Research

# Anterior cingulate cortex projections to the dorsal hippocampus positively control the expression of contextual fear generalization

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**Fear generalization is one of the main symptoms of posttraumatic stress disorder. In rodents, the anterior cingulate cortex (ACC) and the hippocampus (HPC) control the expression of contextual fear memory generalization. Consistently, ACC projections to the ventral HPC contribute to contextual fear generalization. However, the roles of ACC projections to the dorsal HPC (dHPC) in fear generalization are unclear, although the dHPC is required for the retrieval of recent contextual fear memory. To investigate these roles, we examined the effects of optogenetic silencing and stimulation of these projections in contextual fear generalization at the recent and remote time points. Mice underwent contextual fear conditioning and, at 1 or 28 d later, were tested in the conditioned chamber, a novel context, or a similar context. Optogenetic activation of these projections induced higher freezing in mice in the novel context compared with the control group at a recent (1-d), but not remote (28-d), time point following conditioning, suggesting that activation of this pathway enhances contextual fear generalization. In contrast, optogenetic inactivation of these projections induced lower freezing in the similar context compared with the control group at a recent, but not remote, time point, suggesting that inactivation of this pathway impaired contextual fear generalization. These observations suggest that the ACC to the dHPC projections positively regulate the expression of contextual fear generalization when contextual fear memory is recent.**

Posttraumatic stress disorder (PTSD) is a mental disorder associated with traumatic memory, including fear memory. Some aspects of PTSD may be modeled using Pavlovian fear conditioning, which generates conditioned fear memory, reflecting an association between a conditioned stimulus (e.g., a conditioned context) and an unconditioned stimulus (for example, an electrical footshock). One of the main symptoms of PTSD is fear generalization, in which a fear conditioned human/animal exhibits fear responses to similar stimuli; that is, they fail to discriminate the conditioned stimulus from similar stimuli.

Contextual fear generalization is observed when contextual fear memory is remote; that is, animals fail to discriminate the conditioned context and a similar context at a remote time point (Biedenkapp and Rudy 2007; Wiltgen and Silva 2007; Wiltgen et al. 2010; Yokoyama and Matsuo 2016; Qin et al. 2019). Recent contextual fear memory depends on the dorsal hippocampus (dHPC), whereas remote contextual fear memory is dependent on cortical regions including the anterior cingulate cortex (ACC) (Frankland et al. 2004; Gafford et al. 2013). Importantly, the ACC regulates the expression of contextual fear generalization; silencing or stimulating the activity of the ACC blocks or enhances, respectively, contextual fear generalization (Cullen et al. 2015; Einarsson et al. 2015; Bian et al. 2019; Ortiz et al. 2019). Interestingly, the ventral HPC (vHPC) contributes to contextual fear generalization in a similar manner as the ACC (Cullen et al. 2015; Bian et al. 2019; Ortiz et al. 2019). Furthermore, the ACC controls the expression of contextual fear generalization at a remote time point through projections to the vHPC (Bian et al. 2019).

The activity of the dHPC is required for the accuracy of recent contextual fear memory, whereas the vHPC is involved in emotion and stress (Fanselow and Dong 2010; Wiltgen et al. 2010; Alvares et al. 2012). Importantly, bidirectional projections are observed between the dHPC and the ACC (Rajasethupathy et al. 2015). Therefore, it is possible that the projections from the ACC to the dHPC regulate the expression of contextual fear generalization (contextual fear discrimination).

In the current study, we investigated the roles of the ACC-to-dHPC projections in contextual fear generalization. To do this, we examined the effects of silencing and stimulating these projection terminals in the CA1 area of the dHPC in contextual fear generalization at recent and remote time points from contextual fear conditioning.

