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ORIGINAL INVESTIGATION

Contribution of the prefrontal cortex and basolateral amygdala to behavioral decision-making under reward/punishment conflict

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Abstract

Rationale Control of reward-seeking behavior under conditions of punishment is an important function for survival.

Objectives and methods We designed a task in which rats could choose to either press a lever and obtain a food pellet accompanied by a footshock or refrain from pressing the lever to avoid footshock, in response to tone presentation. In the task, footshock intensity steadily increased, and the task was terminated when the lever press probability reached < 25% (last intensity). Rats were trained until the last intensity was stable. Subsequently, we investigated the effects of the pharmacological inactivation of the ventromedial prefrontal cortex (vmPFC), lateral orbitofrontal cortex (lOFC), and basolateral amygdala (BLA) on task performance.

Results Bilateral inactivation of the vmPFC, lOFC, and BLA did not alter lever press responses at the early stage of the task. The number of lever presses increased following vmPFC and BLA inactivation but decreased following lOFC inactivation during the later stage of the task. The last intensity was elevated by vmPFC or BLA inactivation but lowered by lOFC inactivation. Disconnection of the vmPFC-BLA pathway induced behavioral alterations that were similar to vmPFC or BLA inactivation. Inactivation of any regions did not alter footshock sensitivity and anxiety levels.

Conclusions Our results demonstrate a strong role of the vmPFC and BLA and their interactions in reward restraint to avoid punishment and a prominent role of the lOFC in reward-seeking under reward/punishment conflict situations.

Keywords Basolateral amygdala · Lateral orbitofrontal cortex · Punishment · Reward · Ventromedial prefrontal cortex

Introduction

Reward-seeking and punishment-avoidance are the fundamental principles of behavior and decision-making. Reward-seeking behavior is frequently accompanied by aversive punishment such as social punishment, health impairment, or encountering predators in the wild, which results in mental conflict, while choosing action (reward-seeking while receiving punishment) or restraint (reward restraint to avoid punishment). In such situations, behavior is selected based on the evaluation of the outcome value, and an overestimation of reward may be associated with addictive behaviors (Holden 2001; Bubier and Drabick 2009; Ahmed 2018), while prediction of excessive aversion and attenuation of reward-seeking are observed in patients with anxiety disorders and depression (Muscat et al. 1990; Willner et al. 1992; Sugiyama and Kanba 2001).

A reward-seeking choice is easily selected, if the punishment is considered tolerable by the individual but is inevitably suppressed under conditions of strong punishment (Pelloux et al. 2018; Datta et al. 2018). In contrast, we hesitate to make behavioral choices if the intensity of punishment borders between tolerable and intolerable. It is possible that the brain processes underlying behavioral choices differ, depending on the intensity of punishment. Moreover, reward restraint caused by punishment is an important aspect of several diseases, but the neural basis governing punishment tolerance under the reward condition is unclear. We have addressed these questions in the present study.

The prefrontal cortex (PFC) is a core brain region involved in decision-making. The subregions of the PFC, such as the

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ventromedial PFC (vmPFC) and lateral orbitofrontal cortex (lOFC), are also involved in the reward and aversion processes (Schoenbaum et al. 1998; Lacroix et al. 2000; Ishikawa et al. 2008; Burgos-Robles et al. 2013; Sangha et al. 2014; Izquierdo 2017). In contrast, the basolateral amygdala (BLA) is associated with the representation of emotion (Ishikawa et al. 2015; Schoenbaum et al. 1998; Shabel et al. 2011). It probably acts as a source of reward and aversion-related information to the PFC, during decision-making. Reward/punishment-guided behaviors are investigated by using a footshock as punishment. The vmPFC, lOFC, and BLA were observed to be involved in these behaviors under a variety of experimental situations (Bravo-Rivera et al. 2014; Jean-Richard-Dit-Bressel and McNally 2016; Orsini et al. 2015, 2018; Piantadosi et al. 2017). However, little is known about the role of these brain regions in behaviors under conditions in which the outcome reward and footshock are certain for every behavioral choice. Moreover, the vmPFC and lOFC are reciprocally connected with the BLA (Ishikawa and Nakamura 2003; Likhtik et al. 2005; Hoover and Vertes 2007; Price 2007; Ishikawa et al. 2015), but the role of PFC-BLA interactions in reward/footshock-guided behavior is poorly understood.

We designed an action/no-action conflict task, in which rats were required to choose an action (lever press) or inaction (no lever press) in response to a tone stimulus. The subsequent outcomes of action included a reward (a food pellet) and punishment (footshock). During the task, the intensity of the footshock steadily increased and the task was terminated when the lever press probability reached < 25% (last footshock intensity). We investigated whether the last footshock intensity was altered by inactivation of the vmPFC, lOFC, and BLA following treatment with the $GABA_A$ and $GABA_B$ agonists, muscimol and baclofen (M/B). We also investigated the effects of inactivation on lever press probability, under various footshock-intensity conditions. Moreover, we examined whether inactivation altered reward-seeking behavior under the no footshock condition, and its effects on sensitivity to footshock and anxiety levels. Finally, we examined the effect of PFC-BLA disconnection on task performance.

Materials and methods

Subjects

Sprague–Dawley male rats (Clea Japan, Japan) were individually housed at 22 °C with a 12-h light–dark cycle (lights were on from 8:00 A.M to 8:00 P.M.). Rats were allowed at least 2 weeks of ad libitum food (MF, Oriental Yeast, Japan), followed by 1 week of restricted food before training. During training and experiments, rats were maintained at 80–90 % of their ad libitum weight. Water was available continuously during whole experiments. Rats at the age of 15–25 weeks were used in experiments. All experiments and training were conducted during light cycle. The experiments were reviewed and approved by the Yamaguchi University Graduate School of Medicine Committee of Ethics on Animal Experiments. All manipulations and protocols were performed according to the Guidelines for Animal Experiments at Yamaguchi University Graduate School of Medicine and in accordance with Japanese Federal Law (no. 105), Notification (no. 6) of the Japanese Government, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications no. 80-23), revised in 1996.

Surgery

Animals were anesthetized with sodium pentobarbitone (50 mg/kg, intraperitoneal) and placed in a stereotaxic apparatus. Bilateral 26 guide stainless steel guide cannulas (Plastics One Inc., VA, USA) were implanted. The 33 gauge injector cannulas would extend 1 mm below the end of the guides and reach the vmPFC, vlPFC. Target regions of the injectors relative to bregma were as follows (Paxinos and Watson 1998): vmPFC (anterior (A) from bregma, 3.2 mm; lateral (L) to midline, 0.65 mm; ventral (V) from bregma, 4.8 mm), and lOFC (A, 3.4 mm; L, 2.7 mm; V, 4.2 mm), BLA (A, − 3.0 mm; L, 4.8 mm; V, 8.5 mm). The dummy cannulas were inserted into guide cannulas, and the ends of the dummy cannulas were flush with the end of the guide cannulas. Animals were allowed to recover from surgery at least 7 days before retraining of the task. Rats were excluded for analysis if cannulas did not target the region.

Behavioral training and procedure of the action/no-action conflict task

Experiments were conducted in an operant chamber (30 × 30 × 30 cm), with a grid floor enclosed within a sound and light-insulated box. A lever was placed on the wall of the operant chamber, which activated a pellet dispenser (Med Associates Inc., VT, USA) to deliver a 45-mg food pellet (Bio-Serv., NJ, USA), to a pellet dish located 7 cm above the floor. The speaker was located 20 cm behind and 20 cm above the top of the chamber wall equipped with the lever. All experimental events were controlled by the BAITC system (LABTEC, Japan). All events including tone presentation, lever press, and delivery of a food pellet and footshock were recorded in real-time by the BAITC system to the computer using a Spike2 data acquisition system.

