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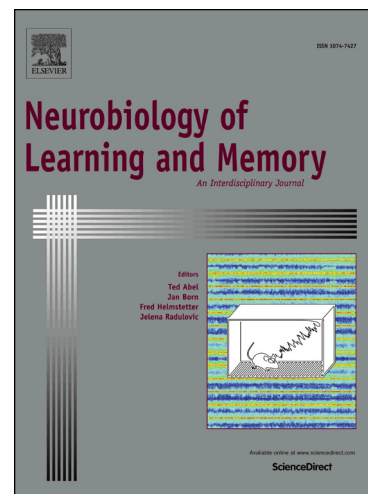
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**Impairment of Decision Making and Disruption of Synchrony between  
Basolateral Amygdala and Anterior Cingulate Cortex in the Maternally  
Separated Rat**

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**ABSTRACT**

There is considerable evidence to suggest early life experiences, such as maternal separation (MS), play a role in the prevalence of emotional dysregulation and cognitive impairment. At the same time, optimal decision making requires functional integrity between the amygdala and anterior cingulate cortex (ACC), and any dysfunction of this system is believed to induce decision-making deficits. However, the impact of MS on decision-making behavior and the underlying neurophysiological mechanisms have not been thoroughly studied. As such, we consider the impact of MS on the emotional and cognitive functions of rats by employing the open-field test, elevated plus-maze test, and rat gambling task (RGT). Using multi-channel recordings from freely behaving rats, we assessed the effects of MS on the large scale synchrony between the basolateral amygdala (BLA) and the ACC; while also characterizing the relationship between neural spiking activity and the ongoing oscillations in theta frequency band across the BLA and ACC. The results indicated that the MS rats demonstrated anxiety-like behavior. While the RGT showed a decrease in the percentage of good decision-makers, and an increase in the percentage of poor decision-makers. Electrophysiological data revealed an increase in the total power in the theta band of the LFP in the BLA and a decrease in theta power in the ACC in MS rats. MS was also found to disrupt the spike-field coherence of the ACC single unit spiking activity to the ongoing theta oscillations in the BLA and interrupt the synchrony in the BLA-ACC pathway. We provide specific evidence that MS leads to decision-making deficits that are accompanied by alteration of the theta band LFP in the BLA-ACC circuitries and disruption of the neural network integrity. These observations may help revise fundamental notions regarding neurophysiological biomarkers to treat cognitive impairment induced by early life stress.

## 1. Introduction

Early life experiences, such as neonatal maternal separation (MS), have been widely believed to exert strong influence on behavioral and brain development in animals and humans (Macri, Chiarotti, & Wurbel, 2008). Previous research has revealed a profound influence of MS on the hypothalamic-pituitary-adrenal (HPA) axis activation in adulthood, one of the essential neuroendocrine stress systems (Engert et al., 2010). Dysregulation of the HPA axis is implicated in the pathophysiology of stress-related disorders, such as depression and anxiety. While separation that occurs during an important developmental period may interfere with appropriate maturation of essential modulatory systems and cause neurophysiological alterations (O'Mahony, Hyland, Dinan, & Cryan, 2011). For example, repetitive separation of rat pups from their mother for three weeks -a widely used model for early life stress- was reported to alter dendritic morphology and spine density in prefrontal cortex (PFC) pyramidal neurons (Bock, Gruss, Becker, & Braun, 2005; Monroy, Hernandez-Torres, & Flores, 2010; Pascual & Zamora-Leon, 2007).

Making a decision under complex and uncertain conditions is a basic cognitive process for adaption that relies on the integration of several executive functions. Despite this, the impact of early life stress on decision-making performance in adulthood has not been investigated. We have previously reported on a decision-making test, the rat gambling task (RGT) (Mu et al., 2015; Xu et al., 2015), and found that, similar to the findings of the most advantageous strategy from the Iowa gambling task (IGT) utilized in human studies, most rats can learn to maximize their food reward during the RGT. Recent human and animal studies have shown that decision-making performance in the IGT/RGT depends on the integrated functions of several sub-regions of the PFC, especially the prelimbic, cingulate (ACC) and

orbitofrontal cortices, as well as the basolateral amygdala (BLA) (Rivalan et al., 2011; Bechara et al., 1999, 2000; Zeeb et al., 2011). Interestingly, regulation of the HPA axis is partly controlled by the PFC and disrupted in various psychiatric disorders including schizophrenia, and anxiety disorders (Arnsten, 2009; Diorio, Viau, & Meaney, 1993; Sullivan & Gratton, 2002). The reciprocal connections between the BLA and medial PFC including the ACC have been reported previously. Indeed, an interconnected neural circuitry, between the BLA and ACC regions, guides behavior in certain types of cost-benefit decision-making tasks (Floresco & Ghods-Sharifi, 2007). Early life experiences have been shown to alter prefrontal cortex structure and function (Baudin et al., 2012); here, we use a maternally separated rat model to examine the decision-making process. This process depends on there being an intact neuronal activation in the ACC, as well as associated neuronal synchronization with the BLA.

Large-scale neural oscillations play a primary role in fundamental cognitive functions (Varela, Lachaux, Rodriguez, & Martinerie, 2001). Oscillations in the theta frequency band (4-10 Hz) are known for their association with a wide range of cognitive functions, including attention (Ishii et al., 1999), encoding of new information (Klimesch, 1999) and increased memory-load (Jensen, 2005). Theta band oscillations are also known to modulate neuronal activity in subcortical, limbic, and cortical structures (Cardoso-Cruz, Lima, & Galhardo, 2013). Coordinated timing of neuronal firing across populations of neurons is necessary to induce plasticity (Markram, Lubke, Frotscher, & Sakmann, 1997). Rutishauser et al. have shown that memory formation in humans can be predicted by close coordination of the phase-locking of spike times to the theta band oscillations of the local field potentials (LFP). This suggests that synchronized oscillatory activity promotes communication

between anatomically distant, yet functionally related structures, during cognitive processes (Rutishauser, Ross, Mamelak, & Schuman, 2010). Our previous studies showed that cisplatin, a chemotherapy drug, could interrupt the phase-locking of ACC neuronal spikes with the ongoing theta band oscillations of the LFP, which was associated with impaired cognitive functions (Mu et al., 2015). MS –related cognitive deficits have not yet been investigated systematically in animals. In our study we performed a series of behavioral assessments in MS rats, e.g. the RGT was used to assess decision-making functions. Simultaneous recording from an array of microelectrodes allowed us to characterize interruption in the phase-locking of single unit spike timings with the ongoing theta oscillations of the LFP in the ACC, and to identify desynchronized theta activity between the BLA and the ACC in MS rats.

## **2. Materials and methods**

### **2.1. Animals**

Female pregnant Sprague-Dawley rats and adult male Sprague-Dawley rats (3-4 months) were used in the present study. Before starting the experiments, the rats were kept in plastic cages in groups with supply of food and water *ad libitum*. The animal holding room was maintained in 12:12 light and dark cycle (light from 07:30 to 19:30), and with a constant room temperature of 25°C. The experiments were approved by Department of Health of the Hong Kong Government [Reference no. (14-41) in DH/HA&P/8/2/5 Pt.2].

### **2.2 Maternal separation**

In this study we chose a separation procedure of 180 min with extension to PND 21, a popular and valid animal model of emotional and cognitive deficits (Vetulani, 2013).

After birth, only male pups were used in the current study. They were randomly

divided into experimental and control groups. The day of delivery was defined as postnatal day 0 (PND 0). In the experimental group, maternal separation was carried out from 10:00 to 13:00 daily from postnatal day 2 to day 21 (PND 2-21). During the separation period, the male pups were kept in single cages with no visual contact with their mothers. After the separation period, the pups were returned to their mothers. In the control group, the male pups were kept undisturbed with their mothers. After 21 days, all pups were weaned and transferred to a new cage with 4-5 rats per cage. They were left undisturbed until experiments in adulthood, except for routine animal care procedures.

### **2.3 Behavioral assessments**

All the behavioral tests were performed on the 3-month old rats in the following order: (1) open field test, (2) elevated plus maze, (3) rat gambling task. Body weight was measured before experiments. The behavioral apparatuses and experimental procedures used in the present study have been described in our previous publications (Mu et al., 2015; Xu et al., 2015).

#### *Open field test (OFT)*

The open field test is a typical rodent behavioral test that assesses locomotor function and anxiety-like exploratory behavior. Rodents tend to stay close to the walls (thigmotaxis) when first exposed to an open field, and the tendency decreases gradually with time, the degree of thigmotaxis during this period of decrease can be considered as the anxiety index of rodents. The test was performed in adult MS and control rats when 3 months-old. In brief, the rats were placed individually in the center of a 40 × 40 cm square drawn in the middle of a black square arena (80 × 80 × 40 cm) and then allowed to explore the field freely for 5 min. Spontaneous exploration behavior in the OFT were observed and recorded by ANY-maze (Stoelting

Co., Wood Dale, IL, US).