## Results

### Optogenetic activation of the ACC-to-dHPC projection pathway enhances the generalization of contextual fear memory

Remote, but not recent, contextual fear memory shows generalization of a conditioned context. To understand the roles of the ACC-to-dHPC projections in contextual fear generalization, we first examined the effect of optogenetic activation of these projections on the generalization of recent contextual fear memory (24-h memory). The mice received a microinfusion of an adeno-associated virus (AAV) vector expressing channelrhodopsin-2 (ChR2) under the control of the CaMKII promoter into the ACC, and the projection pathway was targeted with blue light at the axon terminals in the dHPC (Fig. 1A). Consistent with previous

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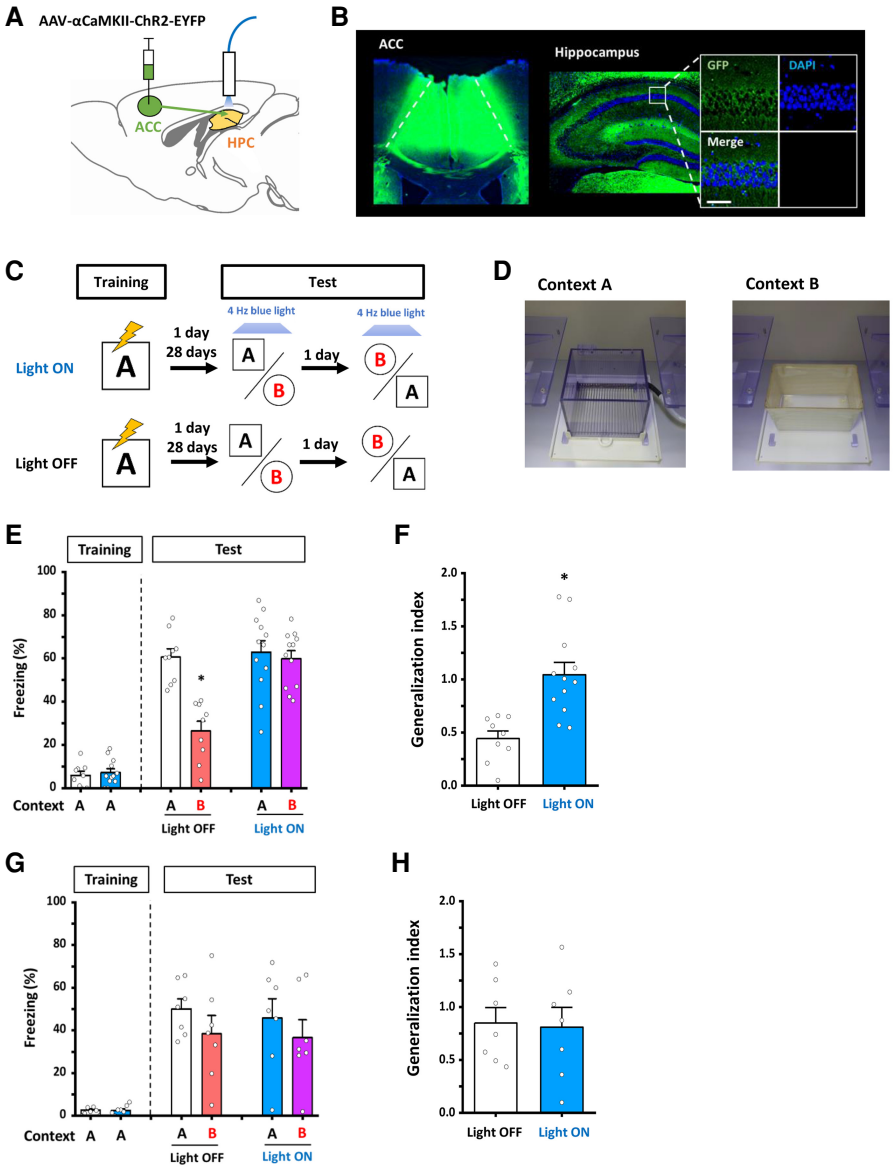


