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ORIGINAL ARTICLE

Amygdala electrical stimulation for operant conditioning in rat navigation

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Abstract

There have been several attempts to navigate the locomotion of animals by neuromodulation. The most common method is animal training with electrical brain stimulation for directional cues and rewards; the basic principle is to activate dopamine-mediated neural reward pathways such as the medial forebrain bundle (MFB) when the animal correctly follows the external commands. In this study, the amygdala, which is the brain region responsible for fear modulation, was targeted for punishment training. The brain regions of MFB, amygdala, and barrel cortex were electrically stimulated for reward, punishment, and directional cues, respectively. Electrical stimulation was applied to the amygdala of rats when they failed to follow directional commands. First, two different amygdala regions, i.e., basolateral amygdala (BLA) and central amygdala (CeA), were stimulated and compared in terms of behavior responses, success and correction rates for training, and gene expression for learning and memory. Then, the training was performed in three groups: group R (MFB stimulation for reward), group P (BLA stimulation for punishment), and group RP (both MFB and BLA stimulation for reward and punishment). In group P, after the training, RNA sequencing was conducted to detect gene expression and demonstrate the effect of punishment learning. Group P showed higher success rates than group R, and group RP exhibited the most effective locomotion control among the three groups. Gene expression results imply that BLA stimulation can be more effective as a punishment in the learning process than CeA stimulation. We developed a new method to navigate rat locomotion behaviors by applying amygdala stimulation.

Keywords Amygdala · Electrical stimulation · Medial forebrain bundle · Neuromodulation

1 Introduction

During the last couple of decades, there have been several attempts to navigate the locomotion of animals or insects by modulating neural signals. To date, various animal and insect models have been proposed for this purpose, including rodents, birds, fish, and insects with different underlying mechanisms of neuromodulation [1–13]. Electrical stimulation has been applied to the optic lobes and muscles of beetles to navigate their flying pathways [4–6]. The antenna nerve of cockroaches was stimulated to provide virtual sensation and guide the movement direction [7, 8]. A study tried to guide the swimming of sharks by applying virtual olfactory stimuli mimicking the smell of blood [9, 10]. The flying control of the pigeons by the electrical stimulation of specific brain regions has also been reported [11–13].

Among the tested animals, rodents are the typical animal model for neuromodulation-based navigation because they are the most commonly used experimental animals in

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neuroscience research and, thus, their nervous system is well known compared with other species [1–3, 14–16]. Various brain regions of rodents have been studied for locomotion control, such as the ventral posterolateral nucleus of the thalamus [17], the dorsal periaqueductal gray [18], the nucleus accumbens [19], the amygdala [19–21], and the medial forebrain bundle (MFB) [1, 2, 16, 22].

Among them, the MFB is the most widely studied motivational substrate of reward, which is found in the mesolimbic dopaminergic pathway passing information from the ventral tegmentum to the nucleus accumbens. Since the activation of this pathway induces a sense of pleasure and reward, several researchers targeted this brain region to train rodents through operant learning paradigms. Operant conditioning has been regularly utilized to train laboratory animals; it enables the rodents to learn new learning based on rewards (food or water) or punishments (electric foot shock) for specific behaviors [23–27]. In operant conditioning, the key element is to associate a specific voluntary behavior with specific consequences responding to external cues such as acoustic tones or visual lights. Several reports have demonstrated that electrical stimulation can be also used as a reinforcing reward and successfully train animals to perform desired behaviors.

Since Talwar et al. showed that the electrical stimulation of the MFB can successfully serve as a virtual cue for forward movement and a reward to steer rats along three-dimensional routes as the researchers intended [1], many relevant studies have followed to miniaturize the stimulation system, optimize the stimulation parameters, or apply various stimulation modalities such as optogenetics and ultrasounds [3, 8, 28–30]. Besides MFB stimulation for reward and forward locomotion, to control the direction of animal movement, the somatosensory cortex is also frequently utilized as a stimulation target for directional cues. In the somatosensory system of rodents, whisker sensation is one of the most important and sensitive sensory inputs for their survival. The rodents detect obstacles in front of them by whisker-mediated vibration and tend to avoid them [31]. Therefore, the barrel cortex, which is the sensory cortex of the whiskers, is conventionally chosen as the stimulation target for directional commands [32]. When the barrel cortex is activated by electrical stimulation, the rat may perceive a signal from the whisker on the contralateral side, consequently tending to turn toward the other side of the whisker. However, rats can be trained to turn to the same side of the stimulated whisker, which means that a new behavior paradigm can be trained using virtual cues and rewards to overcome instinctive behaviors [33, 34].

Although electrical stimulation for rewards and cues is effective to train and navigate the locomotion of rodents, it has some limitations. They include the need to preliminarily and repeatedly train the animals to learn the meaning of reward stimulation and understand the relationship between cue stimulation and the turning direction intended by the experimenter [2]. For example, in the training session, when the animal turns toward the correct direction following the cue stimulation, reinforcing MFB stimulation is given; this training should be repeated until the animal successfully learns the meaning of the virtual stimulation for navigation. Therefore, the training process is time-consuming and must be repeated to prevent the animal from forgetting the paradigm [21].

In the present study, for more effective and faster training of animal navigation, the amygdala was additionally targeted for punishment stimulation. Besides the reinforcement via MFB stimulation, when the animals turned in the wrong direction, amygdala stimulation was performed as a punishment. Amygdala activation is closely involved in fear-related emotions. Since the amygdala consists of two distinct functional subregions, namely basolateral amygdala (BLA) and central amygdala (CeA) [35, 36], each with different functions in fear conditioning [35–38], we attempted to determine which amygdala region is more effective for animal locomotion control by determining the behavioral responses to the electrical stimulation and training efficacies. Furthermore, to determine the effects on memory and learning, gene expression profiling was performed using hippocampal and amygdala tissues after stimulation of each region. Then, the behavioral training for navigation was performed in three different groups: group R (only reward via MFB stimulation), group P (only punishment via amygdala stimulation), and group RP (both reward and punishment via stimulation of, respectively, the MFB and amygdala).

2 Methods and materials

2.1 Electrode implantation surgery

Male Sprague Dawley rats (Samtaco, Osan, South Korea) weighing 250–300 g were used for the study. The animal care and surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC 15-081). All the rats were randomly divided into three groups: group R (n = 5), which received only MFB stimulation for reward; group P (n = 21), which received only BLA stimulation for punishment (BLA was targeted since its stimulation is more effective method for rat navigation than CeA stimulation based on the comparison which will be described in Method 2.3.); group RP (n = 17), which received both MFB and BLA stimulations for, respectively, reward and punishment. The surgical procedures were equivalent except electrode placement.

Rats were anesthetized by intraperitoneal injection of a ketamine/xylazine cocktail (2.6 ml/kg) containing ketamine

(100 mg/ml) and xylazine (10 mg/ml). To confirm that they were deeply anesthetized, we pinched the hind paws and observed their motion response; in the absence of any response, they were placed on a stereotaxic apparatus (Narishige Corp., Tokyo, Japan). During the surgery, a feedback temperature control system (FHC, Inc., Maine, USA) was used to maintain the body temperature at 37 ℃. After shaving hair in the rat head, the scalp was locally anesthetized by subcutaneous injection of 1% lidocaine and then incised along the midline of the skull. The exposed skull was gently rubbed with a swab to strip the remaining micromembranes and blood vessels; next, it was drilled to make holes through which we inserted the electrodes.

