[**Original Research**]

Conditional Activation of Peripheral Sensory Nerves Induces an Aversive State with the Down-Regulation of Neural Functions of the Nucleus Accumbens

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Abstract: The mesolimbic dopaminergic network from the ventral tegmental area (VTA) to the nucleus accumbens (N.Acc.) has been shown to play a crucial role in the reward system. Although it has been documented that conditional activation of sensory nerves induces aversive behaviors, little is known about the mechanism of aversion induced by the sustained activation of peripheral sensory nerves. In the present study, we demonstrated that conditioned place aversion was induced by the conditional activation of sensory nerves via a Gq-Designer Receptors Exclusively Activated by Designer Drugs (DREADD) system. We found a dramatic decrease in the expression level of cAMP response element binding protein (CREB), an activated neuron marker, in the N.Acc. of mice with the conditional activation of sensory nerves. These results suggest that the aversive behavior associated with a decreased pain threshold could be induced by the conditional activation of sensory nerves through the attenuation of neural activity in the N.Acc.

Key words: Sensory neuron, Pain, Aversion, cAMP response element binding protein (CREB), Nucleus accumbens (N.Acc.)

INTRODUCTION

Stimulus-triggered neuromodulative techniques, including optogenetics and chemogenetics, have been developed over the past decade[1, 2]. Chemogenetics uses a small-molecule ligand to activate synthetic G protein-coupled receptors (Designer Receptors Exclusively Activated by Designer Drugs, DREADDs) or ionic conductance (Pharmacologically Selective Actuator Modules, PSAMs), with several downstream effects on neuronal excitability. These approaches may directly influence a specific locus without regulating the overall nervous system.

Recently, it has been revealed that the activation of peripheral sensory neurons using optogenetics with sciatic nerve-injection of adeno-associated viruses encoding an excitatory opsin enabled light-inducible acute pain[3]. Furthermore, we recently reported that activation of peripheral sensory neurons using optogenetics induces a significant decrease of dopamine production, storage and release in the caudate putamen (CP), the lateral part of the nucleus accumbens (N.Acc.) and the medial part of the N.Acc.[4]

Dopaminergic neurons from the ventral tegmental area (VTA) to the N.Acc. are predicted to play important roles in the reward system[5]. In fact, activation of dopaminergic neurons by the administration of morphine, cocaine, or methamphetamine induces rewarding effects[6–8]. On the other hand, it has been reported that chronic pain suppresses the intrinsic activity of mesolimbic dopaminergic neurons, whereas transient activation of the mesolimbic dopaminergic system inhibits hyperalgesia[9]. These previous reports indicate that inactivation of the mesolimbic dopaminergic pathway may be involved in pain-induced aversive states.

In the present study, we investigated whether conditioned place aversion could be induced by specific and conditional activation of peripheral sensory neurons via a Gq-DREADD system along with dynamic changes in neural functions of the N.Acc.

MATERIAL AND METHODS

1. Animals

In the present study, we conducted all procedures in

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accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University. For all of the present studies, we mostly used 7-week-old male C57BL/6J mice (Tokyo Laboratory Animals Science, Tokyo, Japan). These mice were housed in groups of 5 in animal quarters maintained at an ambient temperature of 23 ± 1℃ using a standard 12 h light-dark cycle (lights on 8:00 a.m. to 8:00 p.m.). During the experimental period, food and water were available ad libitum. We minimized the number and suffering of the animals.

2. Artificial activation of sensory neurons by the DREADD system

2-1. *Virus vector*: To achieve cell-type-specific hM3Dq expression, we used the adeno-associated virus serotype 6 (AAV6) vector with a human synapsin 1 promoter (AAV6-hSyn-hM3Dq-mCherry; AAV6-hM3Dq), and the AAV vector without the hM3Dq gene (AAV6-hSyn-EGFP) as a control. The hM3Dq coding sequence was cloned upstream of an mCherry sequence to generate C-terminal mCherry fusion proteins.

