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**Different Effects of Isolation-Rearing and Neonatal MK-801 Treatment on Attentional  
Modulations of Prepulse Inhibition of Startle in Rats**

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## Abstract

### *Rational*

Prepulse inhibition (PPI) is suppression of the startle reflex by a weaker sensory stimulus (prepulse) preceding the startling stimulus. In people with schizophrenia, impairment of attentional modulation of PPI, but not impairment of baseline PPI, is correlated with symptom severity. In rats, both fear conditioning of prepulse and perceptually spatial separation between the conditioned prepulse and a noise masker enhance PPI (the paradigms of attentional modulation of PPI).

### *Objectives*

As a neuro-developmental model of schizophrenia, isolation rearing impairs both baseline PPI and attentional modulations of PPI in rats. This study examined in Sprague-Dawley male rats whether neonatally blocking NMDA receptors specifically affects attentional modulations of PPI during adulthood.

### *Results*

Both socially-reared rats with neonatal exposure to the NMDA-receptor antagonist MK-801 and isolation-reared rats exhibited augmented startle responses, but only isolation rearing impaired baseline PPI. Fear conditioning of the prepulse enhanced PPI in socially-reared rats, but MK-801-treated rats lost the prepulse-feature specificity. Perceptually spatial separation between the conditioned prepulse and a noise masker further enhanced PPI only in normally-reared rats. Clozapine administration during adulthood generally weakened startle, enhanced baseline PPI in neonatally interrupted rats, and restored the fear-conditioning-induced PPI enhancement in isolation-reared rats with a loss of the prepulse-feature specificity. The clozapine administration also abolished both the perceptual-separation-induced PPI

enhancement in normally-reared rats and the fear-conditioning-induced PPI enhancement in MK-801-treated rats.

*Conclusions*

Isolation rearing impairs both baseline PPI and attentional modulations of PPI, but neonatally disrupting NMDA-receptor-mediated transmissions specifically impairs attentional modulations of PPI. Clozapine has limited alleviating effects.

**Keywords**

Attentional modulation; Animal model; Clozapine; Isolation rearing; Neonatal MK-801 exposure; Prepulse inhibition; Schizophrenia; Emotional learning; Precedence effect; Perceived spatial separation.

## INTRODUCTION

Prepulse inhibition (PPI) is the normal reduction of the amplitude of the startle reflex in response to an intense startling stimulus (pulse) when this intense stimulus is shortly preceded by a weaker, non-startling sensory stimulus (prepulse) (Fendt et al. 2001; Ison and Hammond 1971; Li et al. 2009). PPI can be enhanced by selective attention to the prepulse stimulus in both humans and rats (Li et al. 2009). For example, when a prepulse becomes fear conditioned, the conditioned prepulse, but not a conditioning-control prepulse, elicits enhanced PPI, indicating that the fear-conditioning-induced PPI enhancement exhibits a prepulse-feature specificity (Du et al. 2009, 2010, 2011; Zou et al. 2007). PPI can also be enhanced by spatial attention (see below).

In a reverberant environment, listeners with normal hearing have the ability to perceptually integrate the direct sound wave and the reflections of the sound source: attributes of the delayed and correlated reflections are perceptually captured by the direct sound wave (Li et al., 2005), leading to a single fused image whose perceived point of origin is around the location of the leading source. This auditory phenomenon is called the “precedence effect” (for reviews see Freyman et al. 1999; Li et al., 2009; Litovsky et al., 1999). In humans, when both a target sound and a masker are presented by each of the two spatially separated loudspeakers with an inter-loudspeaker delay of 3 ms, due to the precedence effect, only a single target image is perceived as coming from the leading loudspeaker for the target. Also, only single masker image is perceived as coming the leading loudspeaker for the masker. Recognizing the target under the condition of perceived target-masker spatial separation (when the leading loudspeaker is different between target and masker) is significantly better than that under the condition of perceived co-location between target and masker (when the leading loudspeaker is the same for both target and masker) (Li et al., 2009). Note that shifts between the perceived separation condition and the perceived

co-location condition do not substantially change the signal-to-masker ratio in sound pressure level and the sound-image compactness/diffusiveness (Li et al., 2004; Li et al., 2009).

In rats, with the mediation of the posterior parietal cortex (PPC) (Du et al. 2011), when a fear-conditioned prepulse and a noise masker are delivered by each of the two spatially separated loudspeakers with the inter-loudspeaker delay of 1 ms, the precedence effect-induced perceived spatial separation between the conditioned prepulse and the noise masker further enhances PPI by facilitating spatial attention to the prepulse (Du et al., 2009; 2010, 2011). This perceptual spatial separation-induced PPI enhancement also exhibits the prepulse-feature specificity (Du et al., 2009; 2010).

People with schizophrenia exhibit impairments in both baseline PPI (Geyer et al. 2001) and attentional modulations of PPI (Hazlett et al. 2007). Interestingly, impairment of attentional modulation of PPI, but not impairment of baseline PPI, is correlated with symptom severity of schizophrenia (Hazlett et al. 2007). Thus, there is a need to establish animal models for specifically studying the schizophrenia-related deficits in attentional modulations of PPI.

The neurodevelopmental hypothesis of schizophrenia emphasizes detrimental influences of certain early-life factors upon brain maturation in eliciting schizophrenic symptoms during adulthood (Buuse et al. 2003). Isolation rearing is one of the widely used animal models for inducing neurodevelopmental impairments of various cognitions/behaviors, including impaired PPI (Geyer et al. 2001; Weiss et al. 2000) and attentional modulations of PPI (Li et al. 2009), augmented locomotion (Weiss et al. 2000), impaired learning and memory (Li et al. 2007), and declined novel object recognition and attentional set shifting (McLean et al. 2010). All of these behavioral deficits are called “isolation syndromes”, which are related to disturbances of some neurotransmitter systems, including hypofunctions of both the N-methyl-D-aspartate receptor (NMDAR) and the metabotropic glutamate receptor 5 (mGluR5)

during the maturation process (Arndt et al. 2015).

Moreover, the glutamate hypothesis of schizophrenia emphasizes that non-competitive NMDAR antagonists, such as MK-801, can induce schizophrenia-like symptoms in healthy humans (Geyer et al. 2001). The dysfunction of NMDARs is also associated with some deficits in individuals' development (for a review see Lim et al., 2012a). For example, NMDAR mediates glutamatergic transmissions that are associated with brain growth and neural plasticity (Komuro and Rakic, 1993; Wilson et al., 1998), and blockade of NMDAR in rats using MK-801 during early postnatal days (PND 0-14) causes apoptotic neurodegeneration in a wide range of developing brain regions (Ikonomidou et al., 1999), including amygdala, hippocampus (Beninger et al., 2002; Manning et al., 2011), and cerebellum (Jantas and Lason, 2009; Kanungo et al., 2009). This accelerated programmed cell death following the early MK-801 treatment may also be associated with deficiency of neurotrophic signaling. Specifically, the brain-derived neurotrophic factor (BDNF) and the glial cell line-derived neurotrophic factor (GDNF) promote both neuronal growth and survival in the process of brain development and maturation (Huang and Reichardt, 2003; Kang and Schuman, 1995; Kaplan and Miller, 2000; Luz et al., 2016). Neonatal challenge with MK-801 in rats results in a loss of neurotrophic mRNAs encoding BDNF and GDNF, parallel to a disruption of the ERK1/2-CREB signaling pathway (Hansen et al., 2004). It has been confirmed that NMDARs and related mGluR5 are involved in higher-order cognitive process, such as attention (Amitai et al., 2007; Auclair et al., 2009; Ayala et al. 2009; Semenova and Markou, 2007) and learning/memory (Ayala et al., 2009; Fowler et al., 2013; Jia et al., 1998; Palmer et al., 1997; Zou et al. 2007). Phencyclidine (PCP) is another non-competitive NMDAR antagonist with less selectivity than MK-801. It has been reported that in rats, neonatal PCP treatment exacerbates attentional deficits in the five-choice serial reaction time task (5-CSRTT) (Le Pen et al., 2003) and impairs attention shifting (Broberg et

al., 2008; 2009) during adulthood. Up to date, whether early exposure to MK-801 affects attention during adulthood is not clear.