Depending on the groups, insulated tungsten wire electrodes (diameter: bare 127 µm, coated 178 µm; A-M Systems, LLC., Washington, USA) were implanted into deep brain areas as follow (Fig. 1A). First, two different amygdala regions such as the BLA (Anterior–Posterior (AP): − 2.8 mm, Medial–Lateral (ML): ± 5.0 mm, Dorsal–Ventral (DV): − 8.4 mm, n = 8) and CeA (AP = − 1.9 mm, ML = ± 3.6 mm, DV = − 8.2 mm, n = 5) were targeted. Their stimulation could be used as punishments and their effectiveness was compared with behavioral responses, T-maze training, and gene expression to determine which would be used for group P. As will be shown in Result 3.1–3.3, BLA was selected. Second, for the reward, that is group R, the tungsten wire electrodes were implanted into the MFB region (AP = − 2.0 mm, ML = ± 2.0 mm, DV = − 9.0 mm). Third, in group RP, electrodes were inserted into both BLA and MFB. Though electrodes were bilaterally implanted of each targeted brain area, one side of electrodes which was confirmed better performance were selected and used in this experiment to maximize stimulation effectiveness.

The rest of the surgical procedure was the same as below. Two stainless steel screws (diameter: 1.2 mm) were utilized as the electrodes of the barrel field cortex (AP = − 2.0 mm, ML = ± 5 mm) stimulation for directional cues; and another stainless screw was inserted into the cerebellum surface as a ground electrode. All the electrodes were connected to a custom-made connector and fixed with dental cement. After surgery, the rats were allowed to recover for at least 7 days.

2.2 Apparatus for behavioral tasks

For the animal groups including MFB stimulation (group R and group RP), the lever-press training was performed in an operant conditioning chamber (Med Associates, Inc., Georgia, USA) to allow the rats to identify the MFB stimulation as a reward and induce stimulation-seeking behaviors.

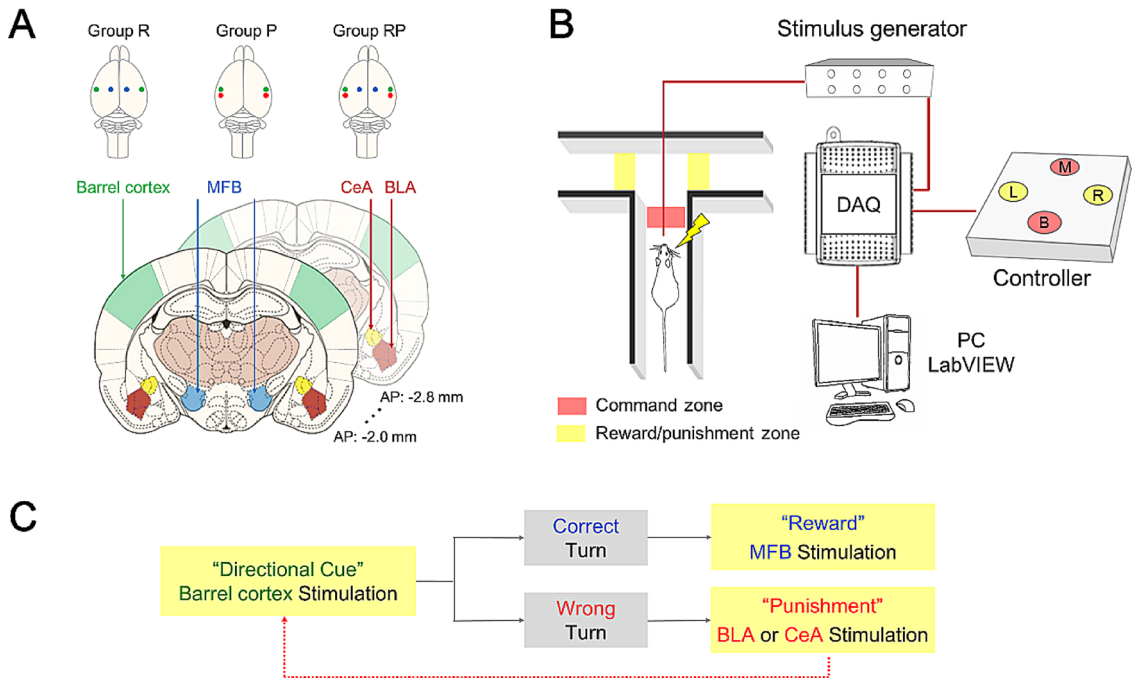

Fig. 1 Concept of rat navigation with reward and punishment electrical stimulation **A** Locations of the implanted electrodes: top views for group R (reward only), group P (punishment only), and group RP (reward and punishment) and coronal brain sections with the electrode placements (Green: barrel cortex, Blue: MFB, Yellow: CeA, Red: BLA). **B** T- maze training setup (Buttons on the controller; L/R: left or right barrel cortex stimulation for directional command, M: MFB stimulation for reward, B: amygdala stimulation for punishment). **C** Protocol example in group RP. (BLA: basolateral amygdala; CeA: central amygdala; DAQ: data acquisition system; MFB: medial forebrain bundle)

The lever-pressing action in the chamber was programmed to deliver the electrical stimulation through the electrodes implanted in the MFB; it was implemented with LabVIEW (version 8.5, National Instruments, Texas, USA) and performed with an STG4008 multichannel electrical stimulator (Multi Channel Systems MCS GmbH, Reutlingen, Germany) via a data acquisition system (USB-66009, National Instruments). The number of lever-press responses was automatically counted for screening the animals successfully trained for MFB stimulation.

T-maze was custom-made for operant training with directional commands (Fig. 1B). It consisted of black Foamex board (width: 9 cm; height: 19 cm), with a command zone and two reward/punishment zones having 5 cm width. When rats reached command zone, barrel cortex stimulation was given as a directional command. When rats covered reward/punishment zone, either MFB or BLA stimulation was given as a reward or punishment. Detailed T-maze training protocols will be explained in Method 2.4 and 2.5.

We used two different forms of electrical current stimulation: tonic electrical stimulation was applied to the barrel cortex, while burst electrical stimulation was performed on the MFB and amygdala. Burst stimulation is known superior to tonic stimulation, especially for activating deep brain regions [28, 39]. The details of the pulse parameters are as follows; the pulse frequency of each stimulation was 250 Hz and 500 Hz, and burst frequency was 50 Hz, and the total length of each stimulation was 200 ms, their amplitude began at 200 μA for the tonic and burst stimulation, and it was increased with 25 μA steps until the animal exhibited these three following behavioral responses according to each stimulation with different brain area. First, tonic stimulation on barrel cortex causes rats' looking around or turning their head. Second, burst stimulation on amygdala induced several anxiety behaviors such as freezing, fleeing, startling and jumping which will be explained in Results 3.1. Third, burst stimulation on MFB enhances rats moving activities showing excited state and tended to go ahead. Mean amplitude of stimulation was 275 μA. The electrical stimulation of 200 ms was repeatedly given until rats leave command zone or reward/punishment zone.