**Figure 1.** Optogenetic activation of the ACC-to-dHPC projection pathway in a novel contextual chamber. (*A*) AAV5-CaMKII-ChR2-EYFP injection into the ACC and optic fiber implantation into the hippocampus (HPC). (*B*) Representative image of the ACC injected by AAV5-CaMKII-ChR2-EYFP (*left*) and projection terminals in the hippocampal CA1 area (*right*). Scale bar, 50 µm. (*C*) Experimental schedule. (*D*) Images of the chambers. (*E*) Effects of optogenetic activation of the ACC-to-dHPC projections on recent fear memory in the conditioned chamber (context A) or a novel context chamber (context B) (Light Off, $n=9$; Light On, $n=12$). (*) $P<0.05$, compared with context A at test. (*F*) Levels of the generalization index at recent contextual fear memory. The generalization index was calculated using the following formula: (freezing percentage in context B)/(freezing percentage in context A). (*) $P<0.05$, compared with Light Off group. (*G*) Effects of optogenetic activation of the ACC-to-dHPC projections on remote fear memory (Light Off, $n=7$; Light On, $n=7$). (*H*) Levels of the generalization index at remote contextual fear memory. Error bars indicate SEM.

observations (Rajasethupathy et al. 2015), we detected projection terminals around hippocampal CA1 pyramidal cells at 3 wk after microinfusion, and blue light was used to stimulate the axon terminals presynaptic to CA1 cell bodies (Fig. 1B). The mice underwent contextual fear conditioning and, 24 h later, were tested in the conditioned chamber (context A) or a novel context chamber (context B). Light On mice were stimulated at 4 Hz (pulse width, 15 msec) using a 473-nm laser during test (Fig. 1C,D). Two-way analysis of variance (ANOVA) revealed significant effects of light (Off vs. On; $F_{(1,38)}=15.692$, $P<0.05$) and context (context A vs. context B; $F_{(1,38)}=17.032$, $P<0.05$), and a light × context interaction ($F_{(1,38)}=11.845$, $P<0.05$) (Fig. 1E). Post hoc Bonferroni's analysis revealed that the Light Off group froze significantly less in context B than in context A, suggesting that the Light Off group showed no generalization of context ($P<0.05$) (Fig. 1E). Interestingly, the Light On group froze comparably in contexts A and B, suggesting that Light On group showed generalization of the context; this group froze even in context B, in which they had not been fear-conditioned ($P>0.05$) (Fig. 1E). Consistently, one-way ANOVA of the generalization index showed a significant effect of light (Off vs. On; $F_{(1,19)}=16.348$, $P<0.05$) (Fig. 1F). The Light On group showed a significantly higher generalization index compared with the Light Off group ($P<0.05$). These results suggest that optogenetic activation of the ACC-to-dHPC projection pathway enhanced contextual fear generalization of recent memory.

We next examined the effect of optogenetic activation of the ACC-to-dHPC projection pathway on the generalization of remote contextual fear memory (28-d memory). The experiment was similar to that outlined in Figure 1C, except that the mice were tested at 28 d after training. Two-way ANOVA revealed no significant effects of light (Off vs. On; $F_{(1,24)}=0.144$, $P>0.05$) and context (context A vs. context B; $F_{(1,24)}=1.774$, $P>0.05$), and no light × context interaction ($F_{(1,24)}=0.024$, $P>0.05$) (Fig. 1G). Both groups showed comparable freezing in contexts A and B, suggesting that they showed contextual fear generalization. Consistently, there was no difference in the generalization index between the Light On and Light Off groups (one-way ANOVA, $F_{(1,12)}=0.0277$, $P>0.05$) (Fig. 1H). These observations are consistent with previous findings showing that contextual fear generalization is observed when contextual fear memory is remote and showed that optogenetic activation of the ACC-to-dHPC projections did not affect contextual fear generalization.