#### *Elevated plus maze*

The elevated plus maze is widely used for testing anxiety-like behavior in rodents and is thought to result from natural aversion to exploring elevated and open areas. In brief, the rats were placed individually in the central square of the plus-maze facing an open arm and then allowed to start exploring the maze freely in a 5 min test. Testing took place between 08:00 and 13:00 in a dimly lit room. Time spent in each arm was recorded using the ANY-maze with entries being defined as 85 % of the area of the animal being present in the area entered. Time spent in open arms, especially the percentage of open arm time versus closed arm time, was evaluated to assess anxiety.

#### *Rat gambling task*

The rat gambling task (RGT) has been developed to test the decision-making capacities of rats via a conflict between immediate and long-term gratification (food reward) (Rivalan, Coutureau, Fitoussi, & Deltu-Hagedorn, 2011). The apparatus and experimental procedures for the gambling task have been described in our prior studies (Mu et al., 2015; Xu et al., 2015). Briefly, the operant chambers (28 × 30 × 34 cm, Imetronic, Pessac, France) consisted of four apertures on the front and one food tray on the opposite wall connected to a dispenser for releasing food pellets as reward (45 mg, TestDiet, USA). Each aperture, and the food tray, were equipped with an infrared detector. To complete this food-driven task, before the training stage, daily food was moderately restricted for 3 days, following one-day fasting, such that, each rat maintained its weight to 90% of their free feeding weight.

During the training stage, each rat was placed in the chamber for a maximum of 40 min each day. Rats gradually learned the association between the nose-poke action



and the release of food pellets. In order to guarantee that the selection of the nose-poke was a conscious choice, the rats were trained to associate a single nose-poke with one food pellet delivery, according to a criterion of at least 100 pellets obtained within a 30-min session, followed by two consecutive nose-pokes with one food pellet delivery with the same criterion. Then, two 5-min sessions were conducted, to habituate rats to the variation of pellet number(s) during the test. The first session was set as two pellets were released after a choice was made and the second set as one pellet. The training phase usually lasted 6-8 days. The number of nose-pokes per min during the final training session and the total duration of the final training session were used to assess the general activity of the rats and their motivation towards performing the task.

The test procedure was conducted on the day following the final training session and lasted for 60 min or was cut off by 250 pellets obtained. Rats were free to make choices among the four apertures (A–D) as it was during the training phase; however, different choices were associated with different outcomes: choice A or B related to two pellets obtained each time as an immediate reward, but had a 50% probability to trigger a 222-s time-out or 25% probability for a 444-s time-out as penalties, during which no pellet could be obtained; choices C or D were associated with less immediate reward (one pellet each time), but also less penalty (25% chance for 12-s time-out, or 50% chance for 6-s time-out). During the punishment, the selected aperture remained illuminated to facilitate the association between this selection and its outcomes. Although the immediate rewards of choices A and B were twice those of C and D, in the long run the theoretical maximum benefit of C and D was five times higher than A and B. The percentage of advantageous choices  $((C + D)/(A + B + C + D) \times 100\%)$  in the last 20 min of the test and the total food rewards obtained across the

test were used to identify the decision-making behavior of rats.

#### ***2.4 Implantation of multiple-channel recording electrodes***

In this study, the ACC area was defined as the cingulate cortex, area 2 (Cg2) and the prelimbic cortex together with the overlying cingulate cortex, area 1 (Cg1) (Paxinos & Watson, 2006), which is a major cortical area of the limbic system, integrating emotion and cognition. The detailed procedures have been described in our previous publication (Wang et al., 2015). In brief, rats were first anesthetized and placed in a stereotaxic frame. A midline scalp incision was made to expose the skull, then two small holes (1-2 mm wide) were drilled above the BLA and ACC in the right hemisphere and the bone and dura were removed. 5 titanium screws were fixed in the skull surrounding the holes with 1 mm depth. Because, almost exclusively, the caudal regions of the BLA ascend projections to the ACC unilaterally (Floresco, & Ghods-Sharifi, 2007), the animals were inserted with two 16-channel microwire electrode arrays (Plexon, Dallas, TX, USA) in the ACC (AP 3.0–3.3, ML 0.6–1.0mm, depth 1.5– 3.5 mm from dura) and the ipsilateral BLA (AP -3.3 to -3.6, ML 5.0-5.3, depth 7-8 mm). The silver grounding wires from the recording electrodes were wrapped around the mounting screws. Then, the recording electrodes were advanced slowly into the brain using a micropositioner until clear neuronal firings in most recording channels were observed on-line (OmniPlex system, Plexon, USA). A mixture of mineral oil and bone wax was packed around the electrode penetration zone. Electrodes were secured to the rat's skull using dental cement until more than half of the 16 channels have clear spike signals. After the surgery, rats were injected with buprenorphine (0.1 mg/kg) as an analgesic, and 1.0 ml sterile saline for hydration, and then placed on a warm heating pad for recovery.

#### ***2.5. Electrophysiological recordings***

After 6-8 days recovery, the rats were recorded in a quiet waking state in their home cages. Two 16-channel headstages (gain  $\times 20$ ) were connected to the implanted electrodes in the ACC and BLA. Both the local field potential and extracellular single unit activity were recorded using a multiple-channel neural data acquisition system (Omniplex-D, Plexon Inc.). Wideband signals were amplified (gain  $\times 1000$ ), high-pass filtered from 0.05 Hz, and sampled at 40 kHz. LFPs were amplified ( $\times 1000$ ), band-pass filtered (0.05-200 Hz, 4-pole Bessel), and sampled at 1 kHz. Spike signals were amplified ( $\times 1000$ ), band-pass filtered (300 Hz to 5 kHz, 4-pole Bessel) and sampled at 40 kHz. The recording lasted for 120 seconds and data were stored on a disk for offline analysis.

## ***2.6. Electrophysiological recording during open field test***

The open field test procedure has been described previously. During the 5 min test, the rats were observed by means of overhead CCD camera connected to video recorders (30 frames/s) and their movements tracked by means of Cineplex (Plexon Inc.). Two LED, one of blue and one of red color, were connected to the headstage for precise recording of the rat's location. The neuronal signals were recorded simultaneously from the freely behaving rats using a multiple-channel neural data acquisition system. The wideband signals, LFPs, and spike signals were stored for offline analysis.

## ***2.7 Electrophysiological data analysis***

### *Data analysis recorded during open field tests*

The behavioral data and electrophysiological data were combined by analysis software (Cineplex Editor, Plexon Inc.) for offline replay. The boundary of the center area was labeled in the software. The transition was identified when a rat moved from the center to the periphery. Whenever the rat transferred from the center to the

periphery, an event was generated by the analysis software to label the neuronal signals. To test for significance in theta band synchronization around the transition points (time 0), we compared data from  $\pm 2$  s around the transition point, for which -2 s to 0 s represented synchronization in the center and 0 s to 2 s represented synchronization in the periphery. The occupation times in the different locations of the open field were calculated by the Neuroexplorer Time spent function. The whole area was divided into a  $40 \times 40$  grid. The boundary of the center area was  $20 \times 20$  grid square.

### *Spectral analysis*

To identify alterations in theta power spectra following maternal separation, we characterized the large scale brain oscillations in the BLA and ACC regions. The LFP traces from the BLA and ACC were band-pass filtered in the theta band (4-10 Hz) using a non-causal zero-phase-shift filter (fourth-order Butterworth). Then the power spectral densities (PSD) were calculated by multi-taper estimates with seven tapers,  $2^{13}$  frequency bins in the range [0, 100 Hz] (NeuroExplorer 5, Plexon, Dallas, TX) and 50% overlapping windows (window durations were  $2^{14}$  data points). The PSD curve was smoothed with a Gaussian filter (15 bins running average). The band power was defined as the area under the curve of the corresponding frequencies, and the band power from each animal were averaged over the 16 channels in the ACC or BLA. 164 data points were used to represent the PSD in theta band. The spectrum units were normalized by the raw PSD, so that the sum of all the spectrum values was equal to the mean squared value of the signal.

### *Synchronized theta oscillations between BLA and ACC*

For the LFP signals, cross-correlation analysis was not affected by changes in the amplitude, but was sensitive only to changes in phase between the two regions. To

explore the time coupling between the BLA and ACC, we used cross-correlograms (Neuroexplorer 5) to evaluate the synchronization of theta activities between the BLA and ACC. LFPs from the BLA and ACC were averaged over the 16 channels separately before aligning, with the LFP in ACC chosen as reference. Pearson correlation values were calculated with lag times from -0.5 to 0.5 s with 2 ms bins. The cross correlation curves were smoothed by a Gaussian filter (5 bins running average). The second positive peak was chosen as a quantitative measure because its location at a lag time of 0.2 s represents theta activity at 5 Hz (Seidenbecher, Laxmi, Stork, & Pape, 2003).