2.3 Terminologies definition

Several terminologies are defined in this paper to quantify learning capability of each training group. First, 'attempt' is defined as follow. When rats try to determine which direction they decide to go after barrel cortex stimulation, their body extends reward/punishment zone but do not pass this zone yet, and may think whether keep going or change their direction. In this case, success or failure did not yet determined and we called this situation as a one 'attempt'. For example, when rats attempt to go wrong direction, they have got punishment and had a chance to correct their decision. Every rat can have three chances of attempts, and if they try over three times and head forward to wrong direction or may not pass the reward/punishment zone, their training was counted as failure. On the other hand, if they correctly pass the reward/punishment zone within three attempts, it is counted as success and proceed to next trial which will be defined as follow. Second, 'trial' includes three or less attempts and defined as rats' final decision. In other word, trial conclude whether rats' final decision is success or failure. Table 1 shows one trial including various cases of success and failure. After finishing each trial, rats were repeatedly located in start point by human hands. Third, 'session' includes ten trials. Two sessions were conducted per day, with a resting time of ten minutes between them. We conducted ten trials for each session, and the success rate curves were obtained up to ten consecutive sessions.

To quantitatively evaluate the efficacy of three groups for rat navigation, the success rate was defined as a percentage of successful trials. The correction rate was calculated to demonstrate the effectiveness of punishment in correcting the wrong decision. This can be utilized to determine which amygdala subregion, BLA or CeA, is more effective as a punishment and should be assigned for group P. For the animals who made wrong decisions at the first attempt, the correction rate was calculated as a percentage of the total success trials after that first failed attempt. By using newly defined terminologies, success rate and correction rate was calculated as below.

$$Success\ rate(\%) = \frac{The\ number\ of\ success\ trials}{The\ number\ of\ all\ trials} \times 100 = \frac{A+B+C}{A+B+C+D+E+F} \times 100$$

$$Correction\ rate(\%) = \frac{The\ number\ of\ success\ trialss\ with\ the\ first\ attempt\ failed}{The\ number\ of\ all\ trials\ with\ the\ first\ attempt\ failed} \times 100 = \frac{B+C}{B+C+D+E+F} \times 100$$

Table 1 The number of cases of success and failure during one trial including three attempts

		Attempt #1	Attempt #2	Attempt #3
Success	A	O		
	B	X	O	
	C	X	X	O
Failure	D	X		
	E	X	X	
	F	X	X	X

2.4 Behavioral experiment protocols in group P

Rats were divided in three groups depending on whether they were being punished, rewarded, or both. Therefore, their T-maze training protocols are different in three ways. First, as a punishment, the electrical stimulation of BLA or CeA was used. Before the T-maze training, the behavioral responses of the stimulation were categorized into 3 anxiety behaviors: (1) startling and jumping, i.e., they jumped with a scream; (2) stepping backward, i.e., they shrank their body and moved backward; (3) fleeing, i.e., they turned their snout and ran suddenly towards an unexpected direction. During the procedure, 23% of the rats exhibited behaviors unrelated to anxiety were excluded for the subsequent training.

After sorting out rats, each rat was placed in the T-maze and trained to turn left or right according to a directional cue stimulation, and according to their decision, punishment would be applied. All the electrical brain stimulations were initiated by pressing the four directional buttons on the controller (Fig. 1B). When the animal reached the command zone, the directional command cue (left or right barrel cortex stimulation) was given by pressing L or R button. For instance, when a rat received electrical stimulation on the left barrel cortex, it was expected to turn left because the rat might feel virtual obstacle on the right side and tend to avoid it. The barrel cortex stimulation was stopped when the head completely passed the command zone and reached reward/punishment zone. As a next step, if the rat turned toward the wrong direction (right), punishment (BLA or CeA stimulation) was given at the wrong direction (right) of reward/punishment zone by pressing B button. If the rat came back to command zone, the directional cue stimulation was applied again to change their wrong decision. If the rat succeed within three attempts, this trial will be counted as success, but no reward would be given. Otherwise, if the rat went through wrong direction within three attempts, this trial was ended and counted as a failure. Moreover, only in case of T-maze training with punishment, the correction rate was also calculated to choose which amygdala sub-region (BLA or CeA) was more adapted for group P. Base on the precedent results, the BLA was selected since it was more effective punishment than the CeA for the navigation control.

2.5 Behavioral experiment protocols in group R and group RP

Unlike group P, the rats in group R and group RP should perform the lever-press training in the operant conditioning chamber before T-maze training. The lever-press training was designed as follows. When rats pressed the lever by chance, MFB stimulation was immediately and automatically given. MFB, a dopaminergic pathway, mediates reward-seeking behaviors [40]. Therefore, if MFB was successfully activated, the rats were expected to repeatedly press the lever to keep getting the reward and feeling happiness. On the other hand, if the implant surgery for MFB stimulation was failed, the rats did not tend to press the lever repeatedly and, thus, were excluded from the experiments. The number of lever-pressing actions per minute was measured over 5 min. The rats that voluntarily pressed the lever more than 30 times per minute were selected for T-maze training with reward stimulation [2]. In the T-maze training for group R, MFB stimulation was performed by pressing M button, when the rats turned toward the correct direction according to a directional command (Fig. 1B). If they turned wrong direction, no reward was given.

In group RP, either BLA stimulation or MFB stimulation was used depending on their decision. Before the T-maze training, rats in group RP also needed not only to conduct lever-press training, but also to confirm behavioral responses of BLA stimulation to decide whether it can be used as a punishment. In the T-maze training for group R and group RP, MFB stimulation was given when the rats turned toward the correct direction according to a directional cue stimulation, while BLA stimulation was given following the same protocol described above (Fig. 1C).

2.6 Histological staining

After the behavioral experiments, rat brains were fixed, sectioned into coronal slices, and two different histological staining for each goal: (1) to confirm the location of the implanted electrodes; (2) to verify neural activation of the targeted brain area from electrical stimulation. If the implanted electrodes were incorrectly located to the targeted brain area, the rats were excluded from the data analyses.

The histological staining was performed in 4 steps: fixation, sectioning, staining, and microscopy. Until sectioning, the experimental protocol was the same, and the staining was divided into two parts: cresyl violet staining and immunohistochemical staining. For fixation, 4% paraformaldehyde was perfused via the vascular system of the animals [41]. The extracted brain was soaked in a 30% sucrose/phosphate-buffered saline solution at 4 °C for 3 days; it initially floated in the solution but sank to the bottom approximately after the first day. Then, the brain was sectioned using a cryostat microtome (CM 3050S, Leica, Wetzlar, Germany). The fixed brain was sliced in the coronal direction with a 40-μm thickness, placed on a slide glass, and stained; first, cresyl violet staining was used to stain the cytoplasm of neurons for identifying the electrode track, and the stained sections were observed with a microscope.

The second histological staining was also conducted to investigate the expression of the c-Fos gene as well as to confirm the location of the implanted electrodes. In general, since the c-Fos expression is used as a marker of increased

neural activity, the information about expressed c-Fos can reveal the stimulated brain region [42]. Therefore, in order to detect c-Fos expression in the BLA region to verity its neuronal activation, immunohistochemical staining was conducted first with a primary rabbit polyclonal antibody binding to the c-Fos protein and, then, with a goat anti-rabbit secondary antibody. The microscopy observation was conducted with a fluorescent upright microscope (BX43, Olympus Corp., Tokyo, Japan).