Thus, NMDAR model is a combination of neurodevelopmental and glutamate hypothesis of schizophrenia. It is of interest and importance to investigate whether the early-life MK-801 administration causes specific deficits in attentional modulation of PPI. Comparing the effects of isolation rearing, this study was to examine the effects of neonatal exposure to MK-801 on the following 4 measures in adult rats: (1) baseline startle, (2) baseline PPI, (3) modulation of PPI by fear-conditioning of the prepulse, and (4) modulation of PPI by perceived-spatial-separation between the conditioned prepulse and a noise masker. Also, the effects of clozapine on these 4 measures were examined.

**MATERIALS AND METHODS**

**Animals and Drugs**

Fifty-four Sprague-Dawley pups (20-30 g) along with their lactating mothers were purchased from the Vital-River Experimental Animals Technology Ltd., Beijing, China. From postnatal days (PND) 7 to 10, all the rat pups were intraperitoneally (i.p.) injected with either MK-801 (0.2 mg/kg, Sigma-Aldrich, dissolved in the acetic acid saline vehicle) or the vehicle (acetic acid saline solution, 1 ml/kg) between 08:00 and 10:00 each day. The pups were remained in the litter along with their lactating mothers until weaning on PND 21.

After weaning, each of the rats was randomly assigned to either the social rearing groups or the isolation rearing (for 8 weeks) group. For isolation-reared rats with saline injection (SAL-IR), each individual was housed in a single transparent plastic cage (48 × 30 × 18 cm). For socially reared rats with either saline injection (SAL-SR) or MK-801 injection (MK-SR), 3 individuals were housed in a cage with the same dimensions.



## Stimuli and Apparatus

The rat's whole-body startle reflex was induced by an intense 10-ms broadband noise burst (0-10 kHz, 100 dB SPL) delivered by a loudspeaker above the rat's head. The prepulse stimulus, which started 100 msec before the onset of startling noise, was a 50-msec three-harmonic tone complex with either low-frequency components (1.3, 2.6 and 3.9 kHz, 60 dB SPL) or high-frequency components (2.3, 4.6 and 6.9 kHz, 60 dB SPL). Note that Sprague-Dawley albino rat have a range of hearing from 250 Hz to 80 kHz with the most sensitivity to tones around 8 kHz (Kelly and Masterton, 1977). The frequency components of the prepulse stimuli used in this study were within the hearing range of rats. The prepulse was delivered by each of the two spatially separated loudspeakers, which were placed horizontally in the frontal field with a 100° separation angle and 52 cm away from the rat's head position. Sound stimuli were digitally generated by Adobe Audition software and converted by a custom-developed sound-delivery system (National Key Laboratory on Machine Perception, Peking University). Calibration of sound intensity was conducted with a Larson Davis Audiometer Calibration and Electroacoustic Testing System (Audit and System 824, Larson Davis, Depew, NY, USA).

## Testing Procedures

The rat was placed in a restraining cage, whose dimensions matched the size of the rat body so that the rat could not reorient its head and body positions on PND 77-79. During the adaptation procedure (Figure 1), the rat was exposed to the background broadband noise which was continuously presented by each of the two horizontal loudspeakers for 30 min, and became adapted to the cage and testing chamber.

----- Insert Figure 1 about here -----

Phase I of the testing procedure started on PND 80 (the day before conditioning, BC, Figure 1). First, the rat received 10 presentations of the startling noise without the prepulse presentation on the broadband-noise background for 5 min. Then, 4 testing blocks were introduced. In each of the testing blocks, 5 trials contained the startling stimulus alone, 5 trials contained a low-frequency prepulse preceding the startling stimulus, and 5 trials contained a high-frequency prepulse preceding the startling stimulus. The inter-stimulus-onset interval between a prepulse and the startling stimulus was 100 msec. The 3 types of trials in a testing block were presented in a random order.

A prepulse (with either higher or lower frequency components) was presented from each of the two horizontal loudspeakers with the inter-loudspeaker onset delay being either +1 msec (left leading) in 2 testing blocks or -1 msec (right leading) in the other 2 testing blocks. Thus, due to the precedence effect (Litovsky et al. 1999;), a single fused prepulse image was perceived at the left loudspeaker in 2 testing blocks and at the right loudspeaker in the other 2 testing blocks. In addition, a background broadband noise was continuously delivered from each of the two horizontal loudspeakers as the masker with the inter-masker onset delay between the 2 loudspeakers being either +1 msec (left leading) in 2 testing blocks or -1 msec (right leading) in the other 2 testing blocks. Thus, a single fused masker image was perceived at the left loudspeaker in 2 testing blocks and at the right loudspeaker in the other 2 blocks. Consequently, there were 4 ( $2 \times 2$ ) combinations of perceived location relations between the prepulse and the masker covered by these 4 testing blocks: 2 blocks with perceptual separation (when the prepulse and masker had different leading position) and 2 blocks with perceptual co-location (when the prepulse and the masker had the same leading position). Trials in each testing block were presented with the inter-trial interval around 30 sec (varied

between 25 and 30 sec). The 4 testing blocks were presented in an order of Latin Design for each of the rat groups.

On PND 81, all the rats underwent both the fear-conditioning and the conditioning-control manipulations. For a rat, the conditioning stimulus (CS) was one of the prepulse stimuli (either lower- or higher-frequency prepulse) delivered by each of the two horizontal loudspeakers with a left/right-leading balance, and the US was a 6-mA rectangular-pulse footshock with the duration of 3 msec provided by a Grass S-88 stimulator (Grass, Quincy, MA, USA, following Zou et al., 2007). For all of the rat groups, fear conditioning was established by 20 temporally synchronized (paired) combination of the low- or high-frequency prepulse with the footshock (footshock started 3 ms before the prepulse ending and co-terminated with the gap, following Zou et al., 2007). Conditioning-control manipulation was conducted by 20 combination between the high- or low-frequency prepulse and the footshock with temporally random (unpaired) prepulse-footshock intervals within the time window of 30 s. The orders of conducting conditioning and conditioning-control manipulations were balanced across rats.

On PND 82 (24 hrs after the fear-conditioning/conditioning-control manipulations), PPI after conditioning (AC) was measured with the same procedure as on PND 80.

On PND 83, all the rats underwent the manipulation of fear extinction, during which the CS was presented every 30 sec (without pairing with the footshock US) for 60 times (3 blocks with 20 presentations in each block and a 10-min inter-block interval).

On PND 84 (24 hrs after the extinction manipulation), PPI after extinction (AE) was measured with the same procedure as used on PND 80.

Phase II and Phase III of the testing procedure was conducted from PND 85 to 87 and from PND 89 to 91, respectively. All the rats were intraperitoneally injected with clozapine

(5 mg/kg) in Phase II and with vehicle in Phase III through the 3 consecutive days in each of the 2 phases. The following procedures were carried out 30 minutes after injection:

On PND 85 and PND 89, testing before fear conditioning (BC) was conducted with the same procedure as used on PND 80.

On PND 86 and PND 90, both the fear-conditioning and conditioning-control manipulations were conducted with the same procedure as used on PND 81.

On PND 87 and PND 91, PPI after conditioning (AC) was measured with the same procedure as used on PND 80.

