r = -0.68$; $p < 0.05$), and (F) the *MR* transcript level in PrL ($r = -0.56$; $p = 0.09$). $n = 5$ per group.

a social context have proven difficult to study due to the challenges of quantitative behavioral and neurological analysis using laboratory animal models such as mice. We investigated the effect of early social deprivation on cognitive behavior in a social context by utilizing recently developed automated behavioral test protocols for group-housed mice [14], and found lasting sex-specific changes in behavior under a social context. Specifically, ED led to competitive subordinance in male mice, enhanced novelty response, subtle changes in executive function, and alterations in the expression of genes associated with neural activation, plasticity, and stress in limbic and frontal cortices.

4.1. Competitive subordinance and novelty response in ED mice

We speculated that testing under a social context would reveal novel phenotypes in the ED model. Most notably, ED showed competitive subordinance in male mice. The C57BL/6J strain has been suggested to be inherently resilient to maternal separation paradigms [16], so the revelation of multiple behavioral phenotypes, including a change in competitive dominance, is indicative of the robustness of our behavioral test protocols.

Among social behaviors, the dominance tendency has been proposed as a promising attribute to study stress-induced psychopathogenesis [17,18]. Indeed, social dominance is the ultimate outcome of physiological advantages, motivation, and emotional control (particularly, anxiety and aggression). In addition to altered psychosocial phenotype, ED mice demonstrated a lasting physical change, i.e., reduced body weight. To the best of our knowledge, this has not been reported following ED in mice, although ED was associated with lower body weight in rats [19]. Similar to changes in competitive dominance, reduced weight was observed only in males. It can be argued that reduced weight is a physical disadvantage that induce competitive subordinance. However, the lack of a significant relationship between body weight and competitive rank (Fig. S6) does not fully support this notion. In addition, importantly, competitive subordinance exhibited by ED mice is unlikely a result of lack of motivation to obtain the reward (water) by competing with opponent mice since ED mice behaved in a manner similar to controls under the low competitive condition in the absence of the control mice. The duration and frequency of total visits made by the ED mice reached that of the control mice approximately 15 min after the start of the competition task, a time when competition for water access was less intense. This observation is consistent with results from another competitive subordinate model, mice exposed to an environmental chemical (dioxin) *in utero* and via lactation [12]. Similarly, no differences in visit frequency were observed during the habituation period and during the first few days of the *Shaping* period, again suggesting that differential motivation cannot account for the low chamber access by the ED mice. Nevertheless, because water drinking is not the only motive for visiting the corner chambers outside the competitive context (i.e., for instance, mice may visit corner chambers out of curiosity, or in order to escape from a fight), we consider visits at the beginning of the low competitive condition to most accurately reflect the motivational level of the subordinate group. In this regard, we found no differences in motivation between groups, suggesting that competitive dominance of the control mice accounted for the difference in corner chamber access under the high competitive condition.

The physical condition of the control and ED mice was also consistent with differences in competitiveness. Bald patches were found on all control mice halfway through the competition task, while the majority of ED mice exhibited no bald patches until the latter half of the test. This may reflect aggressive interactions between the control mice at the beginning of the competition task, when access to the chambers was most desired, whereas the ED mice may have simply avoided confrontation, at least until the latter half of the test.

It is plausible that the control mice would show dominance over the ED mice in other behavioral assays, such as the tube test, especially because the ED mice had a lower body weight than the control mice.

However, because of the major influence of social context, results may differ between the IntelliCage-based competition task and the tube test, in which only two mice are present. In the IntelliCage-based competition task, group-housed condition played a major role in determining competitive ranking, a condition absent in the tube test.

Novelty response in the open field has been reported to be social status-dependent, with dominant individuals exhibiting higher locomotor and exploratory activity [17]. In a previous study [5], however, C57BL/6J mice exposed to ED did not show a significant difference in behavioral profile in a novel open field or in the light/dark exploration test. Conversely, the ED paradigm employed in the present study produced a robust sexually dimorphic phenotype in novelty response behavior. Differences in protocols and parameters might explain this apparent inconsistency. In rats, both enhanced and unaltered behaviors in a novel setting have been documented in adult males and females exposed to ED during neonates, with a tendency for increased anxiety to novelty [20–22]. A sexual dimorphism in early life stress phenotype has been suggested in rats and mice for aggression [23] and hippocampal structural plasticity [24]. Therefore, we suggest that social context may influence expression of the novelty response behavior following ED in mice and explain inconsistencies across studies.

4.2. Alterations in transcript regulation in the hippocampus of ED mice

To explore the neural basis underlying the behavioral differences between ED and controls mice in a competitive social environment, we analyzed expression changes in numerous genes implicated in neural activation, synaptic plasticity, and the stress response. In the present study, the hippocampus, amygdala, and mPFC were chosen as regions of interest as they are known to regulate stress and social behavior [25–27] and have been implicated in modulation of aggression and anxiety-related behaviors such as novelty-seeking and competitive behavior.