The 3-stage training protocol involved food restriction, before surgical cannula implantation. During stage 1, the animals were introduced to the chamber, where pressing the lever triggered the delivery of a food pellet. After the rats learned to

obtain 100 pellets within 60 min, they progressed to stage 2 of training, in which the rats were trained to press a lever in response to a tone (900 Hz) to receive a pellet. Delivery was set at a fixed-ratio (FR) of 1 pellet per lever press. The tone continued for a maximum of 10 s. It stopped when the rat pressed the lever. The tones were presented according to a variable interval schedule, with an average interval of 20 s. The animals were trained until the probability of pressing a lever in response to the tone averaged > 95%. Training was conducted for at least 2 weeks. Daily training was completed when the number of lever press responses reached 100. The maximum training time was set to 90 min, even if the lever presses were fewer than 100 (daily training time was about 30–90 min). Seven days after surgery, the rats underwent retraining for at least 7 days. They were subsequently trained for the action/no-action conflict task (stage 3). At the start of the action/no-action conflict task, 1 pellet was delivered after a tone-evoked lever press (phase 0), without the electric footshock. Exposure to footshock was initiated after the lever had been pressed 5 consecutive times, in response to the tone during phase 0. The intensity of the footshock was increased with each consecutive testing phase, as per the following schedule: phase 1, 0.04 mA; phase 2, 0.06 mA; phase 3, 0.08 mA; phase 4, 0.10 mA; phase 5, 0.12 mA; phase 6, 0.16 mA; phase 7, 0.20 mA; phase 8, 0.24 mA; phase 9, 0.32 mA; phase 10, 0.40 mA; and phase 11, 0.48 mA. Between phases 0 and 3, the phase was increased when 5 lever press responses were obtained at each phase. For all subsequent phases, the phase was increased when 10 lever press responses were obtained at each phase. The maximum number of tone presentations within each phase was set to 40, and the task was terminated if 10 lever press responses were not obtained within a phase (last phase). The maximum duration of the tone was 10 s. It was terminated when the rat pressed the lever. The tones were presented according to a variable interval schedule, with an average interval of 20 s. Task training was conducted for at least 6 days, until the last footshock intensity (last phase) was stable, before inactivation. Although the time required to achieve final-phase stabilization differed for each rat, the stability—once achieved—was maintained. However, the last phase was not always the same, even after stabilization. Sometimes, the last phase was one phase higher or lower (+ or − one phase) than the stabilized phase. Therefore, we considered that last-phase stabilization was achieved, when the last phase remained the same for at least 2 consecutive days, after at least 4 days of training or when the last phase in 3 consecutive days was in the order of: (stabilized last phase) → (+ or − one phase) → (stabilized last phase) after at least 4 days of training.

The rats stopped pressing the lever at the end of the task because of footshock and did not usually press the lever at the following day of training. Therefore, the shock was turned off immediately after task termination, and the lever was manually pressed with a plastic stick by a researcher in the presence of the rat, to indicate that the lever press response would result in the delivery of a pellet, but not a footshock. The rats began to press the lever immediately after the indication that the footshock was turned off. During early training, several manual lever presses were required. However, once the rats learned the association between the manual lever press and the absence of the footshock, only a few (1–5 times) lever presses were required.

The rats usually stayed near the lever during the early phases of the task, even in the absence of the tone. In contrast, during the later phases, they often moved away from the lever, when the tone was absent, but approached the lever, when the tone was present. They repeated the action of placing and release their forelimbs on the lever alternately or kept their forelimbs on the lever, during tone presentation. These behaviors indicate that the rats learned the tone-reward-footshock association, and the tone functioned as a trigger for the rats to make a decision. We used the tone as a cue to measure the latency of decision-making and lever press probability at each phase, in this task.

Behavioral tests

Guide cannulas were implanted as described above. The elevated plus-maze test was carried out at least 7 days after recovery from surgery.

Elevated plus-maze test

The elevated plus-maze and open-field tests were conducted according to the method described by Ishikawa et al. (2014, 2015). The apparatus comprised two opposite open (50 × 11 cm) and closed arms, with 40-cm walls, was elevated to 85 cm above the floor and was dimly illuminated. The arms were connected by a central square (11 × 11 cm). Each rat was placed on the central platform and was allowed to explore the maze for 5 min. The time spent in the open and closed arms, and numbers of arm entries, was measured.

Open-field test

The open-field test was performed 2 days after the elevated plus-maze test. The open field was a circular surface of 60-cm diameter, which was divided into 19 spaces by lines and was enclosed by 50-cm walls. Each animal was individually placed at the center of the field and allowed to freely explore the field for 5 min. The numbers of line crossings (locomotor activity), rearing, and grooming behaviors were manually counted. The proportion of locomotor activity at the center of the field was analyzed.

Lever pressing behavior to obtain one pellet under no footshock condition

One day after the open-field test, the animals were trained to press the lever according to training stages 1 and 2 as described above. They were trained until the probability of pressing a lever in response to the tone averaged > 95%. The experimental conditions were the same as those for the action/no-action conflict task except that a footshock was not delivered.

Flinch-jump test

Flinch/jump responses were evaluated in a chamber box (30 cm × 30 cm × 30 cm) equipped with a stainless-steel grid floor, connected to a shock generator. After a 3-min period of habituation, footshocks (1 s) were sequentially applied every 10 s, with a stepwise increase in intensity (0.05 mA, 0.05–0.7 mA range). The flinch threshold was defined as the lowest shock intensity that elicited a detectable escape response (flinching). The jump threshold was defined as the lowest shock intensity that elicited the simultaneous removal of at least 3 paws from the grid.

All behavioral tests were recorded using a video camera and were subsequently analyzed. The animals used in the behavioral tests including the elevated plus-maze, open-field, and lever press tests to obtain a pellet under the no footshock condition, and flinch-jump test were not the same animals used in the experiment with the action/no-action conflict task.

Inactivation experiments

Bilateral injections of the drug solution or vehicle (saline) were administered to the vmPFC or lOFC or BLA of the rats prior to task performance or behavioral testing. The drug solution consisted of a mixture of M/B dissolved in saline. Each drug was delivered to each hemisphere in a volume of 0.5 μl and a dose of 50 ng. The dummy cannulas were removed for each injection and 30-G injector cannulas were inserted bilaterally into the guides for injection. After an interval of 1 min, the entire volume (0.5 μl) was injected over 2 min. After a 1-min post-injection waiting period, the dummy cannulas were replaced and the rat was immediately placed into the behavioral chamber. For the vmPFC-BLA disconnection experiment, M/B or saline was injected unilaterally into the vmPFC and bilaterally into the BLA (in the contralateral and ipsilateral hemispheres).

The elevated plus-maze and open-field tests are widely used for evaluating behavioral responses in a novel environment, and the flinch-jump test is usually conducted in a novel test box for animals. Since the behaviors of animals who have already undergone these tests should not be evaluated again, we used a between-subject design for these tests. The rats were accustomed to the box used in the action/no-action conflict task or the box used for estimation of lever pressing behavior to obtain a pellet under the no footshock condition. Moreover, the rats were trained and experienced these tasks several times before the inactivation experiment. The effects of the vehicle and drug are frequently compared within the same individual, in this type of experiment (Ishikawa et al. 2008; Churchwell et al. 2009; St Onge and Floresco 2010). Thus, we used the within-subjects design for these experiments.

For the action/no-action conflict task, the cannulas in some animals were bilaterally injected in one of the 3 regions: vmPFC, lOFC, or BLA. Half of these animals received saline injection first, followed by the M/B injection. A reverse order of administration was used in the other half. In contrast, the cannulas of both, the vmPFC and BLA, in rats involved in the disconnection experiment were injected bilaterally in and the effects of bilateral, ipsilateral, and contralateral inactivation of each region (vmPFC or BLA) were investigated. In these animals (n = 7), the effect of inactivation of a single region was investigated first, and the vmPFC of 3 rats were injected first, while the BLA was injected first in the other 4 rats. The order of administration of saline and MB was counterbalanced (saline first, 4 rats; MB first, 3 rats). For the disconnection experiment, ipsilateral injections were performed first, followed by contralateral injections. We examined the effects of inactivation of all injection area patterns per individual rat (ipsilateral, right vmPFC + right BLA, left vmPFC + left BLA; contralateral, right vmPFC + left BLA; left vmPFC + right BLA). The hemisphere in which the drug was injected first was randomized (first ipsilateral injection, 5 right vmPFC + right BLA, 2 left vmPFC + left BLA; first contralateral injection, 3 right vmPFC + left BLA, 4 left vmPFC + right BLA). Since two patterns of each ipsilateral and contralateral injection existed, 2 data readings were averaged and used as the value for that individual. There was an interval of at least 2 days between injections, for all experiments.

Since the animals used in the disconnection experiments received 6 injections at each injection-site, over the course of the experiment, it is possible that the cumulative tissue damage could have affected behavioral performance. We investigated whether there was a difference in last footshock intensity between first and last saline injection over the course of experiment, to determine if cumulative tissue damage affected behavioral performance. No difference was observed between the footshock intensities (vmPFC vs contralateral vmPFC/BLA, $z = -1.60$, $p = 0.11$; BLA vs contralateral vmPFC/BLA, $z = -0.33$, $p = 0.74$). Moreover, rats used in both, one-region injection and disconnection experiments, received a total of 12 injections (6 saline, 6 MB) into the vmPFC or/and BLA. Therefore, we also investigated whether the possibility of the main effect of time on the last footshock intensity after saline injection. However, the Friedman test revealed that

there was no significant temporal change over the course of the experiment ($X^2 = 4.06$, $p = 0.26$).