On PND 88, all the rats underwent the manipulation of fear extinction using the procedures as used on PND 83.

**Data Analyses**

The value of PPI was calculated with the generally used formula:

$$\text{PPI (\%)} = (\text{amplitude to startling noise alone} - \text{amplitude to startling noise preceded by prepulse}) / (\text{amplitude to startling noise alone}) \times 100\%.$$

Analyses of variance (ANOVAs) were conducted using SPSS 15.0 software. The null-hypothesis rejection level was set at 0.05.

**RESULTS**

**Baseline Startle**

The upper panel of Figure 2 illustrates the group-mean amplitudes of the startle reflex in the 3 groups at the BC procedure stage in each of the 3 testing phases. Obviously, baseline startle of the SAL-IR and that of MK-SR groups were larger than that of the SAL-SR group. Clozapine (injected in Phase II) generally reduced baseline startle.

----- Insert Figure 2 about here -----

A 3 (phase stage) by 3 (group) mixed repeated-measures ANOVA showed that the main effect of phase stage was significant ( $F_{(2,16)} = 186.356, p < 0.01$ ), the main effect of group was significant ( $F_{(2,16)} = 16.092, p < 0.01$ ), and the interaction between the factors was significant ( $F_{(4,14)} = 3.390, p < 0.05$ ). Post hoc bonferroni tests showed that (1) in Phase I, baseline startle of the SAL-SR group was significantly lower than that of the SAL-IR group ( $p < 0.01$ ) and that of the MK-SR group ( $p < 0.05$ ); (2) in Phase II (with clozapine injection), there was no group difference (all  $p > 0.05$ ); (3) in Phase III, the baseline startle amplitude of the SAL-SR group was significantly lower than that of the SAL-IR group ( $p < 0.01$ ) and that of the MK-SR group ( $p < 0.05$ ). Also, separate within-subject repeated-measures ANOVAs showed that for each group across testing phases, baseline startle was significantly reduced in Phase II (SAL-SR:  $F_{(2,16)} = 26.959, p < 0.01$ ; SAL-IR:  $F_{(2,16)} = 41.981, p < 0.01$ ; MK-SR:  $F_{(2,16)} = 52.336, p < 0.01$ ).

### Baseline PPI

Under the prepulse/masker co-location condition, baseline PPI was compared between groups at Procedure Stage BC across testing phases. The bottom panel of Figure 2 shows that in Phase I, baseline PPI of the SAL-IR group was lower than those of the SAL-SR and MK-SR groups. After clozapine was applied in Phase II, baseline PPI was increased in both the SAL-IR and the MK-SR groups. These PPI enhancements did not occur in Phase III (with vehicle injection).

A 3 (phase) by 3 (group) mixed repeated-measures ANOVA showed that the main effect of phase stage was significant ( $F_{(2,16)} = 10.178, p < 0.01$ ), the main effect of group was

significant ( $F_{(2,16)} = 8.211, p < 0.01$ ), and the interaction was significant ( $F_{(4,14)} = 3.444, p < 0.05$ ). Post hoc bonferroni tests showed that (1) in Phase I, baseline PPI of the SAL-IR group, but not that of the MK-SR group, was significantly lower than that of the SAL-SR group ( $p < 0.05$ ); (2) in Phase II, baseline PPI of the MK-SR group was significantly larger than those of the SAL-SR group ( $p < 0.01$ ) and SAL-IR group ( $p < 0.01$ ), and (3) in Phase III, baseline PPI of the SAL-IR group, but not that of the MK-SR group, was significantly lower than that of the SAL-SR ( $p < 0.05$ ).

Moreover, within-subject repeated-measures ANOVAs for each group showed that the effect of phase stage was significant in both the SAL-IR group ( $F_{(2,16)} = 3.332, p < 0.05$ ) and the MK-SR group ( $F_{(2,16)} = 15.367, p < 0.01$ ), but not in the SAL-SR group ( $F_{(2,16)} = 0.039, p > 0.05$ ). For these 2 neonatally disrupted groups, post hoc bonferroni tests indicated that the baseline PPI value was significantly larger in Phase II (with clozapine injection) than both that in Phase I (SAL-IR,  $p < 0.05$ ; MK-SR,  $p < 0.01$ ) and that in Phase III (SAL-IR,  $p < 0.05$ ; MK-SR,  $p < 0.01$ ).

**Attentional Modulations of PPI in Phase I**

Figure 3 shows both the effect of fear conditioning of a prepulse on PPI and the effect of perceptual separation either between the noise masker and the conditioned prepulse (CS+, left panels) or between the noise masker and the conditioning-control prepulse (CS-, right panels) on PPI. Before fear conditioning (BC) in each group, since the mean PPI magnitude induced by the low-frequency prepulse was not significantly different from that induced by the high-frequency prepulse (all  $p > 0.05$ , paired  $t$  tests), PPI values induced by the two prepulse types were combined.

----- Insert Figure 3 about here -----

### *SAL-SR group*

For the SAL-SR group, as shown by the top panels of Figure 3, after a prepulse was fear conditioned, PPI induced by this CS+, but not that by the CS-, was enhanced. Also, relative to the perceptual co-location condition, perceived spatial separation between the noise masker and the CS+, but not the CS-, further enhanced PPI. Finally, the extinction manipulation eliminated both the fear-conditioning effect and the perceptual-separation effect.

For PPI induced by the CS+, a 3 (procedure stage: BC, AC, AE) by 2 (separation type: separation, co-location) within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(2,16)} = 12.450, p < 0.01$ ), the main effect of separation type was significant ( $F_{(1,17)} = 5.579, p < 0.05$ ), and the interaction was significant ( $F_{(2,16)} = 6.074, p < 0.05$ ). Post hoc bonferroni tests showed that (1) at Procedure Stage BC, PPI was not significantly affected by separation type ( $p > 0.05$ ); (2) at Procedure Stage AC, PPI was significantly enhanced ( $p < 0.05$ ), and the effect of separation type became significant ( $t_{(17)} = 4.432, p < 0.01$ ); (3) at Procedure Stage AE, the PPI levels under all the conditions reduced to the ones at Procedure Stage BC ( $p > 0.05$ ), including that the effect of perceptual separation became not significant ( $p > 0.05$ ). For PPI induced by the CS-, another 3 by 2 within-subject repeated-measures ANOVA showed that neither each the main effect (procedure stage:  $F_{(2,16)} = 0.380, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.531, p > 0.05$ ) nor the interaction was significant ( $F_{(2,16)} = 1.101, p > 0.05$ ).

### *SAL-IR group*

For PPI induced by either the CS+ or the CS- in the SAL-IR group (Figure 3, middle panels), neither the fear-conditioning effect nor the perceptual-separation effect on PPI was present. Three by 2 within-subject repeated-measures ANOVAs showed that neither the main effects

nor the interaction were significant on PPI induced by either the CS+ (procedure stage:  $F_{(2,16)} = 0.138, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.243, p > 0.05$ ; interaction:  $F_{(2,16)} = 0.486, p > 0.05$ ) or the CS- (procedure stage:  $F_{(2,16)} = 0.188, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.202, p > 0.05$ ; interaction:  $F_{(2,16)} = 0.176, p > 0.05$ ).

***MK-SR group***

For the MK-SR group (Figure 3, bottom panels), fear conditioning enhanced both PPI induced by the CS+ and PPI induced by the CS-, without exhibiting the prepulse-feature specificity.

For PPI induced by the CS+, a 3 by 2 within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(2,16)} = 7.190, p < 0.05$ ), but both the main effect of separation type ( $F_{(1,17)} = 0.515, p > 0.05$ ) and the interaction ( $F_{(2,16)} = 0.346, p > 0.05$ ) were not significant. Post hoc bonferroni tests showed that (1) at Procedure Stage AC, PPI was significantly enhanced ( $p < 0.05$ ); (2) at Procedure Stage AE, PPI levels reduced to the ones at Procedure Stage BC ( $p > 0.05$ ); (3) the perceptual separation effect was not significant at each of the procedure stages ( $p > 0.05$ ).