#### *Spike sorting*

In brief, the single unit spikes were isolated by off-line spike sorter Version 3 software (Plexon Inc.). Spikes were identified when a minimum waveform amplitude threshold of 4 SDs higher than the noise amplitude was reached. All waveforms in each channel were automatically isolated as distinct clusters by principal component analysis (PCA). All isolated single units exhibited recognizable refractory periods (>1 ms) in the inter-spike interval (ISI) histograms.

#### *Computing the spike-field coherence across regions*

Spike-field coherence (SFC) was used to measure the phase synchronization between action potentials and field potential oscillations (Rutishauser, Ross, Mamelak, & Schuman, 2010). To compute the SFC across the BLA and ACC, the spikes recorded in either the BLA or ACC and the averaged LFP from the ACC or BLA were used in the analyses. For every spike, a segment of the LFP data centered on the spike  $\pm$  480 ms were averaged to calculate the spike-triggered average (STA). Then the frequency spectrum of STA (*f*STA) was calculated using multitaper analysis, which used a series of discrete prolate spheroidal sequences (7 tapers) to give estimates of

the PSD. The average of the spectra in each frequency results in the spike triggered power (STP(f)). Finally, the SFC was calculated as the  $fSTA$  over STP(f) as a percentage:  $SFC(f) = [fSTA(f)/STP(f)] \times 100\%$  (Wang et al. 2015). The SFC ranges from 0 to 100%, with 0 representing complete lack of synchronization and 100% representing perfect phase synchronization. The normalization of the SFC is independent of the spike firing rate and the power spectrum of the LFP (Fries, Reynolds, Rorie, & Desimone, 2001).

### **2.8. Statistical analysis**

Results were expressed as mean  $\pm$  SEM. The electrophysiological data were processed off-line using NeuroExplorer 5 (Plexon, Inc.). Statistical comparisons were performed in SPSS v13.0 (SPSS, Chicago, IL, USA). Data were analyzed using Student's t-test where appropriate. For the data with unequal variance, the Mann-Whitney U test was performed as alternative. A value of  $P < 0.05$  was considered statistically significant for all statistical comparisons.

## **3. Results**

### **3.1. Anxiety-like behavior in maternal separation rats**

To examine whether maternally separated rats develop anxiety-like behavior we performed the open field test (OFT). In the OFT, the locomotor functions of the rats were assessed by the total distance moved and the mean speed in the open field. The total distance moved and the mean speed of adult MS rats (mean  $\pm$  SEM: 21.74  $\pm$  1.75 m and 0.08  $\pm$  0.003 m/s) were similar when compared to those of the control rats (mean  $\pm$  SEM: 22.82  $\pm$  1.20 m, 0.076  $\pm$  0.004 m/s) (Fig.1A and B) indicating that maternal separation did not affect normal locomotor function of rats in their adulthood. However, the number of entries into the center and time spent in the center were decreased in MS rats compared to control rats, with significant differences in center

duration between the two groups. Time spent in center is  $23.24 \pm 1.94$ s vs.  $32.30 \pm 4.02$  s in MS and control rats, respectively ( $P < 0.01$ , Fig. 1C). The number of times to enter the center were  $8.78 \pm 0.87$  vs.  $13.25 \pm 0.65$  in MS and control rats, respectively ( $P < 0.01$ , Fig. 1D). Reduced activity in the central area in the open field indicates an increased anxiety level of the MS rats.

In addition, we performed open field tests combined with simultaneous electrophysiological recording in both control (n=6) and MS rats (n=6). The total distance and mean speed moved in the open field were similar between the control and MS rats ( $22.73 \pm 2.774$  m and  $0.080 \pm 0.004$  m/s in control group versus  $18.48 \pm 3.385$  m and  $0.061 \pm 0.011$  m/s in MS group). Total entries into the center were decreased in MS rats when compared to control rats ( $13.50 \pm 0.76$  in control group versus  $7.50 \pm 1.54$  in MS group  $t_{10} = 3.484$ ,  $p < 0.01$ ). Time spent in center was decreased in MS rats when compared to control rats ( $34.67 \pm 4.55$  s in control group versus  $20.60 \pm 3.857$  s in MS group.  $t_{10} = 2.358$ ,  $p < 0.05$ ). Fig. 2A shows examples of maps of occupancy during the open field test from a control rat and a MS rat. The MS rat showed anxiety-like behavior with less time in the center compared to the control rat.

To further confirm the anxiety-like behavior, we performed the elevated plus maze (EPM) test. Consistent with the results in the OFT, no significant difference was found in the total distance moved and mean speeds between the two groups indicating normal locomotor activity of MS rats. However, time spent in the open arm was significantly decreased in the MS rats compared to control rats (mean  $\pm$  SEM:  $58.87 \pm 5.34$  s vs.  $38.34 \pm 6.58$  s in control and MS rats respectively,  $P < 0.05$ , Fig. 1E). When comparing the ratio of time spent in the open vs. closed arm, MS rats showed a significantly decreased ratio compared to control rats (mean  $\pm$  SEM:  $63.32 \pm 8.62\%$

vs.  $35.52 \pm 7.02\%$  in control and MS rats respectively,  $P < 0.05$ , Fig. 1F). These results confirmed that MS rats showed anxiety-like behavior.

### ***3.2 Desynchronized theta activities in the ACC and BLA of MS rats in the center of open field***

We performed cross-correlation analysis to evaluate the theta synchrony between BLA and ACC. The averaged cross-correlograms for control and MS rats are shown in Fig. 2B. The second positive peak represents the synchronized activities of the LFP in the theta band as the lag time is from 0.1 to 0.25 s corresponding to theta band oscillations in 4-10 Hz. In the periphery, MS rats showed decreased theta synchrony compared to control rats ( $0.2032 \pm 0.0029$  in control versus  $0.1728 \pm 0.0027$  in MS rats,  $t_{10}=7.59$ ,  $p<0.01$ ). If, however, we compare the difference when the MS rats were in the center to when they were in the periphery, the theta synchrony decreased significantly when in the center ( $0.0469 \pm 0.0017$  in MS rats,  $t_{10}=39.10$ ,  $p<0.001$ ). These observations are consistent with the findings by Likhtik et al. that increases in theta synchrony with anxiety occur in the periphery of the open field (Likhtik et al., 2014).

### ***3.3. Maternal separation induced behavioral changes in decision-making during the rat gambling task***

One control rat and two MS rats were excluded from the RGT as they were inactive and failed to reach the training criteria during the entire period. Two control rats and three MS rats developed a form of spatial preference behavior for one side of the apertures during training and maintained this side-preference for the whole 60 min test (three preferred to the left side, and the other two preferred to the right side). There was no difference between two groups for this preference behavior (MS, 10% vs. control, 5.9%,  $P > 0.05$ ). These rats were not included in the final analysis as their



preferred choices in the test were not dependent on the decision-making process based on the different outcomes.

No difference was detected in the general activity (numbers of nose-pokes per min) (MS,  $11.91 \pm 0.48$  vs. control,  $10.93 \pm 0.43$ ;  $t_{57} = 0.026$ ,  $P > 0.05$ , Fig. 3A) and motivation (MS,  $25.91 \pm 0.34$  min vs. control,  $25.33 \pm 0.35$  min; t test,  $t_{57} = 1.177$ ,  $P > 0.05$ , Fig. 3B) between the two groups. The performances of the control rats and MS rats during the RGT test are shown in Fig. 3C and D. Three types of decision-making behaviors were identified in both groups. Good decision-makers indicated those who chose randomly at first, then orientated their preference progressively toward more advantageous options to end up making more than 70% advantageous choices during the last 20 min. Poor decision-makers sampled the different options early, then developed a stable preference for the adverse options. They made less than 30% advantageous selections during the last 20 min of the test. Undecided rats did not reveal any stable preference for either option across the whole test.

In the MS group, the proportion of good decision-makers was decreased (MS,  $n = 12$  (44.5%) vs. control,  $n = 22$  (68.75%)) when compared with the control group. On the other hand an increase in the proportion of poor decision-makers was observed in MS rats (MS,  $n = 7$  (25.9%) vs. control,  $n = 3$  (9.375%)). The proportion of undecided decision-makers in MS rats was similar to that observed in controls (MS,  $n = 8$  (29.6%) vs. control,  $n = 7$  (21.875%)). The difference in the proportions of the three ordinal types of decision-making behaviors between the two groups was significant (Mann–Whitney U test,  $U = 314.5$ ,  $P < 0.05$ , Fig. 3E). Mean food reward obtained during the RGT by MS rats was significantly less than for controls (MS,  $102.2 \pm 4.60$  vs. control,  $150.7 \pm 5.415$ ,  $t_{57} = 6.685$ ,  $P < 0.01$ , Fig. 3F).

### ***3.4. Alterations of theta activities in the BLA and ACC after maternal separation***

To characterize the theta rhythm activities of the BLA and ACC, power spectral analysis was performed during 120 s spontaneous activity in the two groups of rats. The distribution of theta band power is shown in Fig. 4A for the ACC and Fig. 4D for the BLA ( $n = 12$  for control and 12 for MS rats). The theta band power in the ACC was decreased significantly in MS rats when compared with controls ( $t_{22} = 2.446$ ,  $P < 0.05$ , Fig. 4B). The theta band power in the BLA during spontaneous conditions ( $t_{22} = 2.178$ ,  $P < 0.05$ , Fig. 4E) was enhanced in MS rats when compared with controls.