For the elevated plus-maze, open-field, and flinch-jump tests, the animals were divided into 2 groups (saline group and MB group). The rats in each group received the respective injections. For the lever press test, half the rats in each group received the saline injection first, followed by M/B injection. A reverse order was used for the other half.

Histology

At the end of experiments, animals were deeply anesthetized with sodium pentobarbitone (100 mg/kg, i.p.) and were immediately perfused transcardially with a solution of 0.1-M phosphate buffer containing 4% paraformaldehyde. The brain was removed and then post-fixed with the same paraformaldehyde solution and dehydrated in 10–30% sucrose solution. Coronal sections (40 μm thickness) were stained with hematoxylin and eosin. The locations of cannulas in the brain were identified with the aid of the stereotaxic atlas (Paxinos and Watson 1998).

For the action/no-action conflict task, 36 rats underwent cannulation. Twenty-six rats received bilateral cannulation in the vmPFC ($n = 4$), lOFC ($n = 12$), or BLA ($n = 10$). The cannulas were misplaced in 7 rats (1 vmPFC, 3 lOFC, 4 BLA). These animals were excluded from all analyses. Ten rats underwent bilateral cannulation in both, the vmPFC and BLA. Three rats were excluded from the disconnection experiment. Because 2 of the 3 rats excluded from disconnection experiments had misplaced only the vmPFC cannulas, the effect of injection into the BLA was analyzed for these 2 rats. In summary, the number of animals analyzed for the effect of bilateral injection into the vmPFC, BLA, and lOFC was 10, 15, and 9, respectively, and 7 rats were analyzed for disconnection effect.

For behavioral tests including the elevated plus-maze, open-field, flinch-jump tests, and the lever press (to obtain a pellet) under no footshock condition, 72 rats underwent bilateral cannulation (25 vmPFC, 25 vlOFC, 22 BLA). Ten rats were excluded owing to misplaced cannulas (2 vmPFC, 2 lOFC, 5 BLA).

We excluded 10 rats from the analysis of estimation of lever press response to obtain a pellet under the no footshock condition (4 mPFC, 1 lOFC, 5 BLA), because the pellet was not delivered properly, owing to technical issues.

Statistical analysis

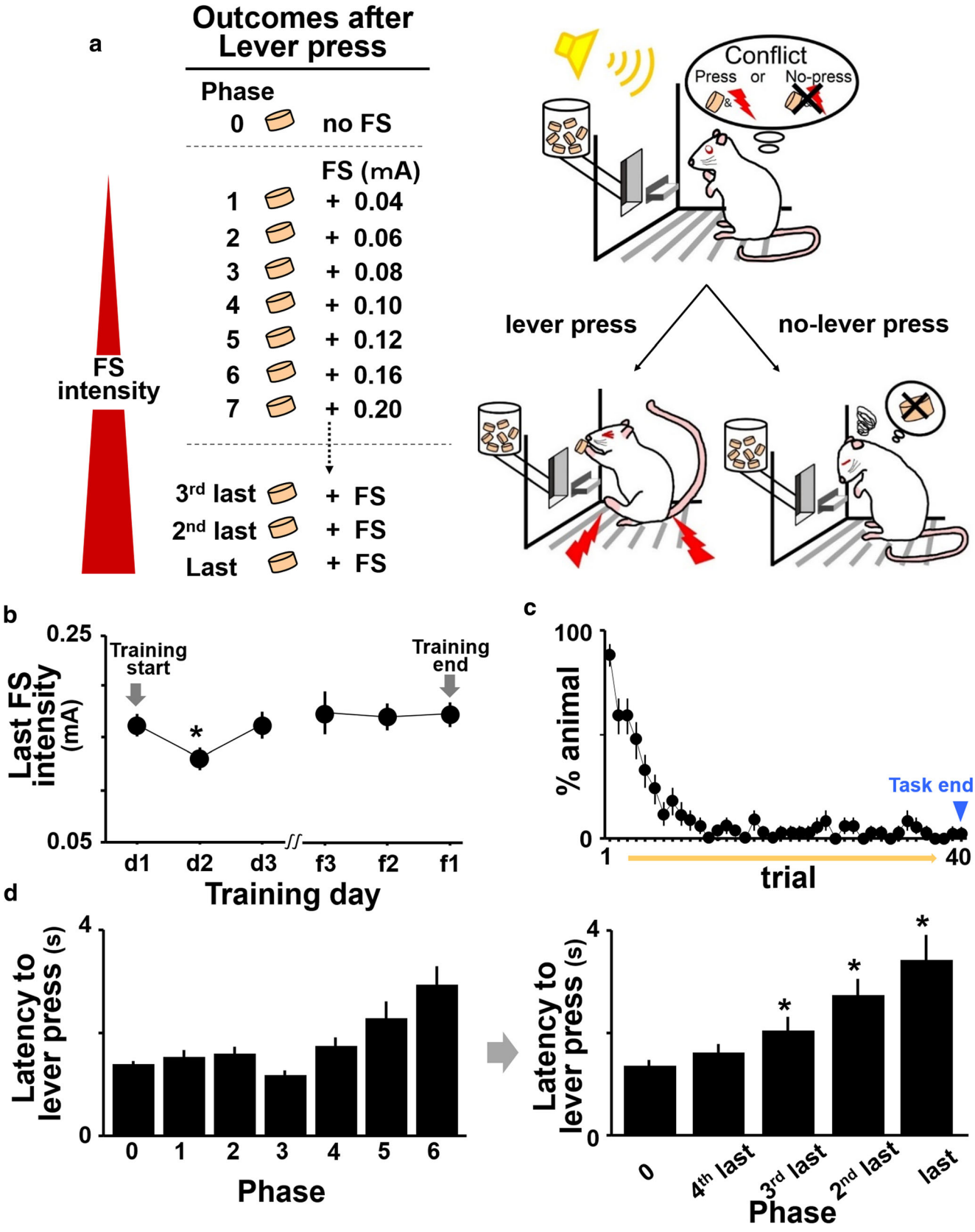
Data analysis was performed with SPSS or StatView software. The normality of distributions was assessed with the Kolmogorov–Smirnov or Shapiro–Wilk test. The last footshock intensity during training days and the latency to lever pressing during each phase was compared with the Friedman test followed by the Wilcoxon signed-rank test. Last-phase footshock intensity, lever press probability in the first 3 and last 3 phases, and the number of lever presses in the no footshock condition were compared between saline and M/B injections with the Wilcoxon signed-rank test. Latency to lever presses in the no footshock condition was compared between saline and M/B injections with a two-tailed paired t test. Behaviors in the elevated plus-maze and the open-field tests were compared with an unpaired t test. Flinch/jump thresholds were analyzed with the Mann–Whitney U test. All data are expressed as the mean ± SEM. Differences were considered statistically significant when $p < 0.05$. Data collection and analysis were not performed blind due to the conditions of the experiments. Animal randomization was not necessary.

Results

Effects of inactivation on the choice made during the action/no-action conflict task

Figure 1 a shows the procedure involved in the action/no-action conflict task. Task training was conducted for at least 6 days until the last footshock intensity was stable. The last footshock intensity on the first few days was unstable ($n = 26$, $X^2(2) = 10.97$, $p = 0.0041$), and last footshock intensity on day 2 was significantly lower than that on day 1 ($z = -2.23$, $p = 0.026$, Fig. 1b) and 3 ($z = -2.60$, $p = 0.0093$). The last footshock intensity gradually stabilized and there was no significant difference in last footshock intensity among the final 3 days of training ($X^2(2) = 0.66$, $p = 0.72$). Moreover, lever press latency became longer as the phase level increased, and the latencies during the last three phases were significantly longer than those during phase 0 ($X^2(4) = 46.60$, $p < 0.001$, Friedman test; 3rd last, $z = -2.92$, $p = 0.0036$; 2nd last, $z = -3.46$, $p = 0.0005$; last, $z = -4.49$, $p < 0.0001$, Wilcoxon signed-rank test; Fig. 1d). During the last phase of the task, several animals pressed the lever during the early trials, but few animals pressed the lever during the later trials (Fig. 1c). Cannula placements are depicted in Fig. 2.