For PPI induced by the CS-, another 3 by 2 within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(2,16)} = 6.102, p < 0.05$ ), but both the main effect of separation type ( $F_{(1,17)} = 1.415, p > 0.05$ ) and the interaction ( $F_{(2,16)} = 0.220, p > 0.05$ ) were not significant. Post hoc bonferroni tests showed that (1) at Procedure Stage AC, PPI was significantly enhanced ( $p < 0.05$ ); (2) at Procedure Stage AE, all the PPI levels reduced to the ones at Procedure Stage BC ( $p > 0.05$ ); (3) the perceptual separation effect was not significant at each of the procedure stages ( $p > 0.05$ ).



## Attentional Modulations of PPI in Phase II with Clozapine Injection

### *SAL-SR group*

For the SAL-SR group (Figure 4, top panels), clozapine did not abolish the fear-conditioning-induced PPI enhancement that exhibited the prepulse specificity. However, the perceptual-separation-induced PPI enhancement disappeared.

For PPI induced by the CS+, a 2 (procedure stage: BC, AC) by 2 (separation type) within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(1,17)} = 5.787, p < 0.05$ ), but neither the main effect of separation type ( $F_{(1,17)} = 0.873, p > 0.05$ ) nor the interaction was significant ( $F_{(1,17)} = 1.247, p > 0.05$ ).

For PPI induced by the CS-, neither the two main effects (procedure stage:  $F_{(1,17)} = 0.732, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.119, p > 0.05$ ) nor the interaction ( $F_{(1,17)} = 0.236, p > 0.05$ ) was significant.

----- Insert Figure 4 about here -----

### *SAL-IR group*

For the SAL-IR group (Figure 4, middle panels), clozapine restored the fear conditioning-induced PPI enhancement when the prepulse was either the CS+ or CS-, without showing the prepulse-feature specificity. Moreover, clozapine had no restoring effects on the modulation of PPI by perceptual separation.

For PPI induced by the CS+, a 2 by 2 within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(1,17)} = 7.654, p < 0.05$ ), but neither the main effect of separation type ( $F_{(1,17)} = 0.693, p > 0.05$ ) nor the interaction ( $F_{(1,17)} = 0.039, p > 0.05$ ) was significant. For PPI induced by the CS-, another 2 by 2 within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant

( $F_{(1,17)} = 4.450, p < 0.05$ ), but neither the main effect of separation type ( $F_{(1,17)} = 0.006, p > 0.05$ ) nor the interaction ( $F_{(1,17)} = 1.227, p > 0.05$ ) was significant.

***MK-SR group***

For the MK-SR group (Figure 4, bottom panels), neither the fear conditioning effect nor the perceptual separation effect on PPI exhibited in Phase II. The 2 by 2 within-subject repeated-measures ANOVAs showed that neither the two main effects nor the interaction was significant for either PPI induced by the CS+ (procedure stage:  $F_{(1,17)} = 0.490, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.001, p > 0.05$ ; interaction:  $F_{(1,17)} = 1.472, p > 0.05$ ) or PPI induced by the CS- (procedure stage:  $F_{(1,17)} = 1.500, p > 0.05$ ; separation type:  $F_{(1,17)} = 1.424, p > 0.05$ ; interaction:  $F_{(1,17)} = 0.002, p > 0.05$ ).

**Attentional Modulations of PPI in Phase III with Vehicle Injection**

***SAL-SR group***

For the SAL-SR group (Figure 5, top panels), similar to the results in Phase I, PPI induced by the CS+, but not that by the CS-, was enhanced after fear conditioning. Also, perceived spatial separation between the masker and the CS+, but not that between the masker and the CS-, further enhanced PPI.

For PPI induced by the CS+, a 2 by 2 within-subject repeated-measures ANOVA showed that both the main effect of procedure stage ( $F_{(1,17)} = 12.234, p < 0.01$ ) and the main effect of separation type were significant ( $F_{(1,17)} = 6.250, p < 0.05$ ), and the interaction was significant ( $F_{(1,17)} = 10.603, p < 0.01$ ). Post hoc bonferroni tests confirmed that (1) at Procedure Stage BC, PPI was not affected by separation type ( $p > 0.05$ ); (2) at Procedure Stage AC, PPI was significantly enhanced ( $p < 0.05$ ), and the effect of separation type became significant ( $t_{(17)} = 5.240, p < 0.01$ ).

For PPI induced by the CS-, another 2 by 2 within-subject repeated-measures ANOVA showed that neither the two main effects (procedure stage:  $F_{(1,17)} = 0.000, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.000, p > 0.05$ ) nor the interaction ( $F_{(1,17)} = 1.313, p > 0.05$ ) was significant.

----- Insert Figure 5 about here -----

### ***SAL-IR group***

For the SAL-IR group (Figure 5, middle panels), neither the fear conditioning nor the perceptual spatial separation enhanced PPI. A 2 by 2 within-subject repeated-measures ANOVAs showed that neither the main effects of procedure stage and separation type nor the interaction were significant for either PPI induced by the CS+ (left panel, procedure stage:  $F_{(1,17)} = 0.280, p > 0.05$ ; separation type:  $F_{(1,17)} = 1.588, p > 0.05$ ; interaction:  $F_{(1,17)} = 0.189, p > 0.05$ ) or PPI induced by the CS- (right panel, procedure stage:  $F_{(1,17)} = 0.072, p > 0.05$ ; separation type:  $F_{(1,17)} = 0.142, p > 0.05$ ; interaction:  $F_{(1,17)} = 0.101, p > 0.05$ ).

### ***MK-SR group***

For the MK-SR group (Figure 5, bottom panels), fear conditioning a prepulse enhanced PPI induced by either the CS+ or CS-. No perceptual separation effect on PPI exhibited.

For PPI induced by the CS+, a 2 by 2 within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(1,17)} = 4.608, p < 0.05$ ), but the main effect of separation type ( $F_{(1,17)} = 1.214, p > 0.05$ ) and the interaction ( $F_{(1,17)} = 0.876, p > 0.05$ ) were not significant. Post hoc bonferroni tests showed that after fear conditioning, PPI was significantly increased ( $p < 0.05$ ), but there was no significant perceptual separation effect ( $p > 0.05$ ).

For PPI induced by the CS-, another 2 by 2 within-subject repeated-measures ANOVA showed that the main effect of procedure stage was significant ( $F_{(1,17)} = 5.265, p < 0.05$ ), but the main effect of separation type ( $F_{(1,17)} = 1.764, p > 0.05$ ) and the interaction ( $F_{(1,17)} = 1.475, p > 0.05$ ) were not significant. Post hoc bonferroni tests showed that after fear conditioning, PPI value was significantly increased ( $p < 0.05$ ), but there was no significant perceptual separation effect ( $p > 0.05$ ).

**DISCUSSIONS**

Although the neural circuit mediating PPI resides in the brainstem (Li and Frost, 2000), PPI can be top-down modulated by higher-order cognitive process (for a review see Li et al. 2009). In this study, the 3 rat groups demonstrated different responding patterns not only in baseline startle and baseline PPI, but also in fear-conditioning modulation of PPI and perceptual-separation modulation of PPI.

**Baseline Startle and Baseline PPI**

The results of this study showed that adult rats with either isolation rearing or neonatal exposure to MK-801 exhibited larger startle amplitudes than normally reared rats. Thus, the two neonatal disruptions (isolation rearing and neonatal exposure to MK-801) generally cause adult rats more stressful than socially reared rats and consequently enhance startle (Du et al. 2009, 2010).