## Optogenetic inactivation of the ACC-to-dHPC projection pathway impairs the generalization of contextual fear memory

We next examined the effect of optogenetic inactivation of the ACC-to-dHPC projection pathway on the generalization of recent contextual fear memory (24-h memory). The experiment was similar to that outlined in Figure 1C, except that the mice received a microinfusion of an AAV vector expressing archaerhodopsin-T (ArchT) under the control of the CaMKII promoter into the ACC,

and the ACC projections were targeted with green light stimulation (532-nm laser) at the axon terminals in the dHPC during test (Fig. 2A,B). Two-way ANOVA revealed the significant effect of context (context A vs. context B; $F_{(1,24)}=39.787$, $P<0.05$), but no significant effect of light (Off vs. On; $F_{(1,24)}=0.232$, $P>0.05$) and no light × context interaction ($F_{(1,24)}=0.0676$, $P>0.05$) (Fig. 2C). Light Off and Light On groups froze significantly less in context B than in context A (Off, $P<0.05$; On, $P<0.05$) (Fig. 2C). It is important to note that the Light On and Light Off groups showed comparable freezing in context A ($P>0.05$), suggesting that the Light On group showed normal retrieval of contextual fear memory; inactivation did not impair the retrieval of contextual fear memory. Consistently, the Light On and Light Off groups showed comparable generalization indices (one-way ANOVA, $F_{(1,12)}=0.238$, $P>0.05$) (Fig. 2D). These results indicated that mice with optogenetic inactivation of the ACC-to-dHPC projections showed no generalization of recent contextual fear memory.

We next examined the effect of inactivation of the ACC-to-dHPC projections on the generalization of remote contextual fear memory (28-d memory). Two-way ANOVA revealed no significant effects of light (Off vs. On; $F_{(1,14)}=0.914$, $P>0.05$) and context (context A vs. context B; $F_{(1,14)}=0.189$, $P>0.05$), and no light × context interaction ($F_{(1,14)}=0.0082$, $P>0.05$) (Fig. 2E). Both groups showed comparable freezing in contexts A and B, suggesting that they showed generalization of contextual fear memory (Off, $P>0.05$; On, $P>0.05$) (Fig. 2E). Consistently, the Light On and Light Off groups showed comparable generalization indices (one-way ANOVA, $F_{(1,7)}=0.0027$, $P>0.05$) (Fig. 2F). These observations suggest that optogenetic inactivation of the ACC-to-dHPC projections failed to block contextual fear generalization when memory is remote.

Inactivation of the ACC-to-dHPC projections showed no effects on contextual fear generalization (Fig. 2). However, the results shown in Figure 2C raised the possibility that the effect of inactivation of these projections on the generalization of contextual fear memory was masked since the mice had no difficulty in discriminating between contexts A and B. Therefore, we next examined the effects of optogenetic inactivation of the ACC-to-dHPC projections using a similar contextual chamber (context C). The experiment was similar to that outlined in Figure 2B, except that the mice were tested in context A or C (Fig. 3A,B). Two-way ANOVA revealed significant effects of light (Off vs. On; $F_{(1,30)}=4.795$, $P<0.05$) and context (context A vs. context C; $F_{(1,30)}=14.251$, $P<0.05$), but no light × context interaction ($F_{(1,30)}=3.989$, $P>0.05$) (Fig. 3C). The Light On group froze significantly less in context C than in context A (Off, $P>0.05$; On, $P<0.05$) (Fig. 3C), while the Light Off group froze comparably in contexts A and C. Consistently, the Light On group showed a significantly lower generalization index than the Light Off group (one-way ANOVA, $F_{(1,15)}=10.993$, $P<0.05$) (Fig. 3D). These observations suggest that contextual fear generalization occurs when contexts A and C are similar, even when memory is recent, and that inactivation of the ACC-to-dHPC projections blocks contextual fear generalization.

We finally examined the effects of optogenetic inactivation of the ACC-to-dHPC projections on remote contextual fear memory (28-d memory) using a similar contextual chamber (context C). Two-way ANOVA revealed no significant effects of light (Off vs. On; $F_{(1,24)}=0.234$, $P>0.05$) and context (context A vs. context C; $F_{(1,24)}=0.251$, $P>0.05$), and no light × context interaction ($F_{(1,24)}=0.707$, $P>0.05$) (Fig. 3E). Both groups showed comparable freezing in contexts A and C, suggesting they showed generalization of contextual fear memory (Off, $P>0.05$; On, $P>0.05$) (Fig. 3E). Consistently, the Light On and Light Off groups showed comparable generalization indices (one-way ANOVA, $F_{(1,12)}=$