Figure 3 shows the lever press probability at each phase (Fig. 3a), including the lever press probability for the first 3 (Fig. 3b) and LAST 3 phases (Fig. 3c), as well as the footshock intensity during the last phase (Fig. 3d) after saline or M/B injection. Since the footshock intensity of the last phase after M/B injection differed from those after saline injection, the lever press probability during the last phase after M/B injection could not simply be compared with those after saline injection. The lever press probability during the same phase should be compared. Therefore, the last 3 phases of saline injection were defined as the "LAST" three phases,



and lever press probability at each phase was compared between saline and M/B injections (Fig. 3c).

Bilateral M/B injection into the vmPFC and BLA shifted the percent lever press-phase curve to the right (Fig. 3a),

◀ **Fig. 1** . Procedures for the action/no-action conflict task. **a** In the inactivation experiment, the task started at phase 0, in which a pellet but no footshock (FS) was delivered after a lever is pressed during tone presentation. Subsequently, a pellet and FS were delivered after the lever press response. To move on to the next phase, the lever needed to be pressed 5 times, in response to a tone, in phases 0–3 and 10 times in phases 4 and above. Maximum tone presentation within a phase was 40, and the task was terminated if the lever press failed to reach 10 responses within a phase (lase phase). **b** The last footshock intensity in the action/no-action conflict task during training days (d) 1–3, and the final 3 training days (f 3–1) **c** Probability that an animal would press the lever in each trial during the last phase. **d** Latency to lever press at each phase and during the last 4 phases Data are shown as mean ± SEM. *$p < 0.05$

elevated the lever press probability during the LAST phase (vmPFC, $z = -2.71$, $p = 0.0068$; BLA, $z = -3.41$, $p = 0.0007$; Fig. 3c), and raised the footshock intensity during the last testing phase (vmPFC, $n = 10$ animals, $z = -2.61$, $p = 0.0091$; BLA, $n = 15$ animals, $z = -3.42$, $p = 0.0006$; Fig. 3d), when compared with saline injection. In contrast, the same curve shifted to the left (Fig. 3b), lever press probability decreased during the LAST three phases (LAST three, $z = -2.37$, $p = 0.018$; LAST two, $z = -2.53$, $p = 0.011$; LAST, $z = -2.08$, $p = 0.037$; Fig. 3c), and the last footshock intensity was lowered ($n = 9$ animals, $z = -2.53$, $p = 0.012$, Fig. 3d) by M/B injection into the lOFC. There was no influence on lever press probability during the first three phases following inactivation of any region (Fig. 3b).

Effects of inactivation on lever pressing behavior to obtain one pellet under no footshock condition

The probability of lever presses in the first 3 phases was approximately 100%. This rate was not altered by inactivation. This indicates that inactivation had no influence on the motivation for reward in the action/no-action conflict task. However, the vmPFC, lOFC, and BLA are reportedly associated with reward-seeking behaviors. It is possible that the contribution of these regions to reward-seeking behavior was different in the footshock and no footshock conditions. To address this question, we investigated the effects of inactivation on reward-seeking under the no footshock condition in animals, who had experienced neither a footshock nor the action/no-action conflict task. In this experiment, rats obtained a pellet but did not receive a footshock after a lever press in response to a tone stimulus. The tone used in this experiment was the same as that used in the action/no-action conflict task. In this test, a total of 200 tones were used, and the number and latency to lever presses were compared between the groups, following saline or M/B injection into the PFC and BLA.

Figure 4 a shows the lever press probability for each of 20 trials after saline or M/B injection. Saline-injected rats exhibited a high probability of lever pressing throughout the test (Fig. 4a). M/B injection into the vmPFC or lOFC decreased the number of lever presses (vmPFC, $n = 19$ animals, $z = -3.19$, $p = 0.0014$; lOFC, $n = 21$ animals, $z = -3.98$, $p < 0.0001$; Fig. 4b) and increased the latency to lever press compared with saline injection (vmPFC, $t(18) = -2.76$, $p = 0.013$; lOFC, $t(20) = -7.63$, $p < 0.0001$). The number of lever presses after M/B injection into the lOFC was fewer than those after M/B injection into the vmPFC ($z = -4.32$, $p < 0.0001$). Animals that received M/B injection into the lOFC showed a longer latency to lever press than those who received an M/B injection into the vmPFC ($t(38) = -5.93$, $p < 0.0001$). M/B injection into the BLA decreased the number of lever presses but this was not significant ($n = 12$ animals, $z = -1.90$, $p = 0.057$), and had no influence on the latency to lever pressing ($t(11) = -0.95$, $p = 0.36$).

Effects of inactivation on aversion-related behaviors

We also investigated whether inactivation of the PFC and BLA altered sensitivity to footshock or basal anxiety levels. Footshock sensitivity was examined with the flinch-jump test, and we compared the threshold of flinching and jumping between saline and M/B injections. The effects of inactivation on anxiety levels and general emotional behaviors were examined with the elevated plus-maze and open-field tests.

The flinching and jumping thresholds were not altered by M/B injection into the vmPFC (saline, $n = 12$ animals; M/B, $n = 11$ animals; flinch, $z = -0.23$, $p = 0.82$), lOFC (saline, $n = 11$ animals; M/B, $n = 11$ animals; flinch, $z = -0.23$, $p = 0.82$; jump, $z = -0.84$, $p = 0.40$; Fig. 5a) or BLA (saline, $n = 9$ animals; M/B, $n = 8$ animals; flinch, $z = -1.25$, $p = 0.21$; jump, $z = -0.84$, $p = 0.40$; Fig. 5a) in the flinch-jump test.

In the elevated plus-maze test, no significant differences were observed in the number of entries into the open (vmPFC, $t(21) = -0.55$, $p = 0.59$; lOFC, $t(20) = 0.95$, $p = 0.35$; BLA, $t(15) = 0.66$, $p = 0.52$) or closed arms (vmPFC, $t(21) = 1.59$, $p = 0.13$; lOFC $t(20) = -1.24$, $p = 0.23$; BLA, $t(15) = -0.61$, $p = 0.55$) between animals injected with saline and those injected with M/B. There was also no significant difference in the duration of time spent in the open (vmPFC, $t(21) = -0.48$, $p = 0.63$; lOFC, $t(20) = 0.92$, $p = 0.37$; BLA, $t(15) = 1.59$, $p = 0.13$) or closed arms (vmPFC, $t(21) = -0.25$, $p = 0.81$; lOFC $t(20) = -1.33$, $p = 0.20$; BLA, $t(15) = -0.61$, $p = 0.55$) between animals injected with saline and those injected with M/B (Fig. 5b).

M/B injection did not alter locomotor activity (vmPFC, $t(21) = 0.31$, $p = 0.76$; lOFC, $t(20) = 0.31$, $p = 0.76$; BLA, $t(15) = -0.24$, $p = 0.81$), the percentage of locomotion in the central area (vmPFC, $t(21) = -1.10$, $p = 0.29$; lOFC, $t(20) = 0.61$, $p = 0.55$; BLA, $t(15) = -0.58$, $p = 0.57$), rearing (vmPFC, $t(21) = 0.32$, $p = 0.75$; lOFC, $t(20) = -1.57$, $p = 0.13$; BLA, $t(15) = 0.27$, $p = 0.79$), or grooming behaviors (vmPFC, $t(21) = -1.10$, $p = 0.29$; lOFC, $t(20) = 0.97$, $p = 0.34$; BLA, $t(15) = 0.00$) in the open-field test (Fig. 5c).