2.7 RNA sequencing

RNA sequencing was conducted to monitor transcriptional changes in the brain regions after BLA and CeA stimulation as compared with control group which was not given any electrical brain stimulation. Two brain regions, the hippocampus and amygdala, were analyzed to find out the effects of the stimulation of the two amygdala subregions, especially in terms of memory and learning. After the T-maze training sessions with BLA and CeA stimulation, the rat brains were extracted, placed on a pre-cooled acrylic brain matrix on ice, and coronally cut with a razor blade. From the brain slices, the hippocampus and amygdala were carefully isolated using fine forceps. The RNA from these tissues was extracted utilizing a TRIzol™ Reagent according to the kit-based method (easy-Blue™ Total RNA Extraction Kit). First, to homogenize the tissues, the TRIzol™ Reagent (1 ml/50–100 mg) was added to them. Second, by the addition of chloroform (200 µl) and centrifugation, the obtained solution was separated into two layers; since most of the RNA was contained in the upper layer, this was transferred for RNA purification. Third, after adding isopropanol (400 µl) and centrifugation, the resulting RNA pellet settled down at the bottom of the solution. After the supernatant was discarded, the RNA pellet was washed with ethanol and dried until looking transparent, completing the RNA-sequencing preparation.

The RNA-sequencing was conducted by Ebiogen Inc. (Seoul, Korea) as follows. The RNA quality was assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, Netherlands), and RNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA). Up- and down-regulated genes were identified utilizing the Excel-based Differentially Expressed Gene Analysis (ExDEGA) software package developed by EBIOGEN Inc. The genes were categorized based on a search performed using the web-based tool Quick Go (https://www.ebi.ac.uk/QuickGO); the gene categories involved in memory, learning, fear, and stress were intensely examined. Moreover, the representative genes were chosen by selecting those with the expression increased or decreased by over 4 times compared to the control animals. The analysis of changed gene expression was focused on identifying the effectiveness of punishment learning via BLA or CeA stimulation.

2.8 Statistics

All the quantitative graph data was presented as mean ± standard error of mean (SEM). Paired *t* test was conducted for statistical analysis with GraphPad Prism 8.0 (GraphPad Software, San Diego, USA). P value < 0.05 was considered statistically significant and indicated in the graph.

3 Results

3.1 Behavioral responses to amygdala stimulation

When the amygdala was electrically stimulated, the rats were expected to feel fearful and anxious, showing anxiety behaviors to avoid the circumstances. After the CeA or BLA stimulation, the responses of the rats were monitored and classified into 4 anxiety behaviors (Fig. 2A): startling and jumping, stepping backward, and fleeing. However, various behaviors which were irrelevant to anxiety behaviors were also occurred. For instance, about 9% of the excited responses, 8% of eye blinking, and 6% of the jaw movement were observed. This would be because of complex neural pathway and it will be covered in Discussion. Those irrelevant-anxiety behaviors and freezing behavior can't be used as a punishment, so they were excluded for further experiment.

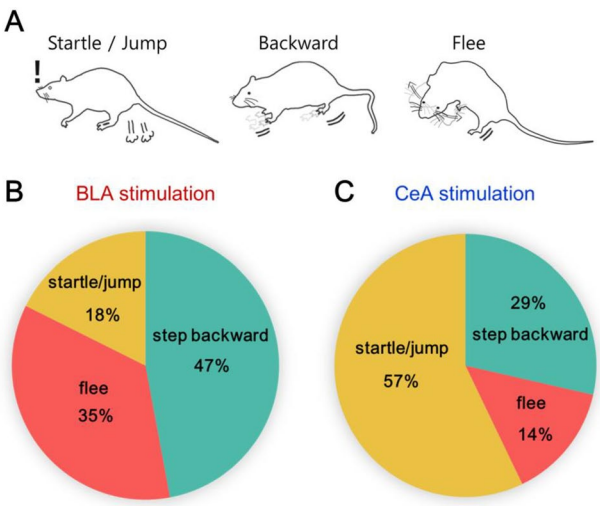

Fig. 2 Anxiety behaviors exhibited by the rats after electrical stimulation of the amygdala: **A** startle and jump (left), step backward (fight), and flee (right). **B** Behavioral responses to electrical stimulation of the basolateral amygdala (BLA) and **C** central amygdala (CeA)

The percentages of each behavioral response to BLA and CeA stimulation are plotted in Fig. 2B, C. Freezing was excluded for the analysis since it was not used for T-maze training. Startling and jumping responses occurred more dominantly when the CeA was stimulated. The animals showed more stepping backward and fleeing responses when the BLA was stimulated. Other distinct behaviors were also observed including jaw movement, eye blinking, or seizure; however, they were not included in the analysis since not directly related to anxiety behaviors.

3.2 Operant conditioning with BLA and CeA stimulation

To evaluate the effectiveness of BLA and CeA stimulation for operant conditioning, the success rate was calculated for each training session. The overall success rate of BLA stimulation was higher than that of CeA stimulation throughout the training sessions (Fig. 3A). The average success rate of BLA stimulation was 79%, while that of CeA stimulation was 38% with an unstable tendency overall. In particular, after 5 training sessions, the CeA-stimulated group showed a rapid decrease in the success rate.

When the animal turned in the wrong direction, BLA or CeA stimulation was applied as a punishment, and the rat was expected to consequently change its turning direction. To evaluate the effect of amygdala stimulation on behavior correction, the correction rate was calculated. The average correction rate of the BLA stimulation ($75 \pm 9\%$) was more than 2 times higher than that of the CeA stimulation ($29 \pm 11\%$) (Fig. 3B); this result indicates that BLA stimulation is more effective than CeA stimulation as a punishment for rat navigation.

3.3 Gene expression after BLA and CeA stimulation

Gene expression in the amygdala and hippocampus was examined through RNA sequencing after T-maze training with punishment by CeA or BLA stimulation. The gene expression was analyzed with ExDEGA (Excel based Differentially Expressed Gene Analysis) which have a function of gene category analysis and customized set analysis. We detected 17,048 genes, which were examined regarding up- or down-regulations as compared with control group (n = 2). The genes with the expression changed over two times after the electrical stimulation were selected and counted for each group (Fig. 4). Overall, for both CeA and BLA stimulation, up-regulated genes were more common than the down-regulated ones after the maze training. However, compared to CeA stimulation, the BLA stimulation induced more up-regulation of the genes in both the amygdala and hippocampus (1320 and 1679 up-regulated genes, respectively, against the 884 and 1534 ones with the CeA stimulation). Based on genes which expression changed more than four, hierarchical clustering was conducted and heatmap was plotted to identify similarities between genes and training groups (Fig. 5). For clusters were categorized and each cluster showed different patterns between amygdala and hippocampus except cluster 2 (Fig. 5C).