The peak frequency increased in the ACC in MS rats ( $t_{22} = 3.747$ ,  $P < 0.05$ , Fig. 4C) but decreased in the BLA in MS rats when compared with controls ( $t_{22} = 2.941$ ,  $P < 0.05$ , Fig. 4F). In the ACC, the peak frequency increased from  $5.62 \pm 0.07$  Hz in control rats to  $6.04 \pm 0.08$  Hz in MS rats. However, the peak frequency in the BLA decreased from  $5.78 \pm 0.09$  Hz in control rats to  $5.42 \pm 0.08$  Hz in MS rats. These data indicated that MS enhanced the theta activities in the BLA, but reduced the theta activity in the ACC.

### **3.5. Desynchronized theta activities in the ACC and BLA of MS rats**

To examine the functional connectivity of the ACC and BLA, we compared the correlation of the theta oscillations of the LFP in control and MS rats. Time varying power spectral analysis of 120 s recordings during spontaneous conditions revealed that the concentrated neuronal activities in the ACC and BLA in control rats became dispersed at theta frequencies in MS rats (Fig. 5A). Cross-correlation analysis allows quantitative evaluation of the synchronized activities of the LFPs. By averaging the cross-correlograms and taking the second positive peak as a quantitative measure (Fig. 5B), a significant difference was detected ( $t_{22} = 4.843$ ,  $P < 0.01$ ; Fig. 5C). The second positive peak represents the synchronized activities of the LFP in the theta band as the lag time is from 0.1 to 0.25 s corresponding to theta band oscillations in 4-10 Hz.

During spontaneous conditions, the correlation value decreased from  $0.317 \pm 0.025$  in control to  $0.159 \pm 0.021$  in MS rats. The results suggest that the BLA and ACC may only loosely interact for dynamic information transfer after maternal separation.

### 3.6. Interrupted spike-fired coherence between BLA and ACC in MS rats

In the current study, SFC was used to quantify the alteration in the spike timing-LFP relationship between the BLA and the ACC in control and MS rats. The SFC value is expressed as a percentage and varies as a function of frequency. The SFC value varies from 0% to 100% with 100% indicating all spikes following a particular phase of oscillation in this frequency, while 0% reflects spikes firing completely at random. The SFC distributions for various frequencies are presented in Fig. 6. We found a significant difference in the average SFC for MS rats in the theta range ( $t_{121} = 3.518$ ,  $P < 0.05$ ; Fig. 6C). SFC values of the ACC spikes coherent with the BLA LFP were decreased in the theta band from  $2.351\% \pm 0.095\%$  in control rats to  $1.747\% \pm 0.150\%$  in MS rats. These results suggest that the spike timing-LFP relationship in the theta band was reduced between the BLA and the ACC in MS rats.

## 4. Discussion

In this study we used a rodent model to investigate the impacts of neonatal maternal separation, during the first three postnatal weeks, on the development of anxiety-like behavior, the impairment of decision-making, and the associated neuronal functional alterations in the ACC and BLA network in adulthood. To the best of our knowledge, the present study is the first to characterize the disruption of theta band oscillations, as well as impairments in the functional coupling between the BLA and ACC circuit, accompanied by anxiety and decision-making deficits in MS rats.

Maternal separation is the most robust and common model for the disruption of the mother-offspring relationship. It can cause acute and long lasting behavioral and

neurochemical phenotype alterations in adulthood, due to repeated activation of stress mediators such as glucocorticoids and catecholamines (McEwen, 2003). Separation during a critical developmental period interferes with appropriate maturation of important modulatory systems. This essential period of intense cellular alteration occurs during a time when the activity in the HPA axis exhibits a dampened profile, known as the stress hypo-responsive period (SHRP). In rodents, MS has been well-characterized to model depression, anxiety and visceral hypersensitivity in irritable bowel syndrome (IBS). Several lines of evidence have shown that early life adversity has a profound impact on emotional and cognitive behaviors, including depression (Aisa et al., 2007), anxiety-like behavior (Ishikawa, Nishimura, & Ishikawa, 2015; van Hasselt et al., 2012), learning and memory deficit (Aisa et al., 2007, Baudin et al., 2012; Sousa et al., 2014), and cognitive flexibility (Baudin et al., 2012). In the present study, MS rats showed general exploratory behavior in the OFT comparative with that of the controls; however, MS rats spent less time in the center area and entered the center area fewer times overall. In the EPM, we observed that in MS rats the amount of time spent in the open arms was decreased suggesting the development of anxiety-like behavior.

Previous studies have shown that high levels of anxiety are related to impaired decision-making both in animals and humans (de Visser et al., 2010; Miu, Heilman, & Houser, 2008, de Visser et al., 2011). Decision-making is the result of the integration of several executive brain areas and has emerged as a crucial theme in neurophysiological studies of cognition. In humans, decision-making has been accurately modeled using the Iowa gambling task (IGT). Recently, we have conducted a decision-making test - the rat gambling task (RGT) - and found that, similar to the findings of the most advantageous strategy from the IGT utilized in human studies,

most rats can learn to maximize their food reward. This is achieved by reasoning and preferring the more profitable options associated with smaller immediate gain but lower risk of punishment (time-out) during the RGT. We showed that prolonged treatment with cisplatin, a chemotherapy drug, induces both anxiety and impaired cognitive functions, including decision-making (Mu et al., 2015). In this study, we reported that maternal separation had no general effect on the motivation to obtain food rewards (duration of the last training session before reach criterion). Three types of decision-making behaviors, namely good, undecided, and poor subjects, could be successfully identified in both control and MS rats. MS rats revealed a lower proportion of good decision-makers, and poor decision making behavior increased in the MS rats. Further, the mean food reward obtained during the RGT was significantly lower in MS rats than controls. These data provide the first evidence that MS can lead to decision-making deficits in rats. Our results from the RGT in MS rats are consistent with previous evidence that lack of maternal care in early life impaired cognitive functions as adults. In human subjects Maier et al. reported that stress induced impairment of goal-directed decision making, with alteration of functional connections between the BLA and PFC (Maier, Makwana, & Hare, 2015). Van Hasselt et al. also showed that rats receiving low amount of maternal care as pups, such as licking and grooming by their mother, developed poor decision-making (van Hasselt et al., 2012).

Brain imaging studies have shown that maternal separation leads to alteration of central activation patterns when compared to controls (Gibney, Gosselin, Dinan, & Cryan, 2009; Zhang et al., 2009). Quantification of c-fos studies have demonstrated that the brain areas related to emotion and anxiety include the ACC, amygdala and hippocampus (Troakes & Ingram, 2009; Zhang et al., 2009). The ACC receives input

from limbic regions such as the thalamus, the hippocampus and the amygdala. A growing body of literature shows that the ACC is critically involved in strategy shifting, behavioral flexibility and goal-directed learning behavior by encoding task-rules (Balleine & O'Doherty, 2010; Birrell & Brown, 2000; Dias, Robbins, & Roberts, 1996; Floresco, St Onge, Ghods-Sharifi, & Winstanley, 2008; Ragozzino, Detrick, & Kesner, 1999; Sul et al., , 2010; Young & Shapiro, 2009). In addition, our series of published observations have characterized chronic visceral pain and the neural electrophysiological activity of the ACC during processing of visceral nociceptive stimulation (Wang et al., 2013, Fan et al., 2009, Cao et al., 2008; Gao, Wu, Owyang, & Li, 2006). Recently we showed that the activation of the ACC is critical for the affective component of visceral pain memory (Yan et al., 2012). Functional integrity of the ACC may allow for the coupling of rats' choices with food rewards to generate an optimal decision-making strategy. Moreover, several subcortical structures have been demonstrated to be involved in differentially mediating the decision-making process, including the basolateral nucleus of the amygdala (BLA) (Floresco et al., 2008). Bidirectional projections between the ACC and the BLA emerge around PND 7, and continue to increase until early adulthood (Cunningham, Bhattacharyya, & Benes, 2002; Ishikawa et al., 2015); separation from PND 2 to PND 21 in the present study may impair the function of this circuit and cause emotional and decision making deficits. By c-fos staining, Ishikawa et al. revealed hypoactivity of the ACC and hyperactivity of the BLA neurons in rats treated with early life stress, with correlation analysis suggesting that imbalanced BLA-ACC activities contribute to increased anxiety-like behavior (Ishikawa et al., 2015).