Moreover, rats with isolation rearing, but not rats with the MK-801 exposure, exhibited a significant reduction of baseline PPI, consistent with previous reports (Du et al. 2009, 2010). It should note that the effects of neonatal exposure to MK-801 on baseline PPI may be both strain (Lim et al. 2012a; 2012b; Uehara et al. 2009, 2010, 2014) and sex (Lim et al. 2012a;

2012b; Nozari et al. 2015) dependent. The absence of effects of the neonatal exposure to MK-801 on baseline PPI may specifically occur in Sprague-Dawley male rats.

### **Modulation of PPI by Fear Conditioning of the Prepulse**

The main purpose of this study was to examine whether neonatal exposure to MK-801 affects attentional modulations of PPI. The results showed that socially-reared rats, either with or without neonatal exposure to MK-801, exhibited the fear-conditioning-induced PPI enhancement. Moreover, the prepulse-feature specificity of this PPI enhancement maintained well only in normally reared rats and disappeared in neonatally MK-801-treated rats. Thus, although neonatal exposure to MK-801 does not abolish the fear-conditioning-induced PPI enhancement, it affects the processing of prepulse features. Also, consistent with previous reports (Du et al. 2009, 2010), in this study, rats with isolation rearing did not exhibit the fear-conditioning-induced PPI enhancement.

Based on previous studies, the mGluR5 is involved in fear-conditioning-induced PPI modulation because administration of MPEP (an mGluR5 antagonist) abolishes this type of modulation (Lei et al., 2014; Zou et al., 2007). On the other hand, Du et al. have discovered that blocking the lateral nucleus of amygdala abolishes fear-conditioning-induced PPI enhancement in normally reared rats (Du et al., 2011). Moreover, some previous studies have shown that isolation rearing not only reduces functions of both NMDARs and mGluR5 during maturation (Arndt et al. 2015) but also impairs cellular signaling pathways of neurons in the basolateral amygdala (Karkhanis et al. 2015) and particularly decreases expression of the immediate-early gene Egr-1 that encodes synaptic-plasticity-related signaling in the amygdala (Okada et al. 2014). Thus, in the future it is important to further examine whether the isolation-rearing-induced impairment of neural processes of the amygdala is the major cause of the absence of the fear-conditioning-induced PPI enhancement.

On the other hand, according to the results of this study, early exposure to MK-801 restores the fear-conditioning-induced PPI enhancement but causes a loss of the prepulse-feature specificity. Previous studies have shown that although the lateral amygdala (LA) is critical to the conditioning-induced PPI enhancement of PPI (Du et al. 2011), neonatal exposure to MK-801 does not substantially impair the functions of the amygdala (Uehara et al. 2014).

Why does neonatal MK-801 intervention specifically impair frequency discrimination after fear conditioning? It has been known that most auditory neurons in the amygdala do not demonstrate selective sensitivity to sound frequencies (Bordi and LeDoux 1992), suggesting that the amygdala itself contributes little to the prepulse-feature specificity in the fear-conditioning-induced PPI enhancement. However, LA receives axonal projections from not only the auditory thalamus (e.g. media geniculate nucleus, MGN) but also the auditory association cortex (AAC/TE3). The LA itself is not sufficient to discriminate the sound frequencies before fear conditioning (Du et al. 2012), but neurons in the primary auditory cortex (TE1) has selective responses to tones with various frequencies from 1 to 40 kHz (Kelly and Sally 1988) and plays a role in modulating amygdala activities *via* AAC (Romanski and LeDoux 1993). Thus, normally fear conditioning a sound enhances functional connectivity between the LA and TE1 *via* AAC (TE3), leading to that the conditioning-enhanced responses of the LA to the sound have the sound-feature specificity (Du et al. 2012), which may be mediated by GABA<sub>B</sub> receptors (Huang et al. 2005).

Moreover, the medial prefrontal cortex (mPFC) receives inputs from AAC and modulates principal neurons in the LA (for a discussion, see Huang et al. 2005). Pharmacological reductions in mPFC GABA transmission impairs discriminative aversive conditioning (Piantadosi and Floresco 2014). In addition, the dysfunction of GABA transmissions in the amygdalo-hippocampal system leads to fear generalization (Bergado-Acosta et al. 2008). It

has been found that neonatal exposure to MK-801 significantly reduces the number of parvalbumin-positive GABAergic neurons in the mPFC (Uehara et al. 2012; Abekawa et al. 2007; Li et al. 2015) and changes the ratio of vesicular glutamate and GABA transporters in the hippocampus during adulthood (Li et al. 2015). In people with schizophrenia, the dysfunctional emotional processing is associated with the abnormal GABAergic activity in the dorsal medial prefrontal cortex (Taylor et al., 2014) and the visual cortex (Tso et al., 2015). Thus, another important issue for future investigation is whether impaired GABAergic transmissions in the mPFC and/or hippocampus following neonatal exposure to MK-801 causes the loss of prepulse-feature specificity in fear-conditioning-induced PPI enhancement.

In people with schizophrenia, the processing the emotional information is impaired (Swart et al. 2013) and the impairment involves an impaired regulation of GABA transmission in the circuits including the prefrontal cortex (Piantadosi and Floresco 2014). Thus, the neonatal MK-801 animal model is useful for further investigating the relationship between impaired attentional modulations of PPI, impaired sound-feature processing, and impaired GABAergic transmissions.

### **Modulation of PPI by Perceived Spatial Separation**

In agreement with previous studies (Du et al. 2009, 2010, 2011), the results of this study showed that in normally reared rats, perceived spatial separation between the noise masker and the conditioned prepulse (CS+), but not the prepulse used for conditioning control (CS-), further enhanced PPI. In humans, this perceived spatial separation of the target and a masker facilitates the listener's selective attention to target signals and improves recognition of the target (Freyman et al. 1999; Li et al. 2004; Zheng et al. 2015). Interestingly, in both rats and humans, the perceptual separation-induced behavioral improvements are normally based on the PPC (Du et al. 2011; Zheng et al. 2015), which also has axonal connections with the

prefrontal cortex (Reep et al. 1994) and plays a role in guiding actions towards spatial goals (Andersen and Cui 2009).

The results of this study also showed that the perceptual separation-induced PPI enhancement did not occur in both isolation-reared rats and neonatally MK-801-treated rats, indicating that the perceptual-separation-induced PPI enhancement is more vulnerable to neonatal interruptions than fear-conditioning-induced PPI enhancement.

For isolation-reared rats, the failure of perceptual separation in enhancing PPI may be related to their impaired attentional shift (McLean et al. 2010) or impaired spatial attention to the conditioned prepulse (Du et al. 2009). As mentioned above, Isolation rearing leads to a broad range of structural and functional abnormalities (including dopamine, glutamate, serotonin) (Leng et al. 2004; Melendez et al. 2004), which may be associated with the deficits in the attentional modulations of PPI.

The ability to perceptually integrate the prepulse stimuli from two separated spatial locations requires calculating the correlation (similarity) of the acoustic features of the leading and lagging sounds (Li et al. 2009). In this study, since neonatally MK-801-treated rats showed deficits in discriminating prepulse frequency during fear conditioning, the feature-processing deficits may affect the perceptual integration of the prepulse signals, leading to the lack of separation-induced PPI enhancement in rats with neonatal MK-801 exposure.

Moreover, neonatal MK-801 exposure causes some neurotoxicity effects in the hypothalamus, amygdala (Otoya et al. 1997), hippocampus (Gilland et al. 1997; Otoya et al. 1997), and posterior brain regions (Gilland et al. 1997). Some studies have shown that in rats MK-801 disrupts spatial processing at both the perceptual and cognitive levels, including both failure to locate the target location (Terry et al. 2011) and failure to detect spatial



novelty (Uziel et al. 1998). Thus, the lack of separation-induced PPI enhancement in rats with neonatal MK-801 exposure may also be associated with impaired spatial ability.