We found region-specific alterations in the transcriptional regulation of the ubiquitous immediate early gene *cFos* in ED mice. Expression of *cFos* was downregulated in the hippocampal CA1 region and upregulated in the BLA. The *cFos* expression level in the hippocampal CA1 region was significantly correlated with competitive rank, and there was also a significant correlation between competitive rank and the *cFos* expression level in BLA. Upregulation of cFos is indicative of increased neural activity as well as plasticity [28]. The expression of *BDNF*, a well characterized neurotrophin associated with synaptic plasticity, was also downregulated in the hippocampal CA1 region of the ED mice. Alterations in hippocampal synaptic development have been reported following maternal separation in rats [29], but there have been no such reports for ED model mice. It has been reported that vulnerability to stress is associated with reduced hippocampal BDNF [30,31], while psychosocial stress such as social defeat reduces *BDNF* expression [32]. High novelty-seeking behavior has been reported to predict susceptibility to depressive-like signs and stress responses induced by social defeat. Moreover, depressive signs were correlated with reduced *BDNF* expression in the hippocampus following social defeat, while rats with low novelty-seeking were less prone to depression and exhibited the increased BDNF expression following social defeat [33]. In the present study, we observed an increased novelty response, subordinance in a competitive environment, and a reduction in *BDNF* transcript expression in the CA1 of ED males. The reduced hippocampal *BDNF* in response to early life stress may contribute to the competitive subordinance observed in ED mice.

4.3. Alterations in transcript regulation in the amygdala of ED mice

Amygdala function is strongly affected by early life stress and maternal care in rats [34], and the present study demonstrated that ED enhances *cFos* expression in the BLA (Fig. 4A). In previous studies, elevated *cFos* in the BLA has been associated with novelty response [35] and anticipatory anxiety [36], and has been observed in a rat post-weaning social isolation model that exhibited hyper-aggressive traits [37]. Notably, *cFos* elevation in the BLA was not shown to be involved in mice naturally selected for aggressiveness [38]. Therefore, *cFos* expression in the BLA may not be a direct reporter for aggressiveness, but instead reflects elevated emotional arousal or reactivity that could in turn alter the propensity for aggression or anxiety depending on condition. The BLA has been identified as a key region regulating social behavior, including social cue processing [39,40] and stimulus-reward processing [41]. Taken together, a sign of enhanced neuronal activity in the amygdala, as evidenced by *cFos* expression, is indicative of heightened emotional reactivity, social anxiety, and abnormalities in processing social cues and reward, which may have contributed to subordinance under competition exhibited by ED male mice.

4.4. Alterations in the mPFC of ED mice

In mice, synaptic efficacy in the mPFC correlated with social hierarchy [42,43]. Correlation between mPFC activity and behavioral dominance or subordinance has been also demonstrated in humans [44]. There are, however, relatively few studies on the effects of early life stress on the mouse mPFC [45,46]. We found a significant decrease in *Map2* expression in the ventral mPFC (PrL region) of ED mice, and a significant correlation was observed between the *Map2* expression level and competitive rank. *Map2* transcripts and protein synthesis are found in dendrites, underscoring the importance of Map2 in dendritic function [47]. The proposed functions include dendritic remodeling associated with synaptic plasticity [48], neuronal outgrowth, and cell death [49]. Glucocorticoids also regulate dendritic spine formation and plasticity [50,51], and prenatal stress has been reported to inhibit neuronal maturation via MR downregulation in the hippocampus [52]. Furthermore, synaptic plasticity disruption induced by social isolation in rats has been shown to be mediated by glucocorticoid [53]. It is, therefore, plausible that synaptic efficacy is reduced in the PrL of ED mice.

We also found elevated *HSD11β2* expression in the IL region of ED mice. HSD11β2 is an enzyme that inactivates glucocorticoid. Increased *HSD11β2* expression might reflect lower glucocorticoid sensitivity in the IL considering that *GR* and *MR* expression levels were unaltered (Fig. 4). The projection from the IL to the BLA suppressed BLA activity and is associated with fear conditioning and stress-related pathology [54,55]. This potential reduction in glucocorticoid signaling may have affected BLA activity in ED mice. It is also noteworthy that a significant correlation was observed between the *cFos* expression level in IL and competitive rank, suggesting potential involvement of IL activity in dominance behavior.

4.5. ED model vs. perinatal TCDD exposure model

Mouse offspring born to dams that were administered a low-dose of an environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during late pregnancy demonstrated subordinance under a high competitive condition [12] similar to the ED model, suggesting a shared neurological basis. In fact, in both studies, the amygdala was suggested to be hyperactivated as evidenced by elevated *cFos* transcript or protein expression. However, in contrast to the ED model, perinatal TCDD exposure resulted in a major abnormality in behavioral flexibility and an implication of decreased mPFC activity as suggested by cFos and Arc immunohistochemistry. The identical behavioral flexibility test revealed that the executive function was apparently unaffected by neonatal ED.

In addition, while the ED model exhibited a suppressed nose poke frequency at the correct corner chambers compared with the control group (Fig. S2E), TCDD-exposed mice exhibited excessive nose poke frequency per visit, indicating increased compulsive repetitive behavior. While additional experiments are required to address the significance

of reduced nose pokes in ED mice, one possibility is that it may reflect an anhedonia-like trait, which is previously reported in ED rats [7].

5. Conclusions

The present study demonstrates that early social deprivation produces lasting changes in social behavior in male mice under a group-housed environment. This observation was successfully obtained by our recently developed robust behavioral test protocols using IntelliCage. The central finding suggests that neonatal ED can affect social behavior later in adulthood, and we have observed alterations of transcript expression of immediate early genes in the three major brain regions associated with stress-related disorders and social behavior. How the transcript expression altered in the hippocampus, amygdala, mPFC, and associated pathways contribute to the behavioral phenotype remains to be clarified. Numerous factors influence behavioral outcome, and some of these differentially expressed transcripts may be specific to the ED model rather than the general characteristics of the subordinate model. Those transcripts that showed significant correlations with competitive rank could be involved in determining behaviors in social hierarchy.

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Conflict of interests

We have no conflicts of interest.

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