Brain rhythms are likely to be a basic mechanism for modulating, filtering, and redirecting information in the nervous system (Buzsaki, 2004). Within individual

brain regions, oscillations can synchronize neurons, creating coherent cell assemblies and favoring plasticity processes depending on the precise timing of pre- and postsynaptic activity (Bi & Poo, 1998). The theta band brain oscillations that are mainly observed in the limbic system, including the hippocampus and the ACC (Paz, Bauer, & Pare, 2008), have been acknowledged to influence extensive cognitive functions. Previous studies have shown that the oscillations of the theta band activity could influence the interactions of the basal ganglia-based and hippocampus-based forebrain circuits during the acquisition and performance of learned behaviors in the rat conditional T-maze task (DeCoteau et al., 2007), and in rats learning new rules on a Y maze (Benchenane et al., 2010). Neuropathological abnormal neuronal theta band activity is associated with a wide range of animal disease states. We have reported that chronic visceral hypersensitivity resulted in enhancement of theta band oscillations in the ACC (Wang et al., 2015). Another study showed increases in theta power in the BLA, but decreases of theta power in the ACC in rats after prolonged tooth loss (Xu et al., 2015).

In this study using an array of microelectrodes we performed simultaneous recording of the BLA and the ACC neural spiking activity and local field potentials during awake conditions. At the end of the electrophysiological recording, the recording sites were checked by Nissl staining, rats with inappropriate electrode placements were excluded from the analysis, the correct recording sites in the ACC are shown in Fig. 7A-D, and in the BLA are shown in Fig. 7E-H. We discovered significant alterations in the theta oscillations in both the BLA and ACC areas in the MS rats. We observed increases in theta band power in the BLA, but decreases in the ACC. The peak frequency of the theta oscillations decreased in the BLA, but increased in the ACC. It has been reported that alterations of oscillation frequencies

may cause changes in the neural spike threshold as well as the spike timing dynamics of individual neurons. The relatively slower oscillation frequencies decreased the spike threshold and increased spike timing variability (Cohen, 2014). It is conceivable that the alterations of the theta band power and peak frequency of the LFP in the ACC and BLA have important physiological implication in MS rats. However, the alterations in the theta oscillation of the LFPs in MS groups measured in the home cage cannot be directly linked with the decision making behavioral effects assessed in the RGT probe tests. In this study electrophysiological recordings were also performed in the rats during the open field test. We showed increases in the theta band synchrony corresponding with anxiety in the periphery of the open field. These observations are consistent with previously reported findings (Likhtik et al. 2014).

Synchronized oscillatory activity facilitates communication and modifies synaptic weights between anatomically distant, but functionally associated, brain regions during learning. In the present study, we observed that cross-correlation analysis revealed that the theta band oscillations of the LFP between the BLA and ACC became desynchronized in MS rats. The desynchronization of the theta band oscillations, induced by maternal separation as pups, suggests that the BLA and ACC may then only loosely interact for dynamic information transfer, which may disrupt neural network assemblies and affect synaptic plasticity. These observations from the large-scale electrophysiological section of this study confirm that impairment of functional coupling between the BLA and the ACC may contribute to behavioral dysregulations in MS rats (Ishikawa et al., 2015).

A human study has demonstrated that theta-frequency phase-locking of single neurons played an important role in memory strength (Rutishauser et al., 2010). Ample evidence suggests that neurons transmit information not only by changing



their firing rates but also in terms of the timing of the spikes corresponding to the ongoing neuronal oscillations (Varela et al., 2001). In the current study, we observed suppressed locking of the ACC spikes to the phase of the theta band oscillations in the BLA of the MS rats. The SFC analysis in our study is independent of the LFP power spectrum and the number of spikes, and is therefore immune to changes in these parameters (Womelsdorf et al, 2007). This allowed us to distinguish between changes in spike-field synchronization and changes in the regularity of oscillatory patterns that are reflected in enhancement of the spectral power of the field. These findings in MS rats are consistent with the observations that damage of either amygdala or prefrontal cortex impairs the capacity to assess and use the value of the predicted outcomes to guide their actions in the Iowa gambling task in humans and monkeys (Baxter et al., 2000).

A limitation of the current study, which produces a potentially relevant confounding issue, is that the electrophysiological recordings obtained were not conducted in the behaving animals during the decision-making task. Directly assessing the effects of MS on the BLA and ACC activities during the event-related choices made by animals should provide useful evidence in future studies.

In summary, neonatal maternal separation induced anxiety-like behavior and poor decision-making in the RGT. In parallel, we observed decreased synchronization between the BLA and the ACC, combined with alteration of theta band activity in the BLA and ACC, which was associated with interrupted SFC between the BLA and ACC. These data are consistent with the theory that oscillatory synchrony, particularly phase synchronization, provides an overall principle for integrating information between different cortical and subcortical regions during complex cognitive processing.

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### Figure Legends

**Fig. 1.** Anxiety-like behavior in maternally separated rats as determined in the open field test (OFT) and the elevated plus maze test (EPM). (A) Total horizontal distance traveled in the OFT. (B) The mean speed traveled in the OFT. (C) Time spent in the center in the OFT. (D) The number of entries into the center in the OFT. (E) Time spent in the open arms in the EPM. (F) Percentage of time spent into open arms versus closed arms in the EPM. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

**Fig. 2.** BLA synchronizes with ACC in the periphery in the MS rats. (A) Example maps of occupancy in the open field for a control rat and a MS rat. The boundary center area of open field is labeled by the white rectangle. Note the longer time spent in the center in the control rats than the MS rats. (B) The averaged cross-correlograms of control rats (upper) and MS rats (bottom) in the periphery (black line) and center (red line). (C) Statistical analysis revealed that the second positive peak, which corresponds to synchronization of theta activity, decreased significantly in MS rats when compared with control rats. In the MS rats, the value decreased significantly when the rats were in the center. Results are presented as mean  $\pm$  SEM. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

**Fig. 3.** The decision-making performance of control and MS rats in the rat gambling task (RGT). (A, B) General activity and motivation between control and MS rats in the RGT. (C, D) The ratio of advantageous choices across the 60 min RGT test identified three types of decision makers in both control and MS rats. (E) The proportions of three types of decision makers in control and MS rats. (F) The food pellets obtained during the test in control and MS rats. Results are presented as mean  $\pm$  SEM,  $n = 27$  for control group and  $n = 32$  for MS group. Statistical significance was determined by student  $t$ -test, \*\*  $P < 0.01$ .

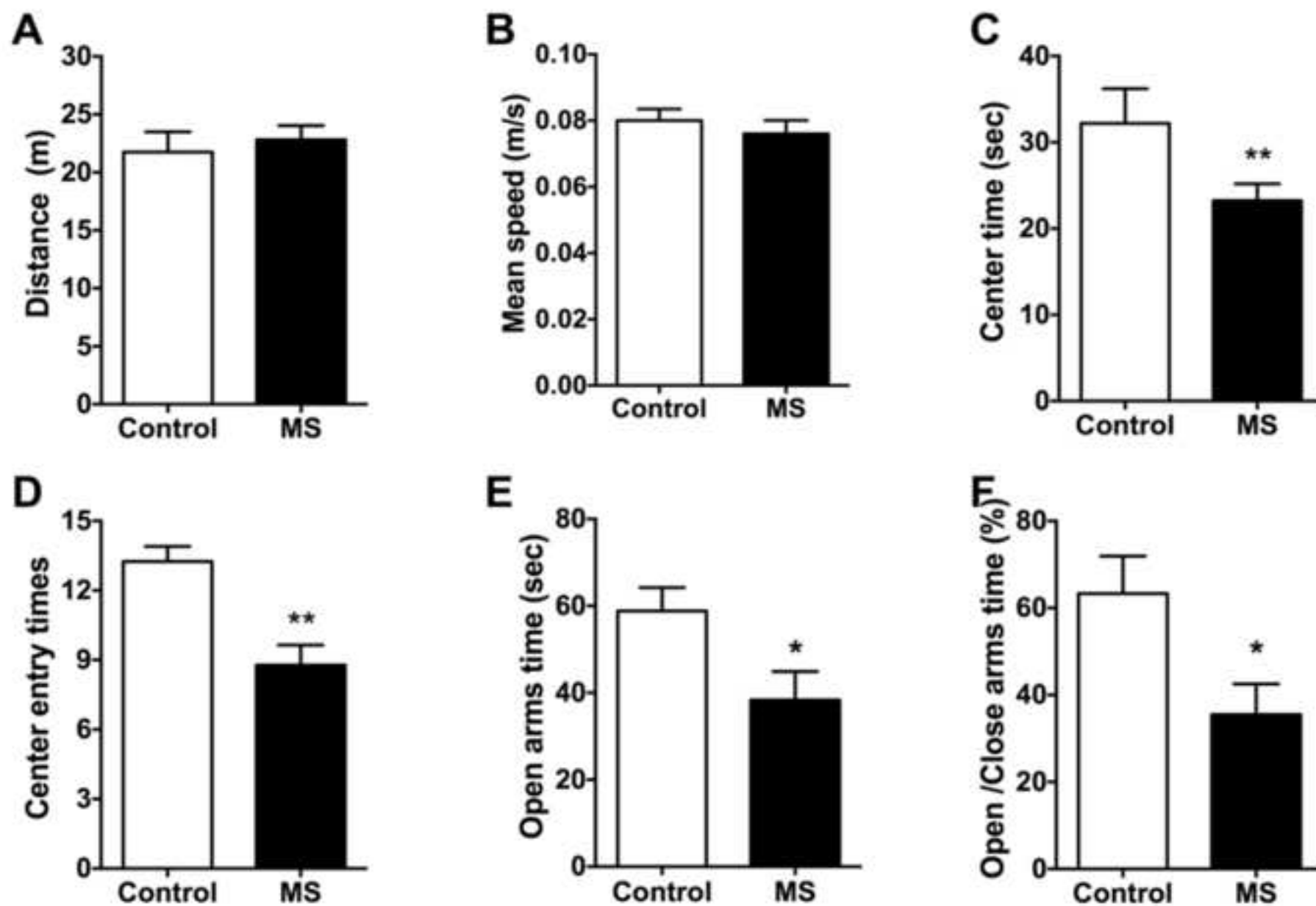
**Fig. 4.** Power spectral density (PSD) analysis of the theta band of the local field potentials (LFP) in the anterior cingulate cortex (ACC) and basolateral amygdala (BLA) in freely behaving control and MS rats. (A, D) Distributions of power spectral density in the theta band (4-10 Hz) LFP recorded from the ACC (top) and the BLA (bottom) in control and MS rats. (B, E) The average of the theta band power (area under curve of the theta band PSD) showed significant decreases in the ACC of MS rats (top), while increased in the BLA (bottom) compared with control rats. (C, F) Peak frequency, which corresponds to the frequency of maximal PSD, revealed a significant increase in the ACC of MS rats (top) but decreased in the BLA (bottom) compared with control rats. Results are presented as mean  $\pm$  SEM, \*  $P < 0.05$ .