People with schizophrenia exhibit impaired selective attention (Damasio et al. 2013; Strauss et al. 2011) and recognition of target speech under masking conditions (Zheng et al. 2015). The neonatal MK-801 animal models are useful for improving animal models to investigate the spatial attention deficits of schizophrenia, especially under adverse listening conditions.

### **Effects of Clozapine on Attentional Modulations of PPI**

In this study, clozapine showed various effects on the four measures (baseline startle, baseline PPI, fear-conditioning-induced PPI enhancement, perceptual-separation-induced PPI enhancement) in different rat groups.

First of all, since clozapine generally decreases the baseline-startle amplitude (this study, Frogley et al. 2012), it has a sedative effect.

In addition, according to the results of this study, clozapine applied during adulthood enhances baseline PPI in both isolation-reared rats and neonatally MK-801 treated rats, but not in normally reared rats, indicating a sensitivity of baseline PPI to clozapine in neonatally disrupted rats (Le Pen and Moreau 2002).

Moreover, the results of this study showed that clozapine had various effects on the fear-conditioning-mediated modulation of PPI in different rat groups: Clozapine did not affect the conditioning-induced PPI enhancement in the normally reared rats, but abolished this PPI enhancement in neonatally MK-801-treated rats. More interestingly, clozapine restored the fear-conditioning-induced PPI enhancement in isolation-reared rats with a loss of the prepulse-feature specificity. It has been well known that the affinities of clozapine at various receptors play an important role in ameliorating schizophrenia-related neurochemical

abnormalities and cognitive/behavioral deficiencies (Sebban et al., 2002; Spagna et al., 2015). For example, clozapine can reduce isolation-rearing-induced deficits in reversal learning in the new-strategy-acquisition phase (Li et al., 2007). Since reversal learning largely depends on the function of the mPFC (Li and Shao, 1998), certain isolation-rearing-induced neurotransmission impairments in the mPFC may be partially ameliorated by clozapine. Moreover, clozapine also reduces isolation-rearing-induced deficits in PPI (Heidbreder et al., 2001; Le et al., 2002; Möller et al., 2011; 2013), object recognition memory (Möller et al., 2013), social interactive behavior (Möller et al., 2013; Möller et al., 2011), and emotional behavior (Koike et al., 2009). Up to the date, it is not clear whether certain isolation-rearing induced neurotransmission impairments in the amygdala can be ameliorated by clozapine. On the other hand, clozapine acts as an antagonist for the GABA<sub>A</sub> receptor (see review O'Connor and O'Shea, 2015). It has been reported that clozapine both decreases extracellular GABA level within the PFC (Bourdelaïs et al., 1994) and reduces the density of GABA<sub>A</sub> receptors in the hippocampus (Farnbach-Pralong et al., 1998). Thus, the loss of prepulse feature in the emotional PPI enhancement after a clozapine treatment in isolation-reared rats may be associated with the impaired GABA transmission in PFC and/or hippocampus. These different effects of clozapine on the fear-conditioning-induced PPI modulation between isolation-reared rats, neonatally MK-801-treated rats, and normally-reared rats further support the view that isolation rearing and neonatal exposure to MK-801 cause different neural impairments.

Finally, the results of this study showed that clozapine impaired normally-reared rats' spatial attention to the conditioned prepulse, because it abolished the perceptual-separation-induced PPI enhancement. Also, clozapine did not restore the perceptual-separation-induced PPI enhancement in either isolation reared rats or neonatally MK-801-treated rats. The results of this study are in agreement with previous studies showing that clozapine does not restore

impairment of spatial memory in neonatally MK-801 administered rats (Liu et al. 2014). Thus, there is a need to develop new antipsychotics that do not hurt the perceptual-separation-induced PPI enhancement.

In people with schizophrenia, clozapine impairs higher-order cognitive processes (Houthoofd et al. 2008) and does not improve spatial memory (Meltzer and McGurk 1999). The specific deficits in perceptual-separation-induced PPI enhancement in neonatally MK-801 treated rats will be useful for establishing a new animal model not only for studying mechanisms underlying schizophrenia but also for examining the efficacy of potential antipsychotics.

## CONCLUSIONS

Using the paradigms of attentional modulation of PPI, we compared the effects of isolation-rearing and those of the neonatal exposure to MK-801 on the following 4 measures: baseline startle, baseline PPI, enhancement of PPI by fear conditioning of the prepulse, and enhancement of PPI by spatial perceptual separation between the conditioned prepulse and a noise masker. The following findings were obtained:

At the level of baseline startle, both isolation rearing and neonatal MK-801 exposure enhance the startle amplitude.

At the level of baseline PPI, only isolation rearing has an impairing effect.

At the levels of fear-conditioning modulation and spatial attentional modulation of PPI, normally reared rats, with the remarkable prepulse-feature specificity, exhibit both the PPI enhancement by fear conditioning and that by perceptual spatial separation. Isolation rearing abolishes these two types of enhancement. Neonatal exposure to MK-801 only abolishes the separation-induced PPI enhancements and reserves fear-conditioning-induced PPI enhancement with a loss of the prepulse-feature specificity. Thus, the early-life MK-801

treatment specifically impairs spatial attention which is associated with failure in discriminating prepulse features, while isolation rearing induces a much wider range of attentional deficits.

Clozapine generally reduces the startle amplitude, and enhances baseline PPI in neonatally-disrupted rats. More importantly, for the first time, this study reveals that clozapine is a “double-edged sword” in treating attentional deficits: it restores fear-conditioning-induced attentional modulation of PPI with a loss of the prepulse-feature specificity in isolation-reared rats but not neonatally MK-801 treated rats, and impairs spatial attention modulation of PPI in normally reared rats.

In conclusion, compared to isolation rearing, neonatal exposure to MK-801 causes different central impairments and can be used for improving animal models of schizophrenia.

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## FIGURE LEGENDS

Figure 1. Experimental timelines for both treatments and testing procedures for the three rat groups. Phase I refers to the testing procedures without clozapine/vehicle treatments. Phase II and Phase II refers to the procedures with clozapine and vehicle treatment, respectively. BC, before conditioning; AC, after conditioning; AE, after extinction.

Figure 2. The upper panel shows the group-mean amplitudes of the startle reflex to the startling noise alone in each of the three rat groups before the fear conditioning/conditioning control manipulations were introduced in Phases I, II and III. The magnitude of the startle reflex was calculated with the device-scale unit. The lower panel shows the amplitudes of PPI in each of the three rat groups before the fear conditioning/conditioning control manipulations in Phase I, II and III. The PPI values were obtained at the procedure stage BC under the perceived prepulse-masker co-location condition.  $*p < 0.05$ ,  $**p < 0.01$ .

Figure 3. Magnitudes of PPI induced by the conditioned prepulse (CS+, left panel) and the conditioning-controlled prepulse (CS-, right panel) in each of the three rat groups at different procedure stages in Phase I. White bars represent the condition when the prepulse was perceptually co-located with the noise masker, while diagonal bars represent the condition when the prepulse was perceptually separated from the noise masker. CS+, the prepulse was fear-conditioned; CS-, the prepulse was conditioning-controlled; SAL-SR, socially-reared rats with neonatal saline injection; SAL-IR, isolation-reared rats with neonatal saline injection; MK-SR, socially-reared rats with neonatal MK-801 injection.  $*p < 0.05$ ,  $**p < 0.01$ .