The up- and down-regulated genes were categorized into memory, learning, fear, and stress to identify the relevant genes, and we selected the gene expressions that changed more than 4 times ($\log_2$ fold change > 2) (Table 2). According to conventional gene expression analysis, the fold change was calculated after $\log_2$ conversion, representing the increase or decrease of gene expression in positive or negative values [43, 44]. Five out of seven memory-related genes were overlapped in learning- and fear-related genes;

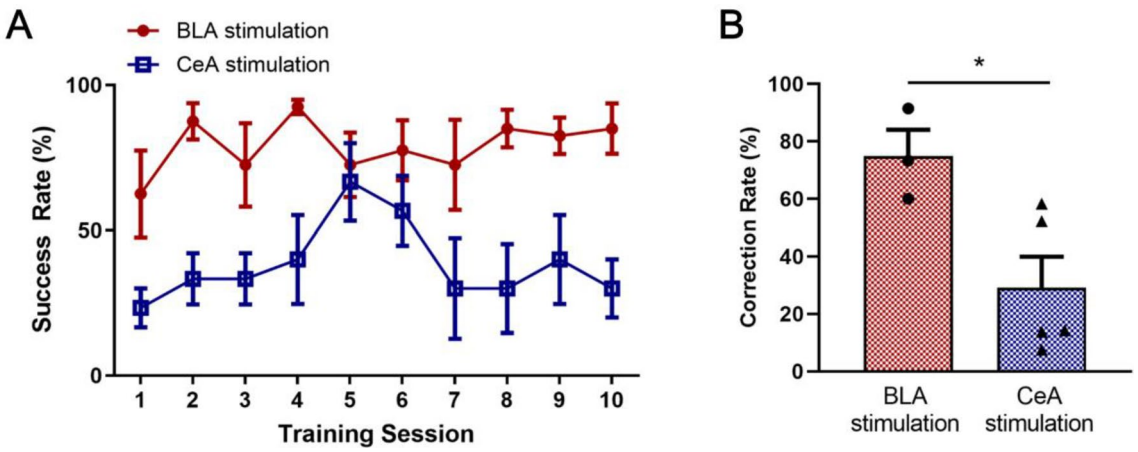

Fig. 3 Comparison of basolateral amygdala (BLA) and central amygdala (CeA) stimulation for T-maze operant training: **A** success rates (n = 4 and 3 for the BLA and CeA stimulation, respectively); **B** correction rates (n = 3 and 5 for the BLA and CeA stimulation, respectively. Paired *t* test, * $p < 0.05$)

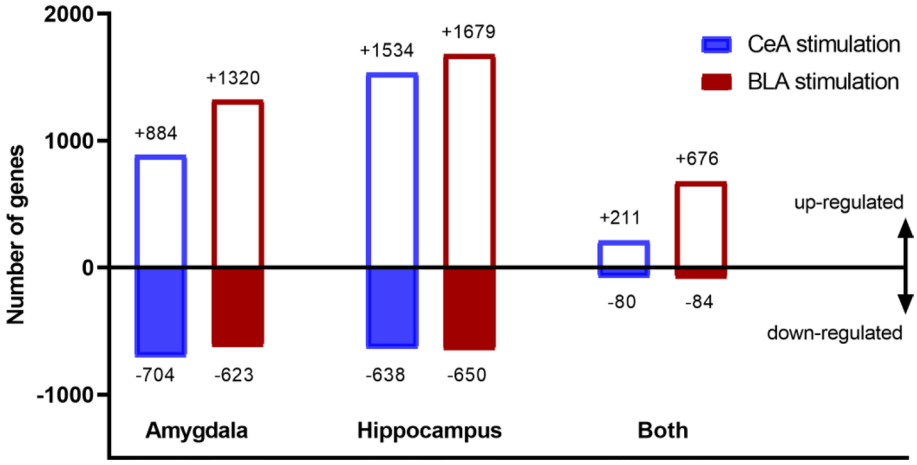

Fig. 4 Overall gene expressions in the amygdala and hippocampus after T-maze training with central amygdala (CeA) or basolateral amygdala (BLA) stimulation

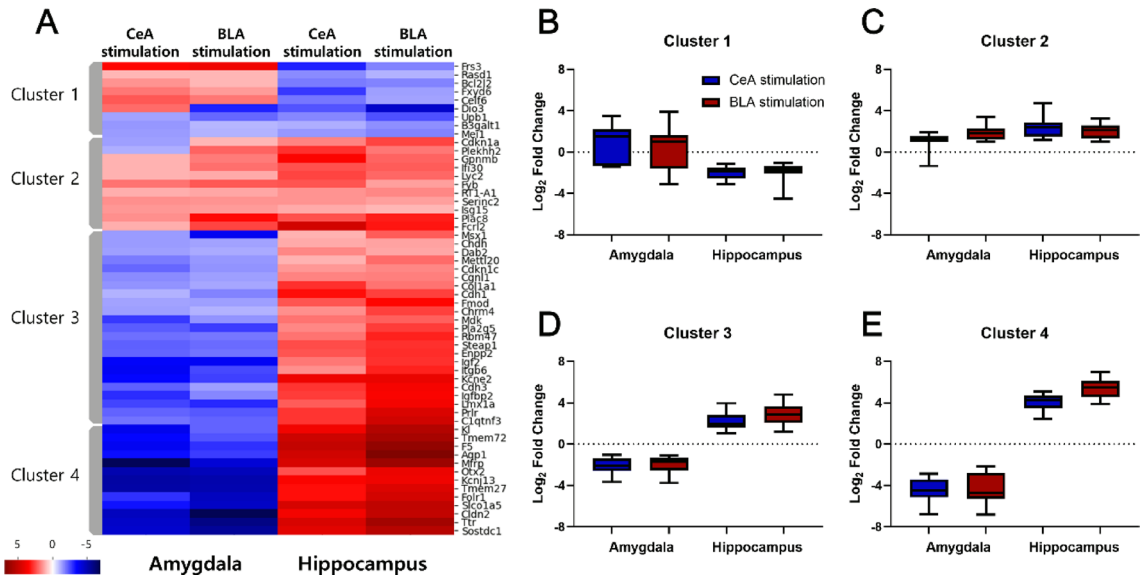

Fig. 5 Categorized gene expressions in the **A** amygdala and **B** hippocampus after central amygdala (CeA) or basolateral amygdala (BLA) stimulation

for instance, Neto1, Lgmn, Arc, and Shank3 could be interpreted as learning- and memory-related genes, while Mdk is a fear- and memory-related gene. The memory-related genes exhibited opposite expression patterns in the amygdala and hippocampus.

To understand the function of gene regulation after the learning process, we screened six representative genes that showed more than two-$\log_2$ fold changes in the expression level compared with the control conditions (Fig. 6). In each case, we examined how their patterns appeared in the amygdala and hippocampus, observing opposite expression patterns in the two brain regions for all of them. Igf2 in the hippocampus was up-regulated in hippocampus after both CeA and BLA stimulation. Igf2 enhances memory retention [45]; especially, its increased expression in the hippocampal region is related to inhibitory avoidance learning. Therefore, Igf2 has been tested in memory-enhancing gene therapy for cognitive enhancement by its injection into the hippocampus [45–47]. Lmx1a, a memory- and learning-related gene, showed similar expression patterns as Igf2. Lmx1a is a transcription factor involved in the proliferation, differentiation, and maintenance of dopamine-producing neurons in the midbrain [48]. A clinical study has demonstrated the influence of the dopamine function related to the Lmx1a-coding gene on training-related working memory improvement [49]. Mdk, a growth factor involved in the development and repair of neural tissues, was up-regulated in the hippocampus. Mdk-deficient infant mice have shown delayed hippocampal development with impaired working memory

Table 2 Changed gene expressions in the hippocampus and amygdala after T-maze training with central amygdala (CeA) or basolateral amygdala (BLA) stimulation