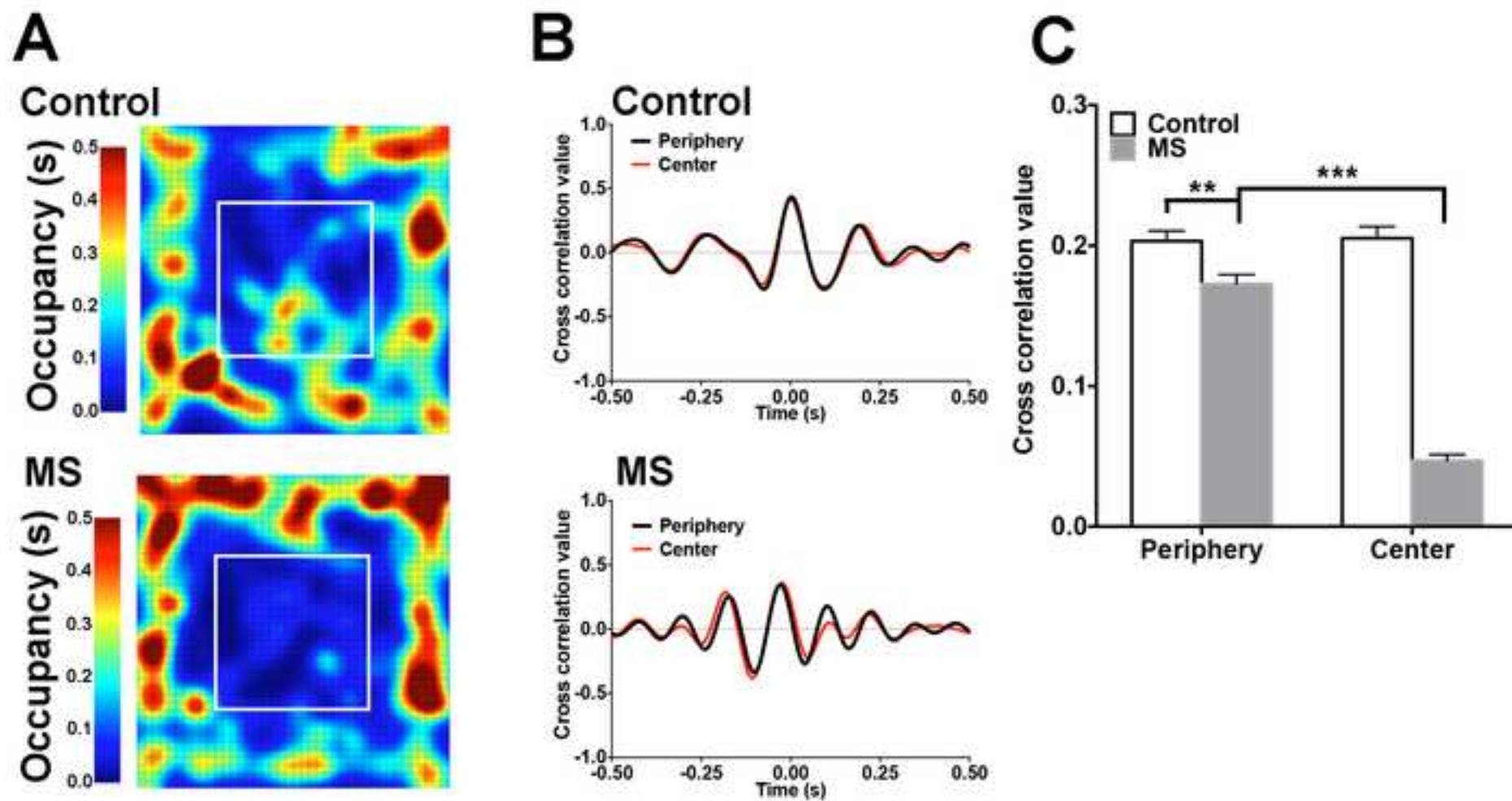
**Fig. 5.** Synchronization of theta band oscillations between the ACC and BLA in control and MS rats. (A) Typical colored power spectrograms (120 sec duration) recorded from the ACC (top) and BLA (bottom) in control rats (left) and MS (right) rats. (B) The averaged cross-correlograms of freely behaving control and MS rats. (C)

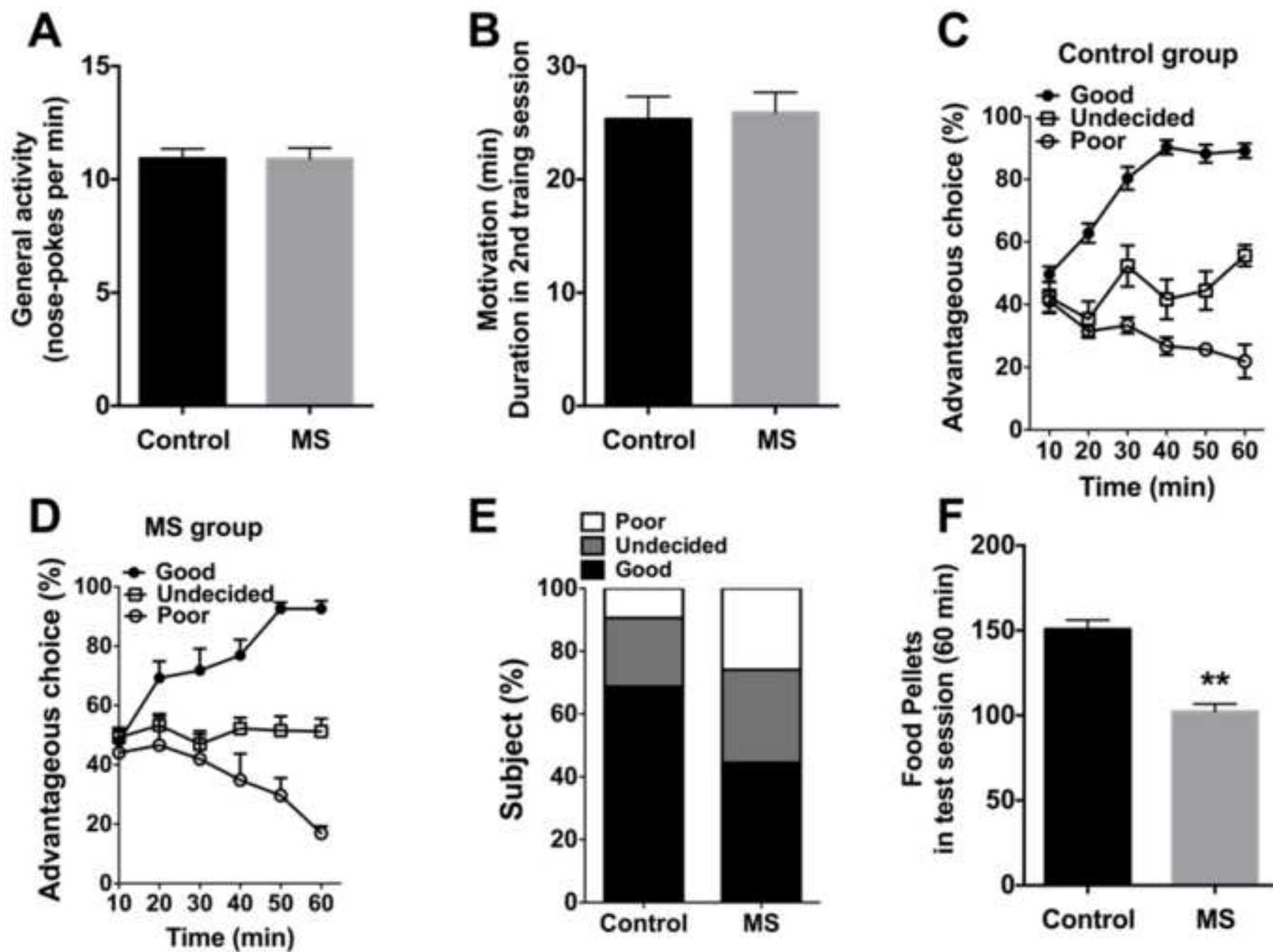
Statistical analysis revealed that the cross-correlation value (the second positive peak shown in B), which corresponds to synchronization of theta activity, decreased significantly in MS rats compared with control rats. Results are presented as mean  $\pm$  SEM, \*\*  $P < 0.01$ .