Figure 4. Magnitudes of PPI induced by the conditioned prepulse (CS+, left panel) and conditioning-controlled prepulse (CS-, right panel) in each of the three rat groups at different procedure stages in Phase II (after clozapine treatment). \* $p < 0.05$ , \*\*  $p < 0.01$ .

Figure 5. Amplitudes of PPI induced by the conditioned prepulse (CS+, left panel) and conditioning-controlled prepulse (CS-, right panel) in each of the three rat groups at different procedure stages in Phase III (after vehicle treatment). \* $p < 0.05$ , \*\*  $p < 0.01$

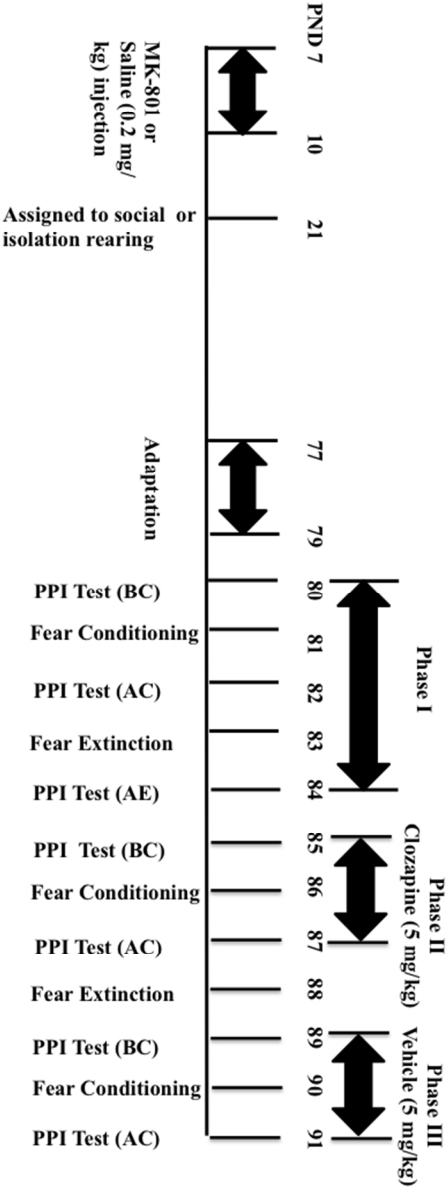


Figure 1. Experimental timelines for both treatments and testing procedures for the three rat groups. Phase I refers to the testing procedures without clozapine/vehicle treatments. Phase II and Phase II refers to the procedures with clozapine and vehicle treatment, respectively. BC, before conditioning; AC, after conditioning; AE, after extinction.

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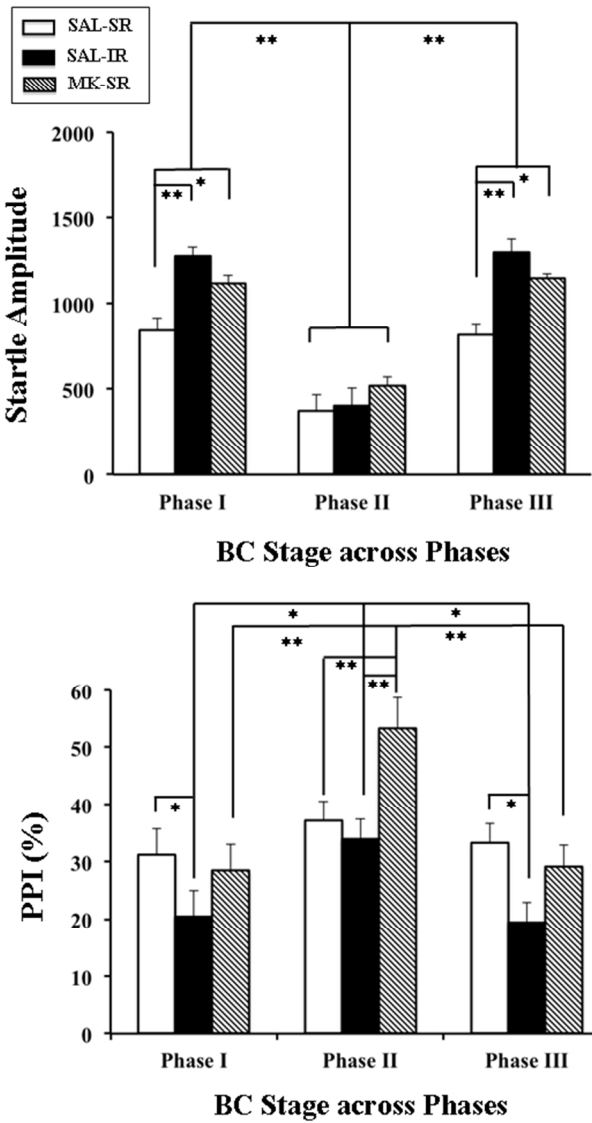


Figure 2. The upper panel shows the group-mean amplitudes of the startling reflex to the startling noise alone in each of the three rat groups before the fear conditioning/conditioning control manipulations were introduced in Phases I, II and III. The magnitude of the startle reflex was calculated with the device-scale unit. The lower panel shows the amplitudes of PPI in each of the three rat groups before the fear conditioning/conditioning control manipulations in Phase I, II and III. The PPI values were obtained at the procedure stage BC under the perceived prepulse-masker co-location condition. \*p < 0.05, \*\* p < 0.01.