Category	Gene	Description	Log_2 fold change			
			CeA stimulation		BLA stimulation	
			Amygdala	Hippocampus	Amygdala	Hippocampus
Memory	Igf2	Insulin-like growth factor 2, transcript variant X3	−3.551▼	1.906	−3.697▼	2.856▲
	Lmx1a	LIM homeobox transcription factor 1 alpha, transcript variant X1	−2.582▼	2.257▲	−2.963▼	3.781▲
	Neto1*	Neuropilin and tolloid-like 1	1.343	−1.526	0.492	−0.915
	Mdk*	Mdkine, transcript variant X3	−2.78▼	1.962	−1.492	2.198▲
	Lgmn*	Legumain	0.582	0.887	1.014	0.564
	Arc*	Activity-regulated cytoskeleton-associated protein, transcript variant X1	1.093	−1.496	1.432	−0.699
	Shank3*	SH3 and multiple ankyrin repeat domains 3	2.812▲	−2.036▼	1.288	−0.611
Learning	Dcdc2	Doublecortin domain containing 2	−0.934	0.543	−0.533	0.629
	Amph	Amphiphysin	0.705	−0.581	1.115	−0.633
	Adrb2	Adrenoceptor beta 2	0.929	1.614	0.584	0.69
	Neto1*	Neuropilin and tolloid-like 1	1.343	−1.526	0.492	−0.915
	Lgmn*	Legumain	0.582	0.887	1.014	0.564
	Arc*	Activity-regulated cytoskeleton-associated protein, transcript variant X1	1.093	−1.496	1.432	−0.699
	Shank3*	SH3 and multiple ankyrin repeat domains 3	2.812▲	−2.036▼	1.288	−0.611
	Stra6	Stimulated by retinoic acid 6	−0.65	2.337▲	−2.543▼	2.513▲
Fear	Mdk*	Midkine, transcript variant X3	−2.78▼	1.962	−1.492	2.198▲
Stress	Myh11	Myosin heavy chain 11	−1.128	1.741	0.521	2.094▲
	Fhod1	Formin homology 2 domain containing 1, transcript variant X2	−0.785	1.855	−0.782	1.302

*: overlapped gene; ▲,▼: log_2 fold change > 2.

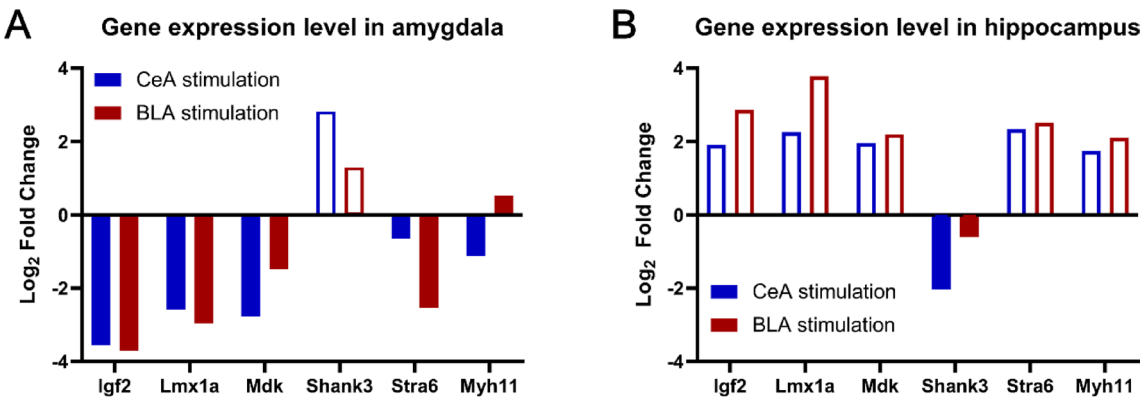

Fig. 6 Representative gene expression levels after central amygdala (CeA) or basolateral amygdala (BLA) stimulation in the **A** amygdala and **B** hippocampus

and increased anxiety [50], and a previous meta-analysis has identified Mdk as a hypo-anxious gene [51]. Therefore, an increased expression of Mdk in the hippocampus after training might have a positive effect on learning.

On the other hand, Shank3 exhibited an opposite expression pattern compared to Igf2, Lmx1a, and Mdk; after CeA or BLA stimulation, it was up-regulated in the amygdala and down-regulated in the hippocampus. Shank3 plays a crucial role in encoding proteins for glutamatergic neurotransmission in the postsynaptic density of neurons [52, 53]. Moreover, it is closely associated with autism-related behaviors [54, 55]. Thus, its reduction in the hippocampus suggests that side effects may occur when learning is performed using only a punishment training like amygdala stimulation. Note that the BLA stimulation resulted in higher expression levels than the CeA one for all these representative genes in the hippocampus. In contrast, the other representative changed genes, Stra6 and Myh11, were less considerable in this study; Stra6 is critical for cellular vitamin A uptake and homeostasis [56], and Myh11 is associated with providing strength and stability to body tissues, especially muscles [57].

3.4 Lever-pressing training for MFB stimulation

MFB was electrically stimulated as a target for reward group (group R and group RP). MFB stimulation was triggered by lever pressing, and rats were trained to press lever. Initially, rats need time to notice that the lever triggers MFB stimulation, and as time passes, they become aware lever-pressing makes them feel happy and be thrilled. Therefore, the number of lever-pressing increased with training days (Fig. 7). The rats that voluntarily pressed the lever more than 30 times per minute were selected for T-maze training, because it may regard that they were addicted to MFB stimulation and were tend to follow instructions to get more MFB stimulation.

3.5 Operant conditioning among three groups

To evaluate the different electrical stimulations for punishment and reward in rat navigation control, the effectiveness of the amygdala stimulation was compared with that of MFB stimulation. In particular, the BLA was selected for the amygdala stimulation since the prior experiment revealed that its stimulation is more effective for animal navigation control than that of the CeA (Fig. 8). Group RP was also included to determine whether the combination of reward and punishment can act synergistically in animal control. The success rate of the BLA stimulation exceeded that of the MFB stimulation in all cases (Fig. 8); it was higher from the first training session and maintained in the successive ones, while the success rate of the MFB stimulation increased slowly and stably during the sessions. Among the three groups, group RP exhibited the highest and most stable success rate; it reached over 80% after only two training sessions and was never below 90%. This indicates that reward and punishment stimulations can be effectively combined to induce a synergetic effect for rat navigation.

3.6 Tissue imaging

Cresyl violet staining was performed to identify the electrode track. When the exact location of the electrode was coronally sectioned, the electrode insertion path could be visualized; the brain region that was actually stimulated could also be precisely confirmed. The brain regions to be identified are different for each Group of rats, and as an example, the electrode track of Group R that received MFB stimulation is representatively shown in Fig. 9A, B. The electrode track was clearly visible in white, and its tip exactly aimed toward the MFB region.

Immunohistochemical staining was also conducted to detect the c-Fos expression in the targeted brain region. Increased c-Fos expression, an indicator of neuronal activity,

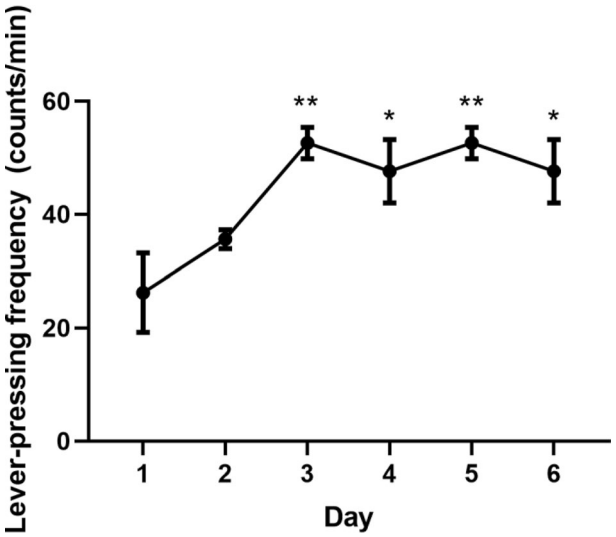

Fig. 7 Rats' lever-pressing frequency. (Paired t test, * $p<0.05$, ** $p<0.01$)

was observed in the BLA region, demonstrating successful neuronal activation by electrical stimulation (Fig. 9C, D).