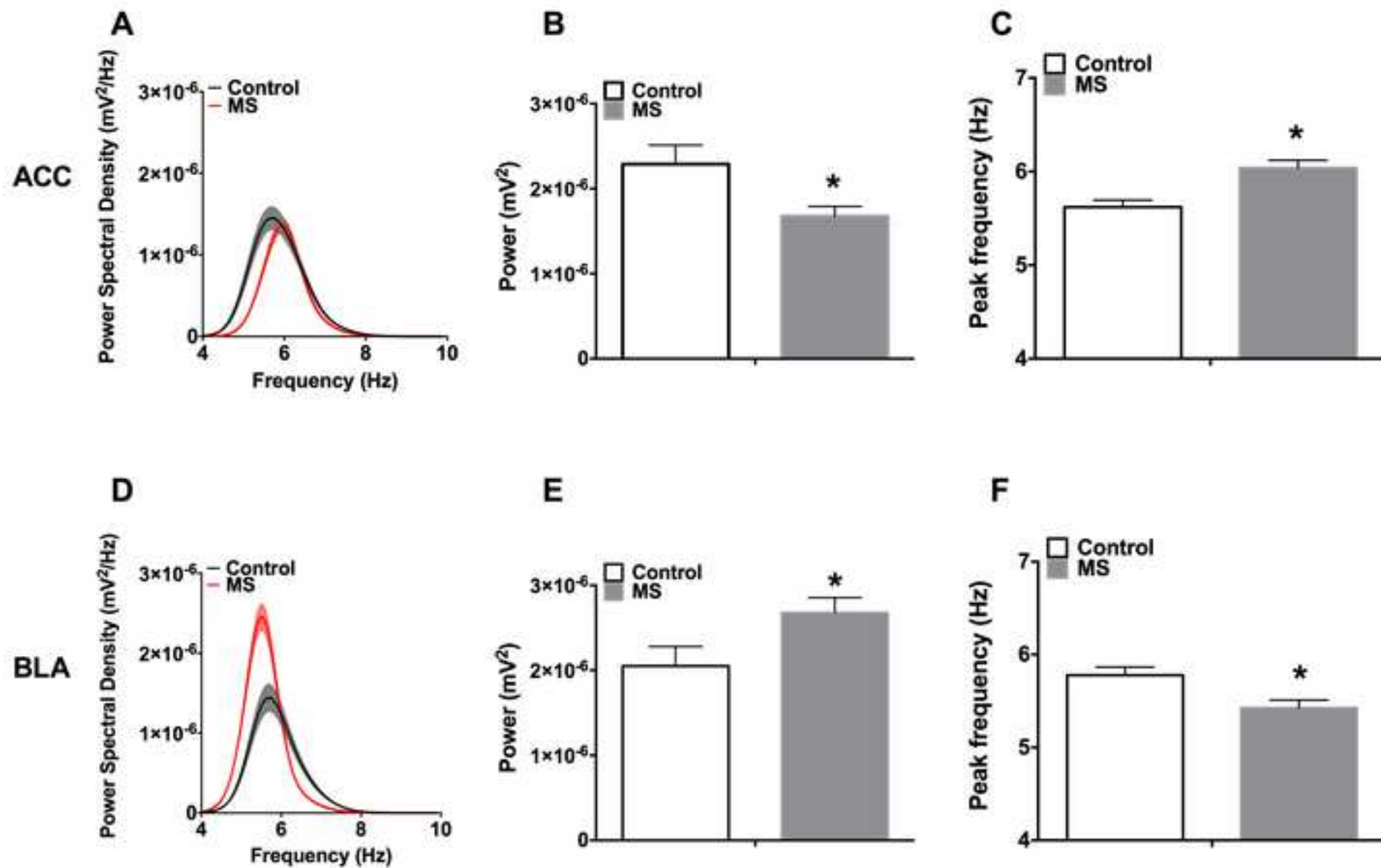
**Fig. 6.** The spike-field coherence (SFC) between the BLA and ACC in control and MS rats. (A) Distribution of the ACC spikes coherent with the LFP in the BLA in control rats (black line) and MS rats (red line). (B) Distribution of the BLA spikes coherent with the LFP in the ACC in control MS rats. (C) Averaged SFC in the theta band showing ACC spikes with BLA LFP decreased significantly in MS rats. Results are presented as mean  $\pm$  SEM, \*  $P < 0.05$ .

**Fig. 7.** Verification of the placement of recording electrodes. (A, B) The correct placements of the recording electrodes in the ACC of a MS rat (A) and a control rat (B) were confirmed by cresyl violet staining of the coronal brain sections. Red arrows point to lesions at the end of electrode tracks. (C, D) Recording sites in the ACC of 12 control rats (solid circles) and 12 MS rats (Hollow circles). (E, F) The correct placements of the recording electrodes in the BLA of a MS rat (E) and a control rat (F). (G, H) Recording sites in the BLA of 12 control rats (solid squares) and 12 MS rats (Hollow squares). Cg1, cingulate cortex, area 1; PrL, prelimbic cortex; BLA, basolateral amygdaloid nucleus, anterior; BLP, basolateral amygdaloid nucleus.