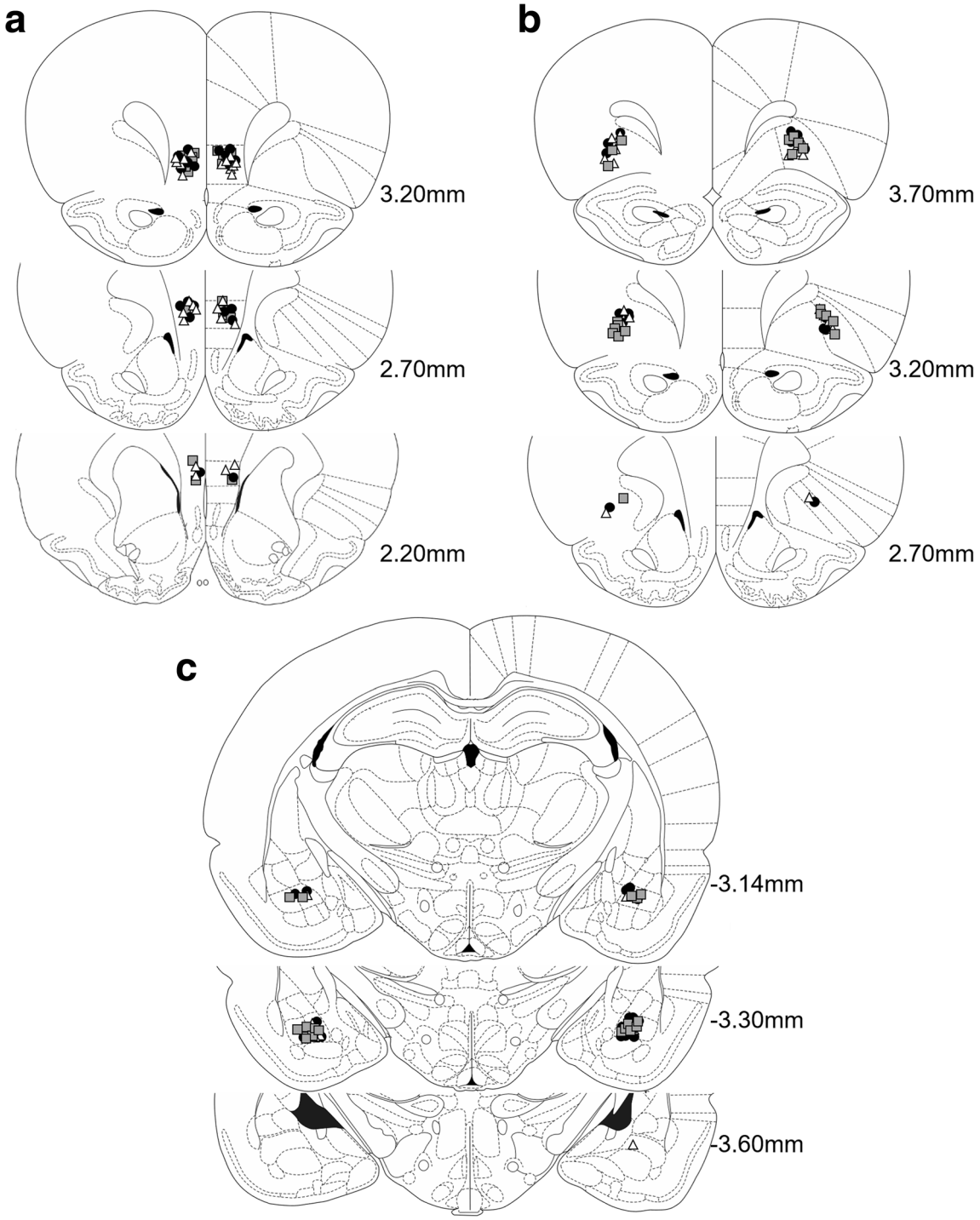


Fig. 2. **a**–**c** Cannula tip placement in the vmPFC, lOFC, and BLA. Black circles indicate injection for the action/no-action conflict task White triangles and gray squares indicate saline and M/B injections, respectively, for behavioral tests including flinch-jump, elevated plus-maze, open-field, and lever press tests. vmPFC ventromedial prefrontal cortex, lOFC lateral orbitofrontal cortex, BLA basolateral amygdala, M/B muscimol/baclofen

Effects of vmPFC-BLA disconnection on behavioral choices in the action/no-action conflict task

Ipsilateral and contralateral inactivation of the vmPFC and BLA shifted the percent lever press-footshock intensity curve to the right (Fig. 6a) and elevated the lever press probability during the LAST phase ($n = 7$ animals; ipsilateral, $z = -2.02$, $p = 0.043$; contralateral, $z = -2.37$, $p = 0.018$; Fig. 6c). To compare lever press probability between saline and M/B injection during the later stages of the same phase, we defined

the last 3 phases after saline injection as the "LAST" 3 phases (Fig. 6c). The probability of lever press in the LAST phase following contralateral inactivation was greater than that following ipsilateral inactivation, but not significantly ($z = -1.80$, $p = 0.072$, Fig. 6d). To compare lever press probability between ipsilateral and contralateral M/B injection at the same phase, the last phase of ipsilateral M/B injection was defined as the "LAST" phase (Fig. 6d). Footshock intensity in the last phase was elevated by contralateral inactivation ($z = -2.39$, $p = 0.017$, Fig. 6e), but not by ipsilateral inactivation ($z = -1.73$, $p = 0.084$, Fig. 6e), compared with that in the corresponding saline injection group. Contralateral and ipsilateral inactivation had no influence on the choice of behavior during the first 3 phases (Fig.6b).

Discussion

Footshock intensity during the last phase of the action/no-action conflict task was elevated following bilateral vmPFC or BLA inactivation and was lowered by bilateral lOFC inactivation. Inactivation of the vmPFC or BLA elevated the probability of lever press in the LAST phase, while lOFC inactivation lowered the lever press probability during the LAST 3 phases. In contrast, lever press probability during the early phases of the task was not altered by bilateral inactivation of any brain region. Sensitivity to footshock and anxiety were also not altered by inactivation of any region. Disconnection of the vmPFC-BLA pathway induced behavioral alterations similar to those induced by vmPFC or BLA inactivation. Our results demonstrated that the vmPFC and BLA regulate decision-making under conflict, while interacting with each other, and the role of the lOFC is the opposite of that of the vmPFC and BLA.