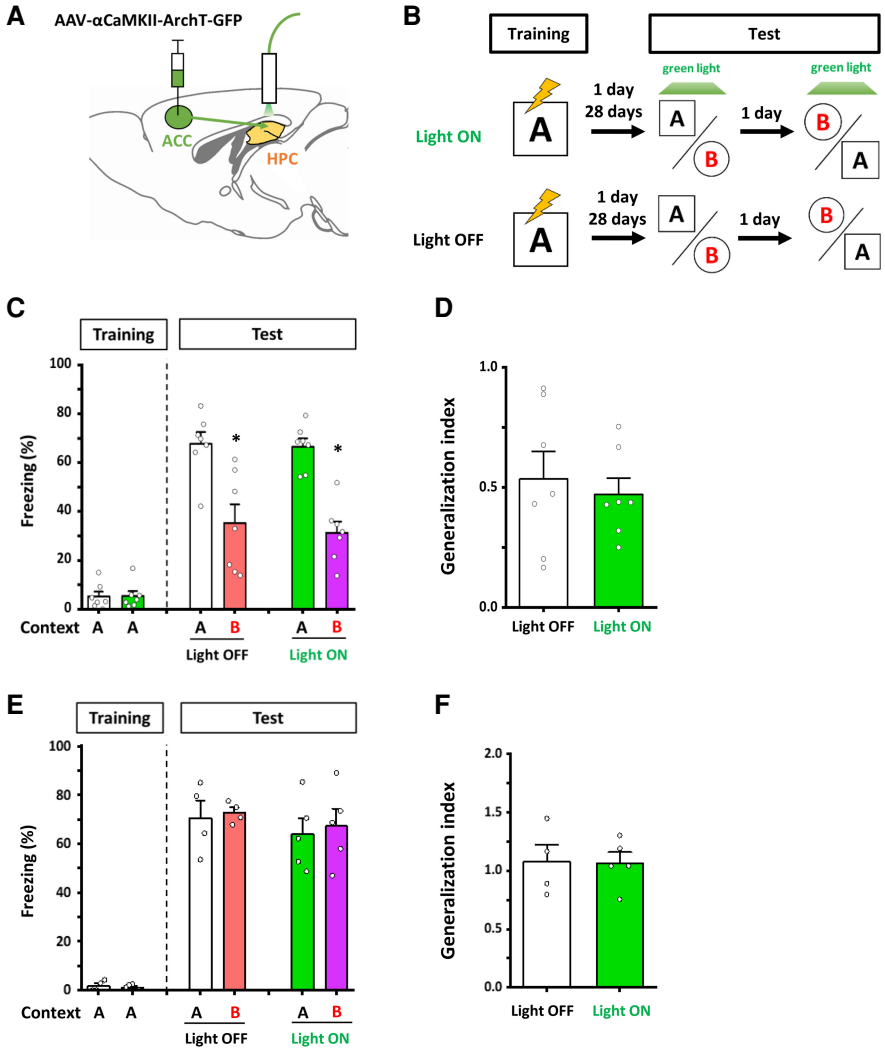


**Figure 2.** Optogenetic inactivation of the ACC-to-dHPC projection pathway in a novel contextual chamber. (*A*) AAV5-CaMKII-ArchT-GFP injection into the ACC and optic fiber implantation into the hippocampus. (*B*) Experimental schedule. (*C*) Effects of optogenetic inactivation of the ACC-to-dHPC projections on recent fear memory in the conditioned chamber (context A) or a novel context chamber (context B) (Light Off, $n=7$; Light On, $n=7$). (*D*) Levels of the generalization index at recent contextual fear memory. (*E*) Effects of optogenetic inactivation of the ACC-to-dHPC projections on remote fear memory (Light Off, $n=4$; Light On, $n=5$). (*F*) Levels of the generalization index at remote contextual fear memory. (*) $P<0.05$, compared with context A at test. Error bars indicate SEM.