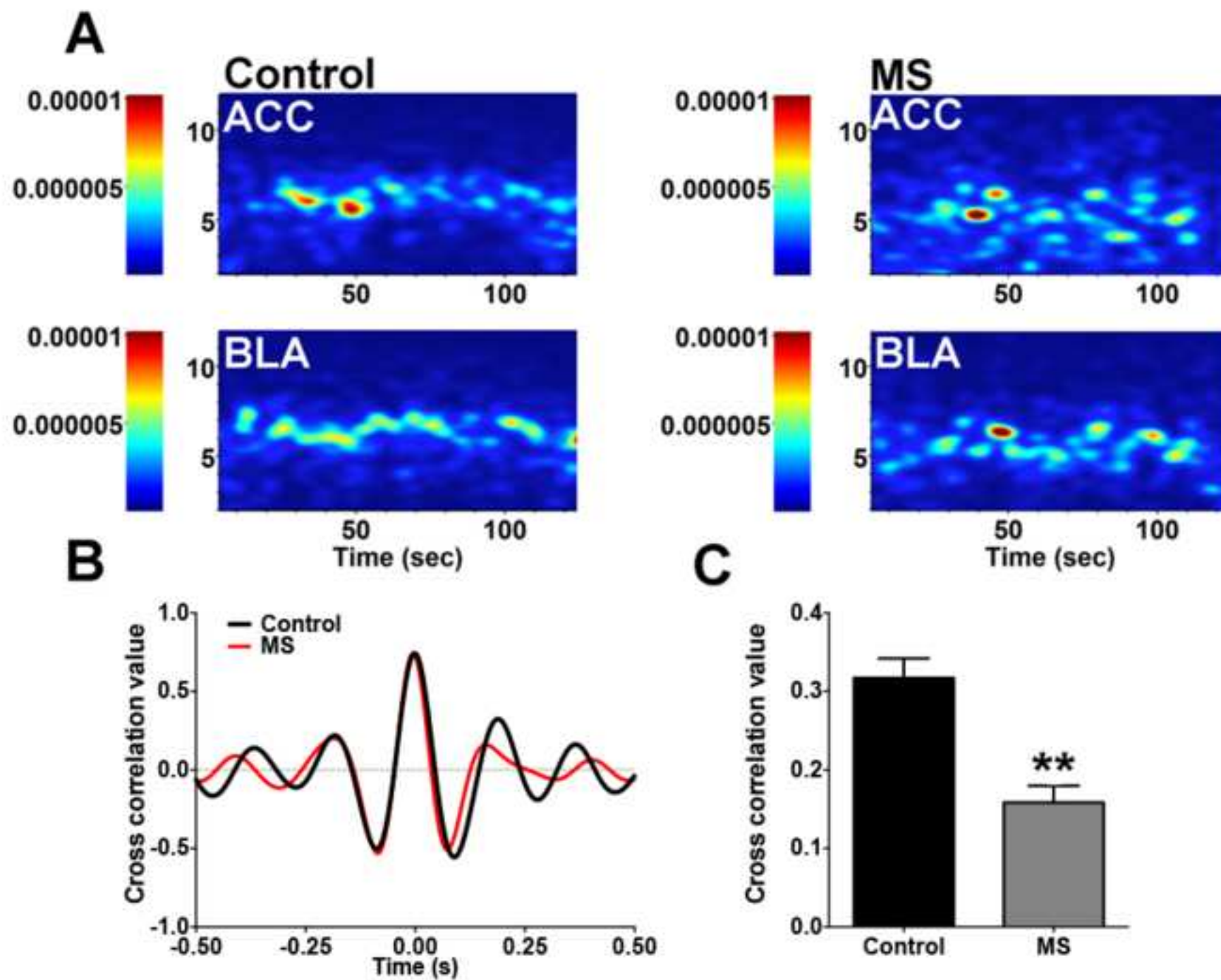


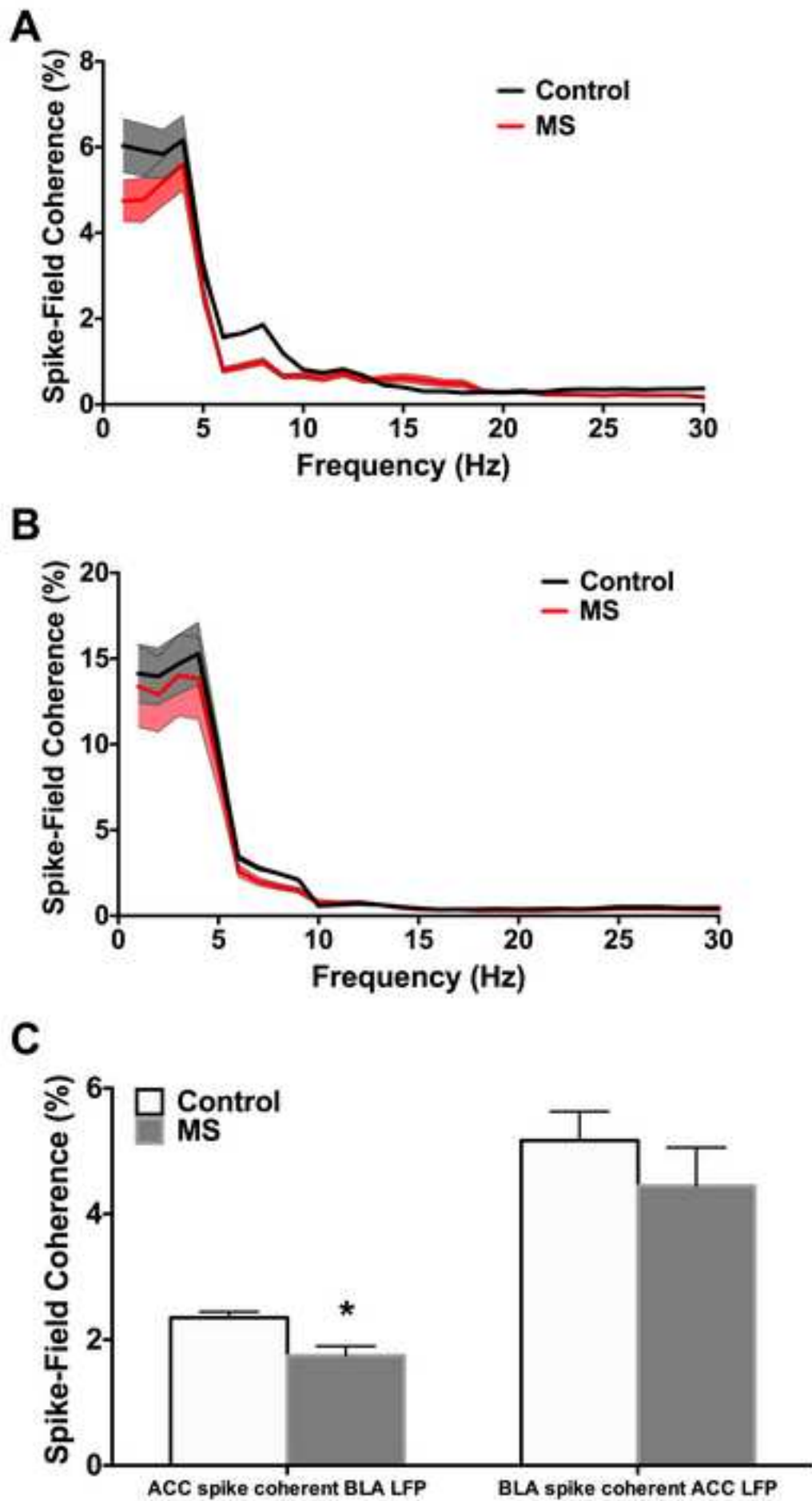


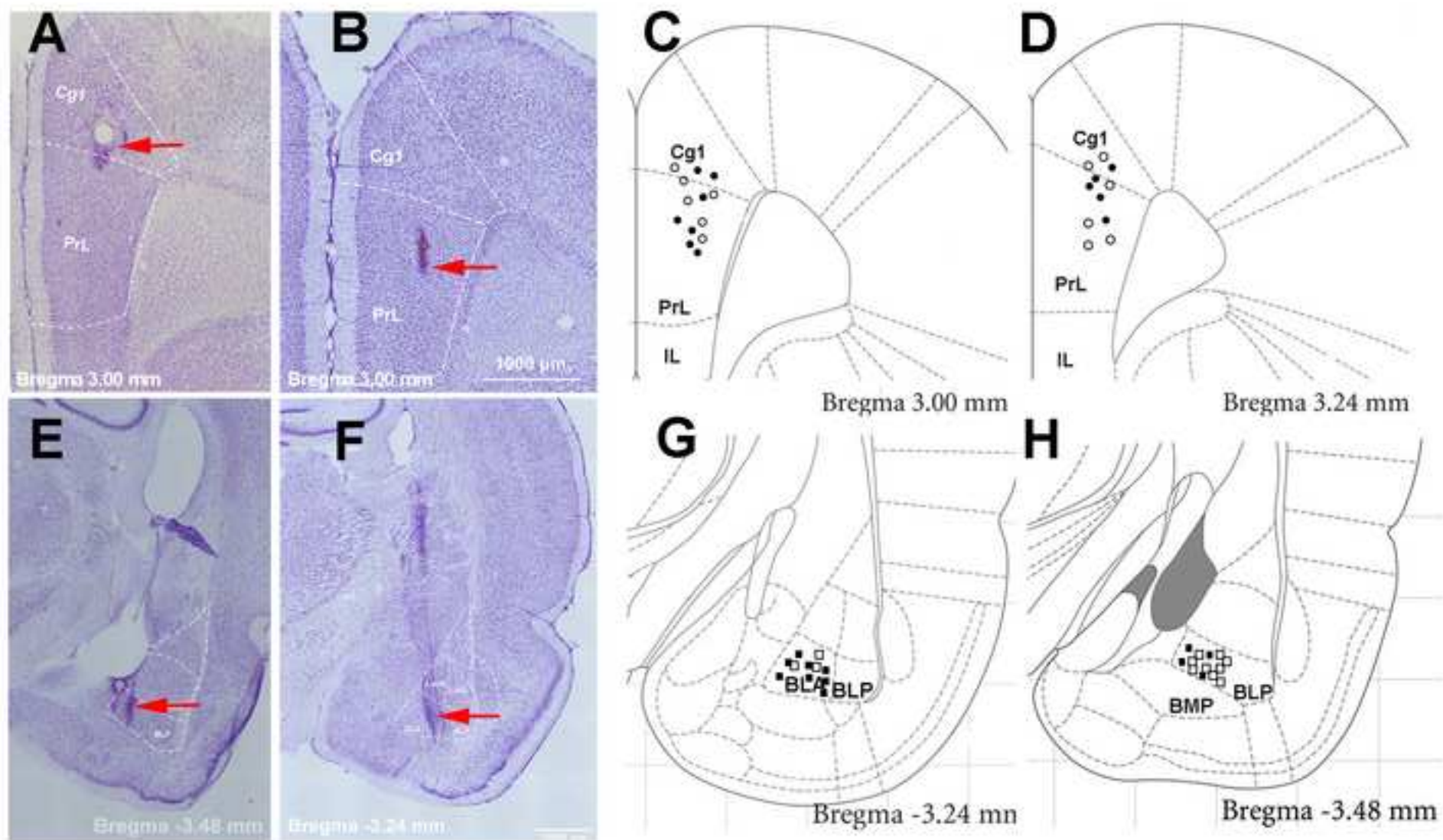












### Highlights

Early life stress causes decision-making deficit in adult rats.

Coherence of ACC spikes to BLA theta oscillations is disrupted in MS rats.

Theta band oscillations between ACC and BLA is desynchronized in MS rats.

ACCEPTED MANUSCRIPT