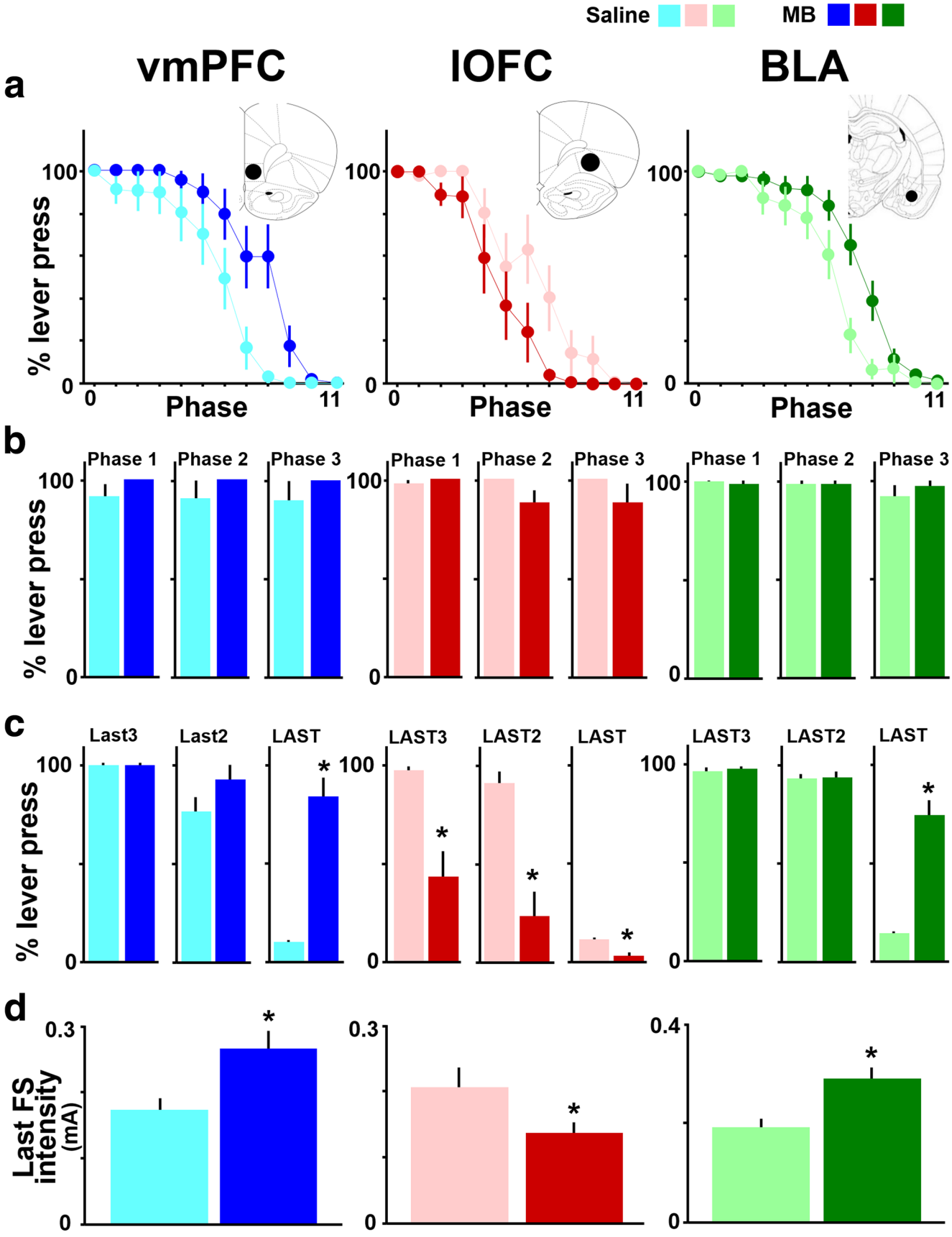
Reward-seeking in various situations

The vmPFC, lOFC, and BLA played no role in lever pressing during the early phases of the action/no-action conflict task (Fig. 3) but contributed to lever pressing for obtaining a pellet in the no footshock condition (Fig. 4). In this experiment, rats that performed lever presses in the no footshock condition had experienced neither a footshock nor the action/no-action conflict task (Fig. 4). Therefore, the lever-press behavior of these animals reflects a simple conditioned response, which is reinforced by reward alone. In contrast, rats involved in the action/no-action conflict task had learned the association between a lever press and outcomes, including the pellet and footshock before the inactivation experiment (Figs. 1, 3, and 6). The behavior of these animals during the task should reflect their choice based on an estimation of outcomes. Interestingly, the roles of the vmPFC and BLA in reward-seeking during the footshock condition were opposite to those in the no footshock condition. These results indicate that the neural basis of reward-seeking behavior varies depending on the situation. In contrast, the tone-lever-pellet association was learned well by both the "conflict task (reward + footshock)" group and "reward + no footshock" group, despite the difference in the training periods for each animal. Therefore, it is possible that the lever press was a goal-directed or habituated behavior, depending on the animal.

Selecting a reward accompanied by a negative factor involves several decision-making processes, which evaluating the risk (St Onge and Floresco 2010; Zeeb and Winstanley 2011; Ogawa et al. 2013; Stopper et al. 2014), delay (Mobini et al. 2002; Winstanley et al. 2004; Rudebeck et al. 2006; Mar et al. 2011), effort (Walton et al. 2002; Ostrander et al. 2011), and risk of punishment involved (Amemori and Graybiel 2012; Orsini et al. 2015). These conditions are simulated experimentally, when animals are made to choose a small reward + small negative or a large reward + large negative. The vmPFC, lOFC, and BLA are involved in the decision-making processes in these tasks, but the roles of these regions in large-reward choices differ among tasks. For instance, inactivation or the presence of lesions in the rat vmPFC and BLA increased large-reward choices in the gambling task (Zeeb and Winstanley 2011; Zeeb et al. 2015) and decreased large-reward choices in delay (Churchwell et al. 2009) and effort (Walton et al. 2002; Ostrander et al. 2011) discounting tasks. In contrast, inactivation of the rat orbitofrontal cortex, including the lOFC, has no clear effect on choice in the gambling (Zeeb and Winstanley 2011), delay (Churchwell et al. 2009), and effort (Rudebeck et al. 2006) discounting tasks. However, lesions in this region decrease high-reward choices in the punishment discounting task (Orsini et al. 2015). Since the brain probably weighs the relative costs and benefits associated with the available options to make a beneficial decision, the mechanism governing reward-seeking behaviors may differ, depending on the type of decision and outcomes of the choice.

Roles of the vmPFC

The vmPFC contributed to reward-restraint choices in the later phases, but not in early phases of the action/no-action conflict task. McNally (2016) reported that inactivation of the mPFC had no influence on reward/punishment-guided lever press behavior, when rats learned the lever press-footshock association by fixed footshock intensity on a FR-10 schedule, when levers were reinforced with a pellet at a variable 30-sec interval schedule, and when footshock was not delivered in the test session. These observations indicate that the functions of the vmPFC depend on the situation, even for the same type of decision-making task (reward-punishment conflict task). On the other hand, inactivation of the vmPFC, slightly dorsal to our target region, increases large reward + risk of punishment


Saline
MB
a
vmPFC
lOFC
BLA
% lever press
100
0
Phase
0
11
b
Phase 1
Phase 2
Phase 3
c
Last3
Last2
LAST
LAST3
LAST2
*
d
Last FS intensity (mA)
0.3
0.4

◀ **Fig. 3** . The effects of inactivation on performance in the action/no-action conflict task. **a** Probability of lever pressing during each phase in saline and muscimol/baclofen (M/B) injected rats. **b** Comparison of lever press probability in the first 3 phases. **c** Comparison of lever press probability in the LAST 3 phases between saline and M/B injections. **d** Comparison of footshock intensity in the last phase between saline and M/B injections. Data are shown as mean ± SEM. *$p < 0.05$ compared with saline

choices, when the punishment probability steadily increases, but decreases risky large-reward choices when the punishment probability steadily decreases, indicating impairment of adaptability (Orsini et al. 2018). Judging by the data reported by that study, the probability of large-risky choices is altered by mPFC inactivation, if the probability of punishment was less than 100%, and did not differ between the inactivation and vehicle injection groups, if the punishment probability is 100%. In our preliminary experiment, inactivation of the dorsal-mPFC, which is almost the same as target region in their study, had no influence on choice-making behaviors in the action/no-action conflict task (data not shown), which is consistent with their study. The dorsal-mPFC could be associated with the ability to adapt to change, when faced with punishment. In contrast, vmPFC inactivation increased lever pressing only during the LAST phase, but not during the other phases, indicating that role of the vmPFC is not restricted to adaptation. The vmPFC might contribute to adaptation to change according to the intensity of punishment, and/or regulation of choice-making behavior during the later phase of the task.

Roles of the lOFC

The lOFC contributed to reward-seeking choices in the later phases of the task. The lOFC reportedly regulates reward/footshock-guided behavioral choices. For instance, Orsini et al. (2015) developed a task in which animals chose either to receive 3 food pellets and a footshock (at a variable probability) or to receive a food pellet without a footshock. In this task, lesions of the lOFC increased safe/small reward choices