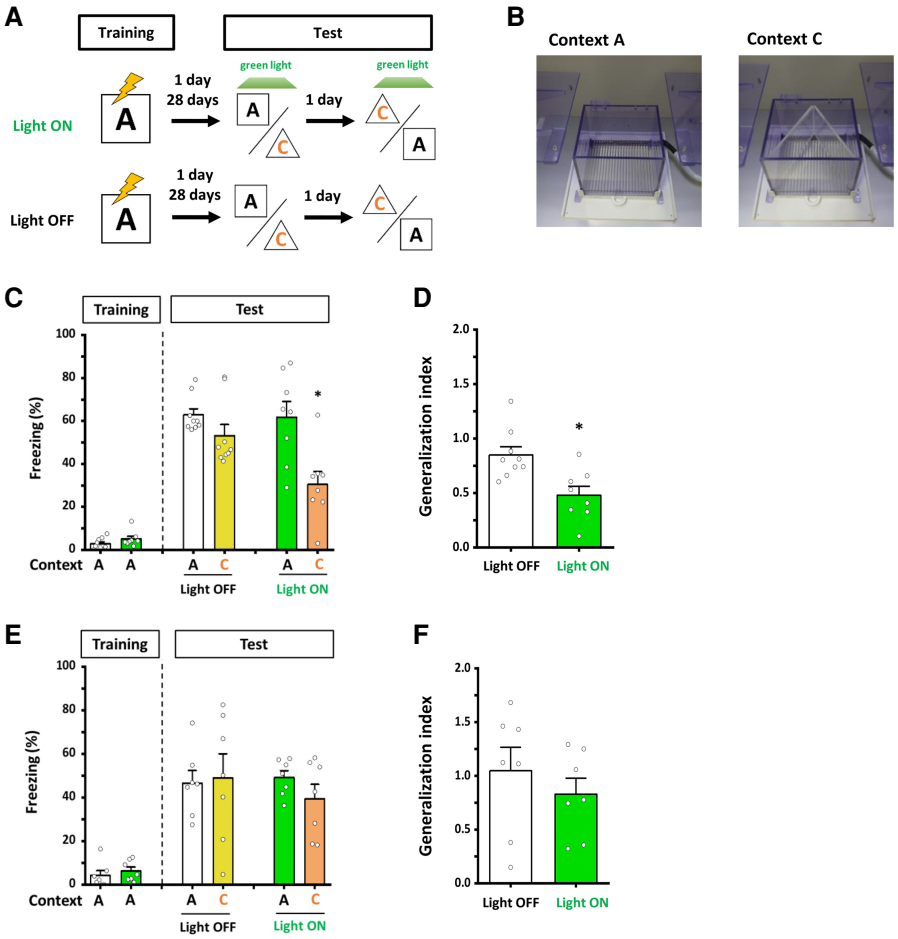


**Figure 3.** Optogenetic inactivation of the ACC-to-dHPC projection pathway in a similar contextual chamber. (*A*) Experimental schedule. (*B*) Images of the chambers. (*C*) Effects of optogenetic inactivation of the ACC-to-dHPC projections on recent fear memory in the conditioned chamber (context A) or a similar context chamber (context C) (Light Off, $n=9$; Light On, $n=8$). (*) $P<0.05$, compared with context A at test. (*D*) Levels of the generalization index at recent contextual fear memory. The generalization index was calculated using the following formula: (freezing percentage in context C)/(freezing percentage in context A). (*) $P<0.05$, compared with Light Off group. (*E*) Effects of optogenetic inactivation of the ACC-to-dHPC projections on remote fear memory (Light Off, $n=7$; Light On, $n=7$). (*F*) Levels of the generalization index at remote contextual fear memory. Error bars indicate SEM.

0.692, $P>0.05$) (Fig. 3F). These observations suggest that optogenetic inactivation of the ACC-to-dHPC projection failed to block contextual fear generalization when memory is remote.