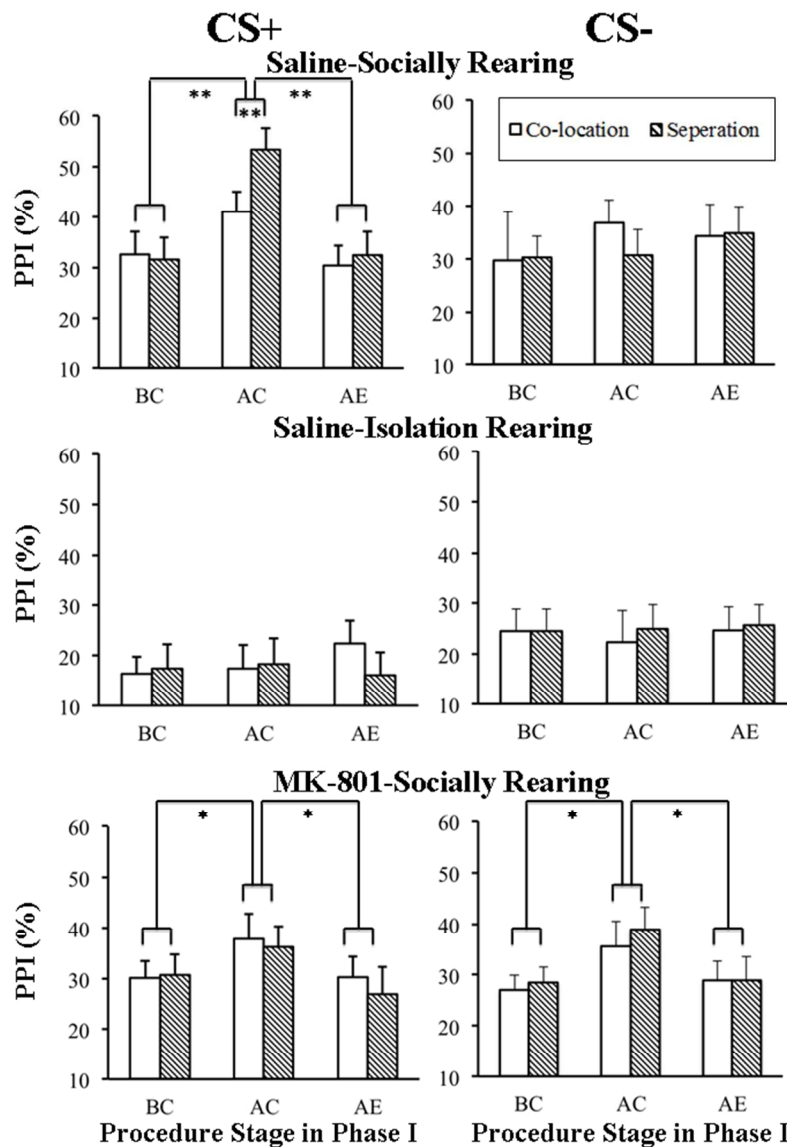


Figure 3. Magnitudes of PPI induced by the conditioned prepulse (CS+, left panel) and the conditioning-controlled prepulse (CS-, right panel) in each of the three rat groups at different procedure stages in Phase I. White bars represent the condition when the prepulse was perceptually co-located with the noise masker, while diagonal bars represent the condition when the prepulse was perceptually separated from the noise masker. CS+, the prepulse was fear-conditioned; CS-, the prepulse was conditioning-controlled; SAL-SR, socially-reared rats with neonatal saline injection; SAL-IR, isolation-reared rats with neonatal saline injection; MK-SR, socially-reared rats with neonatal MK-801 injection. \* $p < 0.05$ , \*\*  $p < 0.01$ .

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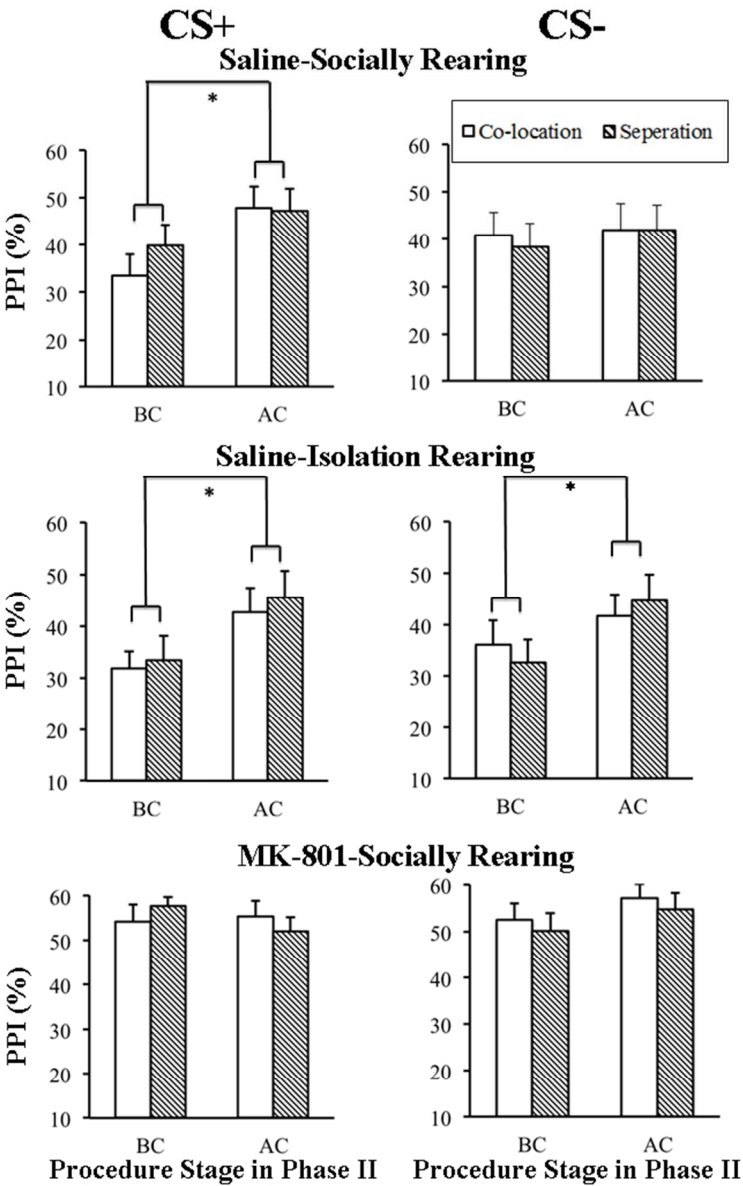


Figure 4. Magnitudes of PPI induced by the conditioned prepulse (CS+, left panel) and conditioning-controlled prepulse (CS-, right panel) in each of the three rat groups at different procedure stages in Phase II (after clozapine treatment). \*p < 0.05, \*\* p < 0.01.

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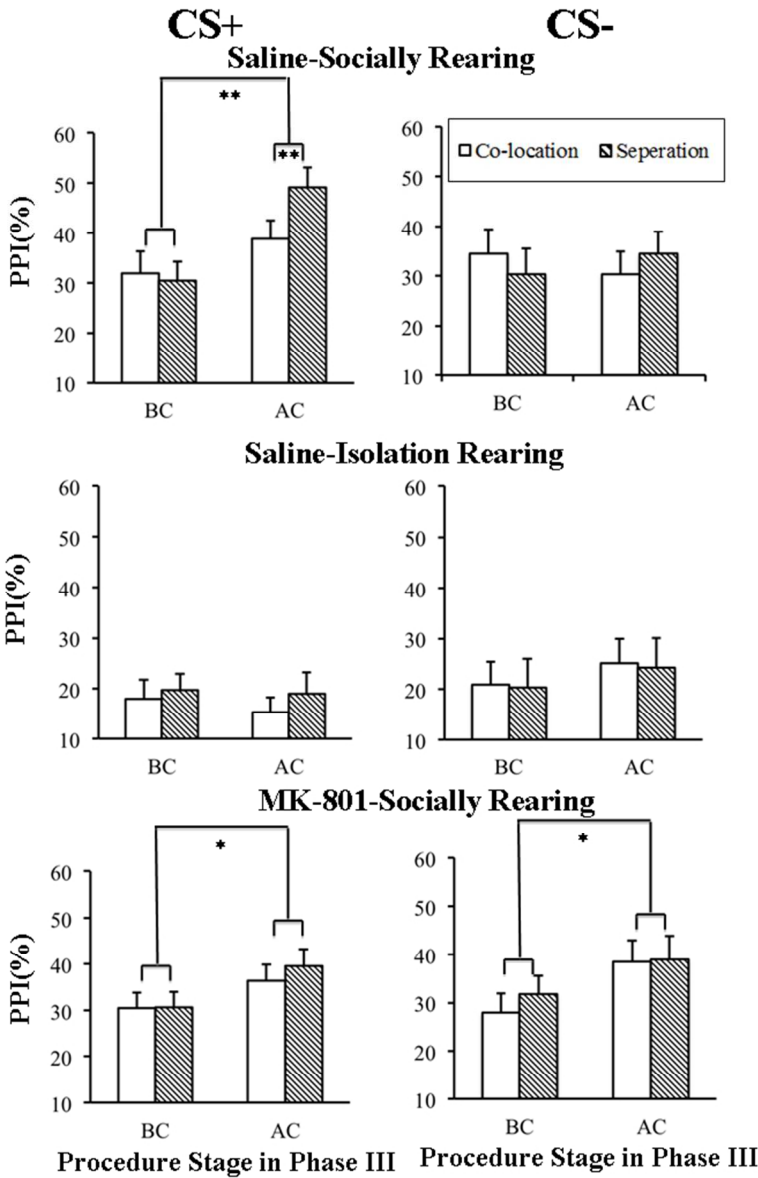


Figure 5. Amplitudes of PPI induced by the conditioned prepulse (CS+, left panel) and conditioning-controlled prepulse (CS-, right panel) in each of the three rat groups at different procedure stages in Phase III (after vehicle treatment). \*p < 0.05, \*\* p < 0.01  
190x275mm (96 x 96 DPI)