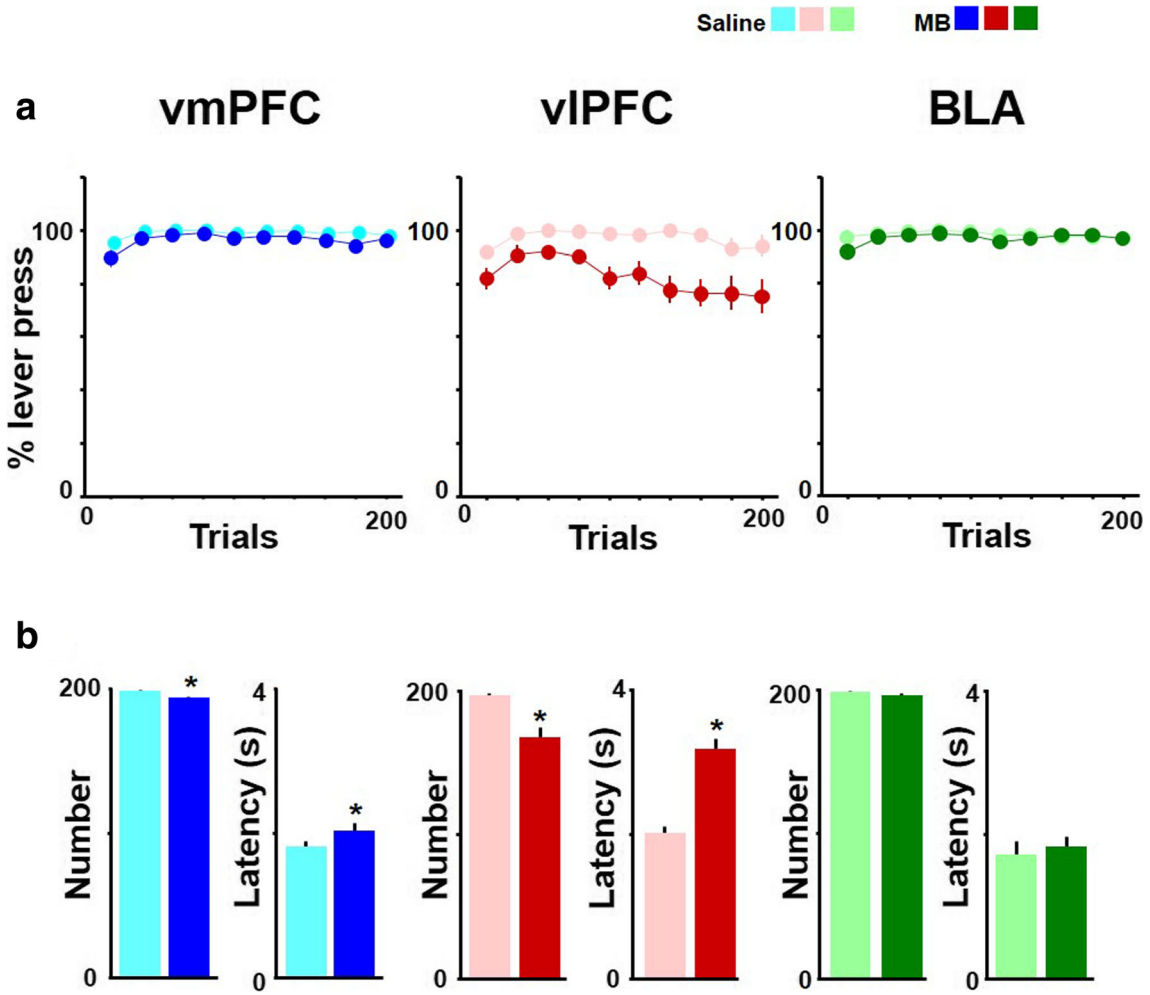
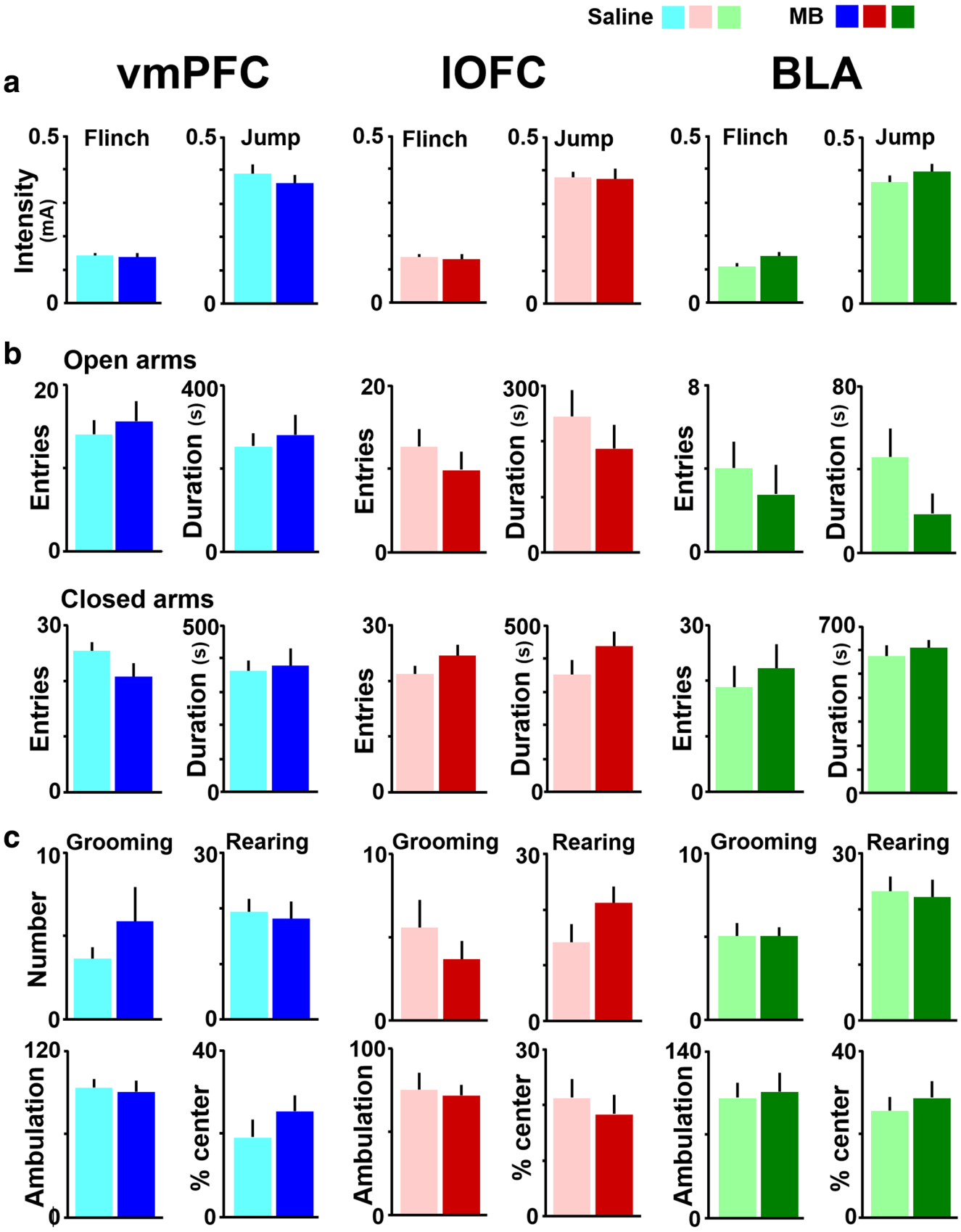


Fig. 4 . The effects of inactivation on lever presses for pellets in the no footshock condition. **a** Probability of lever press for 20 trials in saline and muscimol/baclofen (M/B) injected rats. **b** Comparison of the number and latency to lever presses (to obtain a pellet) between saline and muscimol/baclofen injections Data are shown as mean ± SEM.*$p < 0.05$ compared with saline



in the task. The authors' observations are consistent with our results in that the dysfunction of the lOFC increases

◀ **Fig. 5** . The effects of inactivation on aversive behaviors. **a** The flinching and jumping thresholds in the flinch-jump test. **b** The number and duration of open and closed-arm entries in the elevated plus-maze test. **c** Instances of grooming, rearing, and total line crossing, and the probability of line crossing in the central area in the open-field test Data in bar graphs are shown as mean ± SEM

footshock-avoidance choices. In contrast, inactivation of the OFC increases lever press behavior, which is reinforced by providing a food pellet at a variable interval schedule and footshock at a FR-10 schedule, which contradicts our results, in that OFC inactivation increases footshock-acceptable behavior (Jean-Richard-Dit-Bressel and McNally 2016). The OFC might have a flexible role in reward/punishment-guided behavioral choices. Zeeb et al. (2010) revealed that the function of the lOFC in a delay-discounting task, in which delay was cued (from behavioral response to reward delivery), differs from that in a condition, which lacks a cue. Although the lOFC is involved in various decision-making processes, its function might be influenced by the predictability of the outcome or timing of outcome delivery.

Roles of the BLA

Inactivation of the BLA increased reward-seeking choices during the later phases of the action/no-action conflict task. Dysfunction of the BLA is also reported to induce a similar behavioral change in several conditions. For instance, lesions in the BLA increased the choice of high-reward pellets and footshock (at a variable probability) (Orsini et al. 2015). BLA inactivation increased lever presses to obtain sucrose (FR-1) and a footshock (50% probability) (Piantadosi et al. 2017). These results suggest that dysfunction of the BLA might increase footshock-acceptable behavior in conditions in which a reward can be obtained.