4 Discussion

Generally, MFB stimulation has been utilized as a reward for rat navigation because they start seeking the stimulation and following directional commands during T-maze training. However, this method requires time-consuming lever-press training sessions to make the animals recognize it as a reinforcement. In the present study, amygdala stimulation was newly proposed as a punishment for rodent navigation control. Since the activation of the amygdala is closely related to fear and anxiety behaviors, we tried its electrical stimulation as a punishment to correct the wrong directional navigation. Compared with operant training based only on reward stimulation, amygdala stimulation has various advantages. For instance, the pre-training session is unnecessary because amygdala stimulation promptly triggers a fearful response, motivating rats to avoid repeating incorrect directional movements. This mechanism operates effectively due to the innate instinct of animals to survive, which is embedded in their sense of fear. Moreover, while the operant training with MFB stimulation showed a gradual learning curve, the BLA stimulation showed a higher success rate from the early training sessions. Also, when amygdala stimulation and MFB stimulation were combined, the training was even more effective in terms of both accuracy and training time.

The electrical stimulation of the amygdala induced various behavioral responses, specifically anxiety-related behaviors, which obviously varied depending on the stimulated targets in its subregions. The responses to BLA stimulation were mostly stepping backward and fleeing, while those to CeA stimulation were startling and jumping. However, it was also reported that irrelevant anxiety behaviors such as an excitable reaction, forward movement, jaw movement, and eye blinking can be induced by amygdala activation, indicating that the amygdala is involved in divergent neural pathways [58] (Fig. 10). Three plausible underlying mechanisms might explain why the animals reacted differently to the BLA and CeA stimulation. The first one is about the several unidirectional outputs from BLA to the ventral hippocampus (vHPC), bed nucleus of the stria terminalis (BNST), and CeA (Fig. 10). Neural information from BLA to vHPC can modulate anxiety-related behaviors [59, 60]. On the other hand,

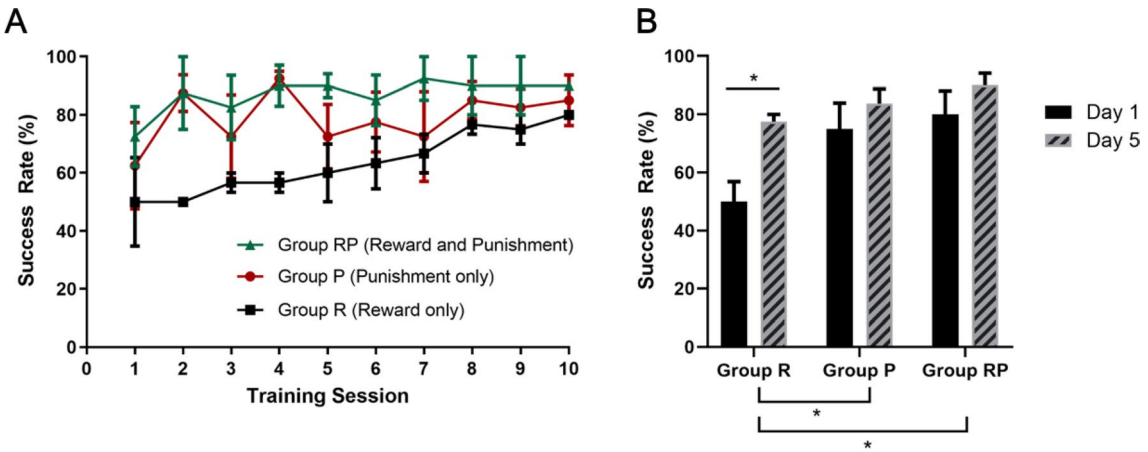

Fig. 8 Comparison of each group in T-maze operant training: **A** success rates for group P (only punishment, n = 4), group R (only reward, n = 3), and group RP (both punishment and reward, n = 3) (BLA: basolateral amygdala; MFB: medial forebrain bundle), and **B** average success rates for each group (Paired t test, * $p<0.05$)

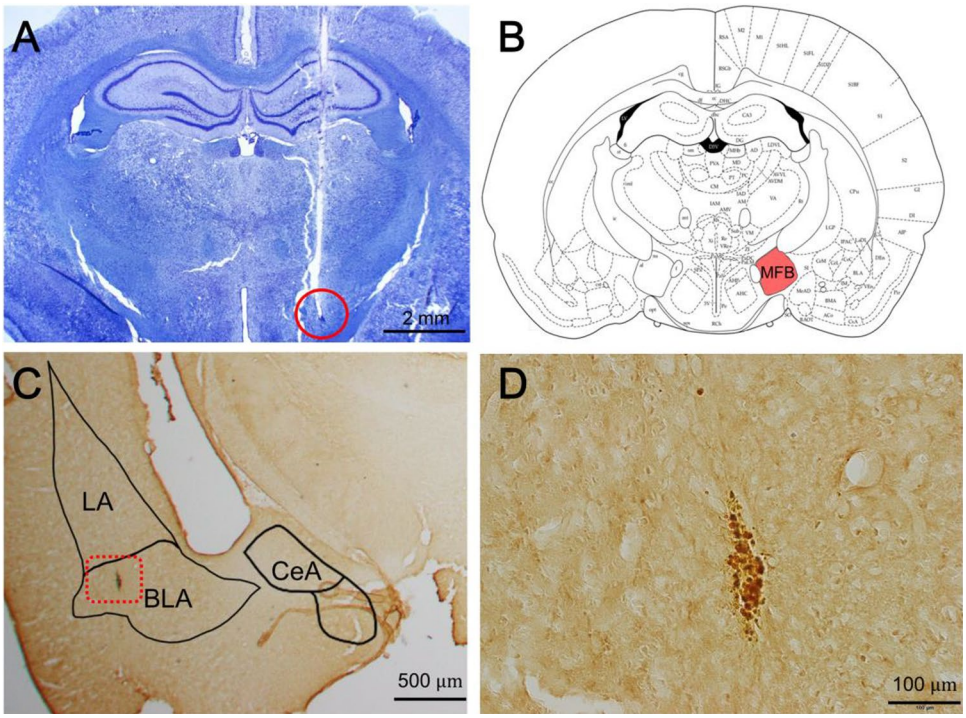

Fig. 9 Location of the electrode track for MFB stimulation: **A** cresyl-violet-stained coronal brain section including the electrode track; **B** rat brain atlas. **C** Immunohistochemically stained section of the amygdala, showing the electrically stimulated area in the BLA region (high-resolution image in **D**). (LA: lateral amygdala; BLA: basolateral amygdala; CeA: central amygdala)

the BLA-to-BNST pathway could mediate anti-anxiety responses, while the activation of the BLA–CeA one may induce both anxiety and anti-anxiety responses [61]. The opposite functions of which have been demonstrated using Cre-dependent viral techniques modulating the specific inputs and outputs of the given cell type [58, 62]. Second, the BLA and CeA are associated with, respectively, fear emotions and pain [63–68]. The sense of fear leads to stepping backward or fleeing, while startling and jumping would be related to pain responses. This explanation well matches our results shown in Fig. 2. Third, recent reports suggested that the amygdala is associated with not only fear but also reward; they confirmed that its lesions can impair reward-induced behaviors [69–73]. Moreover, some studies have argued that an increase in the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor in lateral amygdala neurons is required for a conditioned response to both fear and reward signals [74–76]. In addition, the amygdala stimulation on either on BLA or CeA can induce adverse effects on the animals. Therefore, it is needed to carefully monitor the animals' behavior to minimize pain and destress during any experiment with amygdala stimulation.