## Discussion

In the present study, we investigated the roles of the ACC-to-dHPC projection pathway in contextual fear generalization. To study this, we examined the effects of optogenetic activation and inactivation of these projections on the expression of contextual fear generalization of recent and remote memories in the conditioned context and in a novel or similar context. Optogenetic activation of these projections at the axon terminals in the dHPC enhanced contextual fear generalization when the mice were exposed to a novel context at a recent (1-d) time point without affecting fear generalization at a remote time point. In contrast, optogenetic inactivation of these projections blocked contextual fear generalization when the mice were exposed to a similar context only at a recent time point, although this inactivation showed no effect on the retrieval of contextual fear memory. In other words, optogenetic activation or inactivation of this pathway impairs or improves, respectively, the discrimination of the conditioned context and novel/similar contexts. These observations suggest that the ACC-to-dHPC projections positively regulate the expression of contextual fear generalization when contextual fear memory is recent.

Activity of the dHPC is required for the retrieval of recent contextual fear memory (Frankland et al. 2004; Wiltgen et al. 2010); inactivation of the dHPC blocks contextual fear memory retrieval in the conditioned context. Interestingly, our observations indicate that optogenetic inactivation of the ACC-to-dHPC projections did not affect the retrieval of fear memory in the conditioned context (but see Rajasethupathy et al. 2015), but improved contextual discrimination (conditioned vs. similar contexts). In contrast, optogenetic activation of these projections enhanced contextual fear generalization; this activation increased fear responses in a novel context without affecting these responses in the original context. Similarly, optogenetic stimulation of these projections in a novel context 1 d after conditioning increased freezing, suggesting that this activation promotes contextual fear generalization (Rajasethupathy et al. 2015). These observations suggest that the ACC-to-dHPC projections contribute to contextual fear generalization, but not the retrieval of contextual fear memory.

Chemogenetic silencing or stimulation of the activity of the ACC or vHPC blocks or enhances, respectively, contextual fear generalization only when contextual fear memory is remote. Furthermore, a previous study showed that the ACC controls the expression of contextual fear generalization through projections to the vHPC (Bian et al. 2019). Conversely, our results indicate that optogenetic inactivation of the ACC-to-dHPC projections did not affect contextual fear generalization at a remote time point, although this inactivation enabled the discrimination of the original and similar contexts at a recent time point. These findings raise the possibility that the ACC-to-vHPC projections are required for contextual fear generalization when contextual fear memory is remote.

Importantly, PTSD patients show fear generalization (Dunsmoor and Paz 2015). In the present study, optogenetic activation of the ACC-to-dHPC projections induced contextual fear generalization even when contextual fear memory was recent. Therefore, overactivation of this neural circuit may contribute to acute fear generalization in patients with PTSD.

The dentate gyrus and CA3 areas in the HPC contribute to pattern separation and completion; Memories can be fully reactivated using partial recall cues through pattern completion, whereas distinct memories can be formed and reactivated through pattern separation by representations of the temporal and spatial relationships that comprises the episodes (Nakazawa et al. 2002;

McHugh et al. 2007; Nakashiba et al. 2012). The present findings suggest that activation of the ACC-to-dHPC projections may impair the expression of pattern separation. These projections may affect pattern separation by modulating the output from the dentate gyrus/CA3 areas. In the future, it will be interesting to investigate further the modulatory roles of these projections for pattern separation and completion using established mouse models of pattern separation and completion (Nakashiba et al. 2012).

In the present study, we found that the activation or inactivation of projection terminals from the ACC to dHPC facilitates or reduces contextual fear generalization, respectively, when contextual fear memory is recent. Our findings suggest these projections play a regulatory role in contextual fear generalization.

## Materials and Methods

### Mice

All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (Japan Neuroscience Society and Tokyo University of Agriculture). The Animal Care and Use Committee of Tokyo University of Agriculture (authorization no. 300048) approved all of the animal experiments that were performed in this study. All surgical procedures were performed under Nembutal anesthesia, with every effort to minimize suffering. Male C57BL/6N mice were obtained from Charles River. The mice were housed in cages of 5 or 6, maintained on a 12-h light–dark cycle, and allowed ad libitum access to food and water. The mice were at least 8 wk of age at the start of the experiments, and all behavioral procedures were conducted during the light phase of the cycle. All experiments were conducted by researchers who were blinded to the treatment condition of the mice.