Motivation for reward, anxiety, and punishment sensitivity

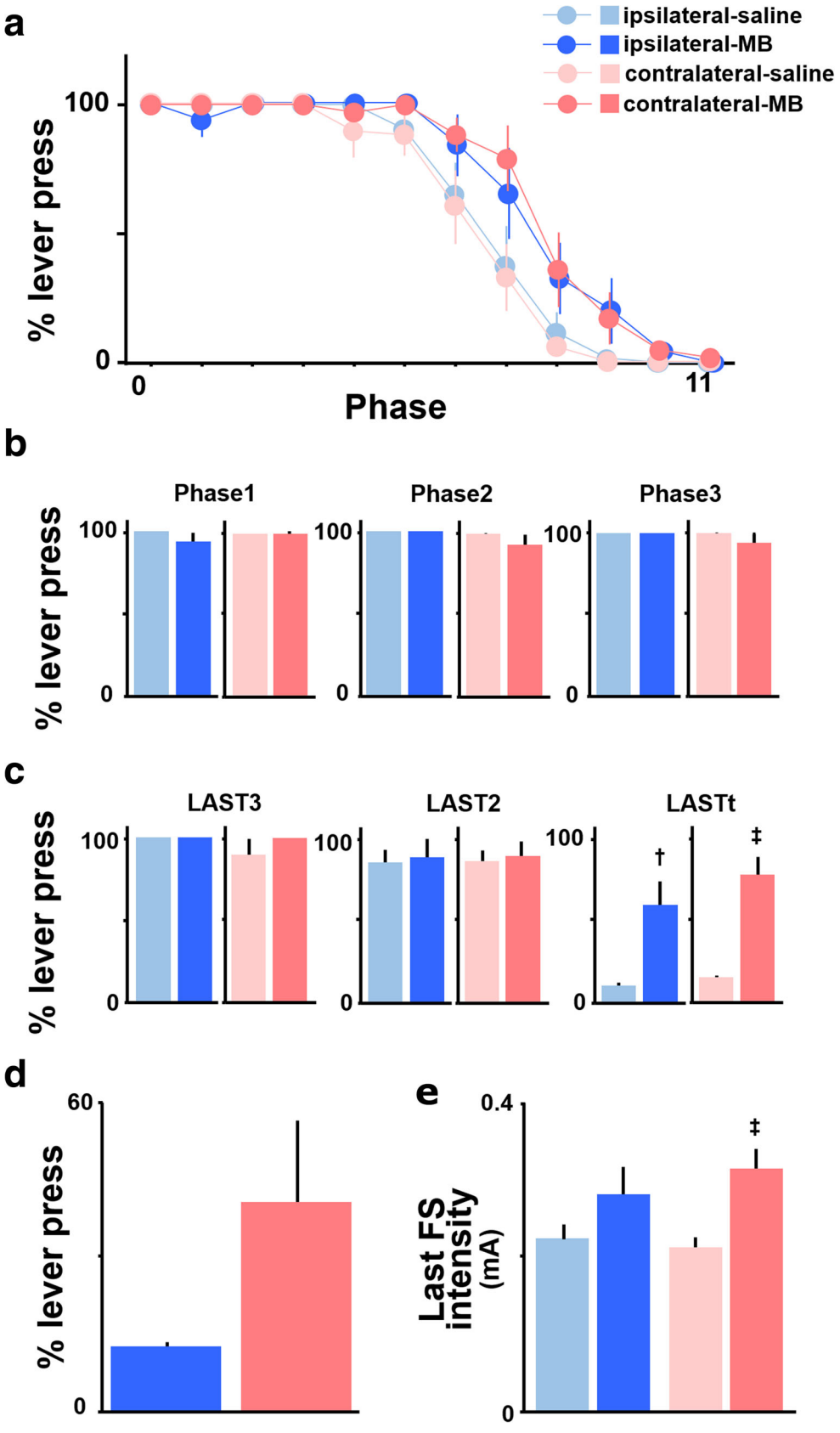
Motivation for reward, anxiety levels, and punishment sensitivity are important factors influencing choice-making behaviors in the action/no-action conflict task. Although the effects of lesions or inactivation of the PFC and BLA on these factors have been investigated, the results slightly differ, depending on the experimental procedure, type, and concentration of drugs used. Therefore, we investigated the effect of inactivation on these factors, using the same injection condition that was used in the action/no-action conflict task.

It has been reported that inactivation of the vmPFC or BLA decreases cue-evoked lever press response for rewards in the discriminative stimulus task (Ishikawa et al. 2008). In our study, vmPFC inactivation decreased lever presses for obtaining a pellet, and BLA inactivation did not significantly decrease but tended to decrease. These results indicate that an increase in lever presses in the vmPFC or BLA inactivation condition is not caused by heightened reward motivation. On the other hand, inactivation of the lOFC significantly decreased lever pressing in our study. Although lOFC inactivation did not decrease lever pressing in the early phases of the task, the possibility that a decrease in lever pressing in the later phases of the task is caused by a reduction in reward motivation cannot be ruled out.

Animals exhibiting high anxiety in the elevated plus-maze test exhibit impairment in decision-making (de Visser et al. 2011; Cao et al. 2016). It has been reported that BLA inactivation or vlOFC lesion does not increase anxiety levels in the elevated plus-maze test (Lacroix et al. 2000; Moreira et al. 2007), which is consistent with our report. On the other hand, the effect of mPFC lesions on anxious behavior in the elevated plus maze differed according to the interval between surgery and the test, and the size or location of the lesion within the mPFC (Jinks and McGregor 1997; Lacroix et al. 2000; Klein et al. 2010). We found that inactivation of the vmPFC by our experimental condition did not increase anxiety in the elevated plus maze. The open-field tests in our study also revealed that inactivation of the vmPFC, vlOFC, and BLA did not increase anxiety. Behavioral changes induced by inactivation cannot be attributed to a change in anxiety levels during the task. On the other hand, the roles of the PFC and BLA in determining footshock sensitivity have not been investigated well. However, infusion of the NMDA antagonist, D,L-2-amino-5-phosphonopentanoic acid, into lesions of the BLA or vmPFC did not alter footshock sensitivity, as estimated by the flinch-jump test (Quirk et al., 2000; Roesler et al., 2000). We also found that inactivation of any of region did not alter behavior in the test, indicating that inactivation was not responsible for alteration in footshock sensitivity.

Interactions between the vmPFC and BLA, and the lOFC and BLA

In the action/no-action conflict task, the vmPFC and BLA regulated behavioral choices, while interacting with each other. Neural projections between the vmPFC and BLA in rats are mainly ipsilateral, but some vmPFC-BLA contralateral pathways also exist (Vertes 2004; Hoover and Vertes 2007). Therefore, contralateral inactivation can disconnect a majority of the vmPFC-BLA pathways, while leaving a few intact. In contrast, ipsilateral inactivation disconnects ipsilateral vmPFC-BLA pathways in one hemisphere, but the ipsilateral pathways in the other hemisphere remain intact. Since disconnection of the vmPFC-BLA pathways following contralateral inactivation is greater than that following ipsilateral inactivation, the effects of contralateral inactivation would be greater than those of ipsilateral inactivation, if vmPFC-BLA pathways are involved in task performance. Our results indicate


a
ipsilateral-saline
ipsilateral-MB
contralateral-saline
contralateral-MB
% lever press
100
0
0
11
Phase
b
Phase1
Phase2
Phase3
% lever press
100
0
c
LAST3
LAST2
LASTt
% lever press
†
‡
d
% lever press
60
0
e
Last FS intensity (mA)
0.4
0
‡

◀ **Fig. 6** . The effects of disconnection between the vmPFC and BLA by ipsilateral and contralateral muscimol/baclofen (M/B) injection on performance in the action/no-action conflict task. **a** Probability of lever press at each phase in saline and M/B injected rats. **b** Comparison of lever press probability in the first 3 phases between saline and M/B injections. **c** Comparison of lever press probability in the LAST 3 phases of saline injection between saline and M/B injections. **d** Comparison of footshock (FS) intensity in the last phase between ipsilateral and contralateral inactivation. **e** Comparison of last footshock intensity between saline and M/B injections Data are shown as mean ± SEM. $^{\dagger}p < 0.05$ compared with ipsilateral saline injection. $^{\ddagger}p < 0.05$ compared with contralateral saline injection. $^{*}p < 0.05$. vmPFC ventromedial prefrontal cortex, BLA basolateral amygdala

that vmPFC-BLA interactions contribute to behavioral choice during later phases of the task.

It has been reported that mPFC-BLA pathways regulate behavioral choices in delay-discounting tasks (Churchwell et al. 2009; Paine et al. 2013). Moreover, because the effect of inactivation or lesions of the mPFC and BLA on behavioral choice in gambling (Zeeb and Winstanley 2011; Zeeb et al. 2015) and effort-based decision-making tasks (Walton et al. 2002; Ostrander et al. 2011) is similar, the vmPFC and BLA might also interact in these decision-making conditions. The vmPFC-BLA pathway may contribute to various types of decision-making. On the other hand, the OFC is also reciprocally connected with the BLA, but the function of OFC-BLA pathways in decision-making is not fully understood. A few studies have reported on the functional interaction between the lOFC and BLA. For instance, disconnection of the lOFC-BLA pathways by contralateral inactivation impairs context-induced cocaine-seeking behavior (Lasseter et al. 2011), and blockade of projections from the lOFC to the BLA decreases reinstatement of cocaine-seeking behavior (Arguello et al. 2017). Contralateral inactivation of the OFC and BLA impairs reversal of learning-guided behavioral responses (Churchwell et al. 2009), and contralateral lesions of the OFC and BLA retard updates to behavioral choices following reward devaluation in a gambling task in rats (Zeeb and Winstanley 2013). Although OFC-BLA pathways contribute to reward-guided behavior and learning, the contribution of the OFC and BLA to decision-making in several conditions, such as gambling (Zeeb and Winstanley 2011), effort-based (Ostrander et al. 2011), delay-discounting (Churchwell et al. 2009), and punishment-discounting tasks (Orsini et al. 2015), is dissociated. Neural pathways between the lOFC and BLA may be functionally disconnected in some decision-making conditions.

In conclusion, the vmPFC, lOFC, and BLA contribute to decision-making during the action/no-action conflict task. The vmPFC and BLA regulate reward restraint to avoid punishment. In contrast, the lOFC contributes to reward-seeking behavior, when faced with aversive punishment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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