The success and correction rates obtained in this study shows that BLA stimulation is more effective than CeA stimulation as a punishment for rat navigation. This result

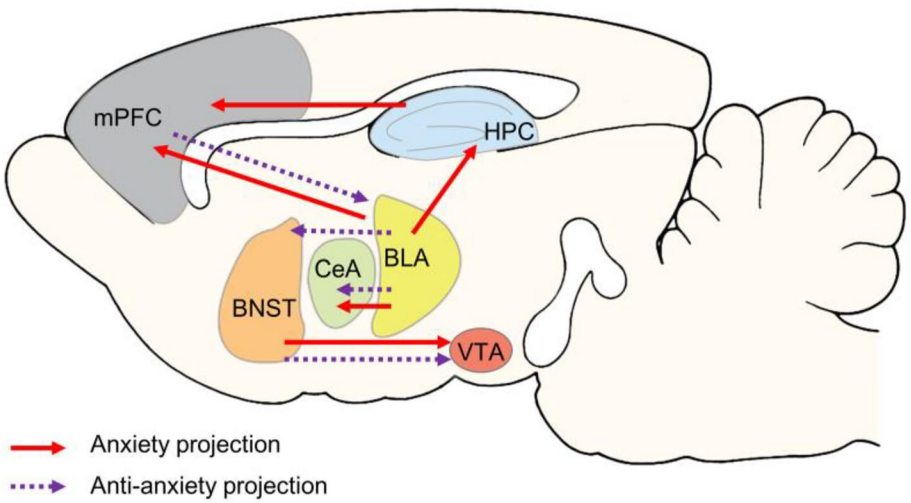

Fig. 10 Brain regions including the amygdala and its projections (BLA: basolateral amygdala; BNST: bed nucleus of the stria terminalis; CeA: central amygdala; vHPC: ventral hippocampus; mPFC: medial prefrontal cortex; VTA: ventral tegmental area)

might be attributed to the different stimulation responses between the two amygdala regions. As described above, the response to CeA stimulation was usually startling and jumping, which made the rats move too fast to control and change their directional behavior. On the other hand, the most common response to BLA stimulation was moving backward or escaping. The rats just contracted their body slightly and tried not to receive the stimulation again, as if they were trying not to be wrong again and correct their wrong directional decisions; consequently, they had more chances of learning commands after BLA stimulation. From these reasons, we can conclude that BLA stimulation is superior to CeA stimulation for rat navigation control.

RNA sequencing is an intensive technique for the detection of gene expression associated with specific neural changes. From the results of gene expression and regulation, we can understand the role of gene expression in forming memories. Memories can be interpreted as synaptic formation of neuronal cells, which is induced by protein translation from messenger RNA [77, 78]; in other words, increased experience-dependent gene expression related with synaptic changes implicates memory formation in the neuronal networks. In this study, we considered the gene expression related to not only memory but also learning, fear, and stress to understand the effect of punishment learning via amygdala stimulation. Several reports indicate that the amygdala and hippocampus can synergistically influence long-term memory formation [79, 80]. Some studies reported that gene expression in the amygdala is related to learning under stress [77, 81]. Differential gene expression between the hippocampus and amygdala has been observed after spatial learning under stress [81]; more specifically, the expression level of memory consolidation-related genes increased in the hippocampus after learning behavioral tasks, while its increase in the amygdala occurred only in animals that learned the tasks under high levels of stress. In the present study, the level of up-regulated gene expression in both the amygdala and hippocampus was higher after BLA stimulation than after CeA stimulation. We could explain this by inferring that the rats who received punishment via BLA stimulation were in a more stressful condition and learned better than with CeA stimulation. The stress-related genes in the amygdala and the learning- and memory-related ones in the hippocampus were more up-regulated after BLA stimulation than after CeA stimulation (Fig. 5). Along with behavioral responses, these gene expression results also imply that BLA stimulation can be more effective as a punishment in the learning process than CeA stimulation.

Even if we seriously studied amygdala to use as a punishment from various experiments, there are still some limitations in our study. First, amygdala stimulation sometimes induced freezing, prolonging the training sessions. Moreover, continued amygdala stimulation might cause psychopathy, such as post-traumatic stress disorder (PTSD) [82]. Therefore, as the number of our experimental trials increased, some rats became slower in motion, presumably due to their fear and anxiety memories from the T-maze training and the PTSD symptoms. Second, amygdala stimulation can induce both negative and positive emotions, as reported in many previous studies for the electrical stimulation of the left amygdala [83, 84]. Moreover, in this present study, 9% of amygdala stimulation induced excited conditions. This is because the amygdala is involved in both anxiety and anti-anxiety responses; therefore, two opposite behaviors can be induced by amygdala stimulation even though amygdala is precisely targeted. These limitations could be the reasons for the instability in the success rate of amygdala stimulation in T-maze training. Nonetheless, this study shows an interesting idea that amygdala stimulation can work as a behavioral demotivator and also that together with MFB stimulation, they function as carrot and stick. Considering the further application on rat navigation control, optogenetic stimulation can potentially be utilized for specific activation or inhibition of neural pathways. Most of the CeA is composed of GABAergic inhibitory neurons, while the BLA mainly consists of glutamatergic neurons [67, 85]. However, the electrical stimulation method stimulates all the neurons located near the electrodes regardless of their cell type. This means that even if the electrical stimulation is assigned to activate a specific brain region, the targeted brain activities can be suppressed rather than excited. Therefore, optogenetic stimulation can be a good alternative to control rat navigation, in that this technique can stimulate distinct cell type or neural pathway.

5 Conclusion

We developed a new method to navigate rat locomotion behaviors by applying amygdala stimulation. The amygdala subregions BLA and CeA were intensively examined. Along with T-maze training with BLA or CeA stimulation, RNA sequencing was conducted to investigate the regulation of relevant genes in terms of memory, learning, fear, and stress. The BLA stimulation was more effective as a punishment than the CeA stimulation, and its effectiveness was also higher than that of the conventional method using MFB stimulation as a reward. The success rate of the MFB stimulation exhibited a stably increasing tendency but could not exceed that of the BLA stimulation. Finally, the combination of MFB and BLA stimulation for both reward and punishment had a clear synergetic effect on operant conditioning of rat navigation; this new approach showed not only the highest and most stable success rate but also the fastest operant training.

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Author contributions YL: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Methodology, Writing—original draft. SK: Investigation, Methodology. YKC: Investigation, Methodology. CK: Investigation, Methodology. JWC: Conceptualization, Supervision, Funding acquisition, Project administration. SBJ: Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Project administration, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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