### Virus injection and cannula implantation

AAV5-CaMKIIα-ChR2-EYFP (titer $4.1 \times 10^{12}$ genome copies [gc]/mL) and AAV5-CaMKIIα-ArchT-GFP ($5.2 \times 10^{12}$ gc/mL) were generated by and acquired from the University of North Carolina at Chapel Hill Vector Core.

For virus injections, the stereotaxic injection of AAV vectors was performed in a biological safety cabinet. The mice were anesthetized with Somnopentyl (50 mg/kg, i.p.; Kyoritsu Seiyaku Corporation) and placed in a stereotaxic frame. The skull was exposed and a small portion of the skull over the ACC was removed bilaterally with a drill. AAVs (0.2 µL/site at a speed of 0.1 µL/min) were injected into the brain using a glass capillary pipette (+0.8 mm anteroposterior, ±0.2 mm mediolateral, −1.5 mm dorsoventral from the dura) pulled with a micropipette puller (Sutter Instruments P-87). After injection, the glass pipette was left in place for another 5 min before being slowly lifted up and removed. The mice were sutured, and an antibiotic ointment was applied. The mice were then kept on a warm heater for recovery.

The mice were allowed to recover for at least 1 wk after surgery. For optic fiber implantation, the mice were anesthetized with Somnopentyl (50 mg/kg, i.p.; Kyoritsu Seiyaku Corporation), and using standard stereotaxic procedures, a stainless-steel guide cannula (22 gauge) was implanted into the dHPC. The stereotaxic coordinates for dHPC placement based on the brain atlas of Franklin and Paxinos (1997) were as follows: −1.6 mm, ±1.6 mm, and −1.1 mm. The mice were allowed to recover for at least 1 wk after surgery. Successful transduction of the dHPC was confirmed histologically by native EGFP fluorescence. Only mice showing bilateral EGFP expression in the dHPC were included in subsequent data analyses.

### Contextual fear conditioning task

The mice were handled for five consecutive days prior to the commencement of contextual fear conditioning. The mice were trained in conditioning chambers (17.5 × 17.5 × 30 cm; O'Hara & Co., Ltd) that had a stainless-steel grid floor through which a footshock could be delivered. Training consisted of placing the mice in the chamber and delivering an unsignaled footshock (2-sec duration, 0.4 mA) at 148 sec later. Then, the mice were returned to their home cage at 30 sec after the footshock. At 1 or 28 d after training, the mice were placed back in the training context (context A), a novel context (context B; opaque white walled cage, 12 × 16 × 11 cm) (Fig. 1D), or a similar context chamber (context C; triangular chamber, 17.5 × 17.5 × 30 cm) (Fig. 3B) for 5 min and freezing was assessed (test). Blue light stimulation was delivered at 4 Hz, 15 msec pulse width using a 473-nm laser (Lucir COME-LB473/200) (Fig. 1), and green light stimulation was delivered using a 532-nm laser (Lucir COME-LG532/200) (Figs. 2, 3), during test. The generalization index was calculated using the following formula: (freezing percentage in context B or context C)/(freezing percentage in context A).

### Microscopy

The mice were perfused transcardially with 4% paraformaldehyde. Brains were then removed, fixed overnight, transferred to 30% sucrose, and stored at 4°C. Coronal sections (30 µm) were cut in a cryostat. Fluorescent images were acquired using a confocal microscope (Leica TCS SP8) with a 40× objective or a fluorescence microscope (Keyence BZ-X710). Confocal 2-µm *z*-stack images were obtained using LAS AF software (Leica). Equal cutoff thresholds were applied to all slices.

### Data analysis

One- or two-way factorial ANOVA followed by post hoc Bonferroni's comparisons were used to analyze the effects of light and context. All values in the text and figure legends represent the mean ± standard error of the mean (SEM).

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