2-2. *AAV injection*: To selectively express hM3Dq receptors in sensory neurons, AAV6-hM3Dq was microinjected into the sciatic nerve of C57BL/6J mice[3]. Mice were anesthetized under 3% isoflurane at the start of the surgical procedures. The recombinant AAV was microinjected into the sciatic nerve through an internal cannula (Eicom Co., Kyoto, Japan) at 1 μL/min for 4 min (4 μL total volume). After the surgical procedures, mice were allowed to recover in individual housing for at least 2 weeks, during which period they fully expressed AAV vector-derived transgene. To selectively manipulate the activity of sensory nerves, these mice were then intraperitoneally injected with clozapine *N*-oxide (CNO, 3 mg/kg, t.i.d., Abcam plc., Cambridge, UK) for 2 weeks.

3. Immunohistochemistry

As previously reported[9, 10], 2 weeks after AAV injection, mice were anesthetized by the inhalation of 3% isoflurane with oxygen, and intracardially perfusion-fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After perfusion, the dorsal root ganglion (DRG) and sciatic nerves were quickly removed and post-fixed in 4% paraformaldehyde. They were then permeated with 20% sucrose in 0.1 M PBS and 30% sucrose in 0.1 M PBS for 1 day with agitation. Transverse sections (8 μm thick) were cut with a cryostat (Leica CM1510; Leica Microsystems, Heidelberg, Germany) and blocked in 3% normal goat serum with 0.1% triton in 0.01 M PBS for 1 h at room temperature. The DRG and sciatic nerve sections were incubated with primary antibodies: anti-peripherin (goat polyclonal 1:50, Santa Cruz Biotechnology), anti-myeline (1:300, Molecular Probes) and anti-mCherry (rabbit polyclonal 1:1000, Abcam). Following washes, the samples were incubated with an appropriate secondary antibody conjugated with Alexa 488 or 546 (Invitrogen). The sections were mounted with Dako fluorescent mounting medium (Dako, Glostrup, Denmark). Fluorescence of immunolabeling was detected using a light microscope (BX-61; Olympus, Tokyo, Japan) and photographed with a digital camera (MD-695; Molecular Devices).

4. Measurement of thermal thresholds

We measured the thermal thresholds as described previously[9]. Briefly, thermal response thresholds were used to assess manipulation of the sciatic nerve. Following exposure to the testing chambers, mice were tested individually by exposure to a focused, radiant-heat light source (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA, USA). We considered only quick hind paw movements (with or without licking of the hind paws) away from the stimulus, and not movements associated with locomotion or weight-shift, as a withdrawal response. Under these conditions, we measured the latency of paw withdrawal in response to the thermal stimulus.

5. Conditioned place aversion test

We conducted the conditioned place aversion/preference test as previously reported[11]. In the present study, we used a new shuttle box (15 cm wide×30 cm long×25 cm high; O'Hara & Co., Ltd., Tokyo, Japan), which was divided into two equal-sized compartments, made of acrylic resin board. One compartment was white with a textured floor, and the other was black with a smooth floor. The conditioned place aversion test consisted of a pre-conditioning test, conditioning, and a post-conditioning test. For each test, the time spent in each compartment during a 900 s session was recorded with an infrared beam sensor (TimeLD4 for Light/dark transition; O'Hara & Co., Ltd.). After the pre-conditioning test, we performed conditioning sessions (6 days each for CNO and saline) once daily for 12 days. Immediately after CNO injection, mice were conditioned in the compartment in which they had spent the most time in the pre-conditioning test for 1 h. On alternative days, mice received saline and were conditioned in the other compartment for 1 h. The post-conditioning test was performed 1 day after the final conditioning session.

6. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA isolated from the N.Acc. and VTA in mice with aversive behaviors 2 days after the post-test was extracted by a mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Inc.). According to the manufacturer's protocol, 0.5 μg of purified RNA was then reverse transcribed into cDNA using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). We synthesized primers (Table 1) and performed qPCR using a StepOnePlus™ system (Applied Biosystems, Inc.) with Fast SYBR® Green Master Mix (Applied Biosystems, Inc.) and with GAPDH as a normalization control.

7. Statistical analysis

The present data are shown as means ± standard error of the mean (SEM). We analyzed the statistical significance of differences between the two groups by using the unpaired *t*-test with Prism version 5.0 (GraphPad software, La Jolla, CA, USA). We also used the Pearson product moment correlation as appropriate for the

Table 1 RT-PCR primer sequences

Primer	F/R	Sequence(5'-3')
mGlu1	Forward	TCG GCT ACG TAT GCC CTT TC
	Reverse	TGC GTG CAA TAC GAT TGG TT
mGlu2	Forward	GGC CAA GTT CAT CGG CTT TA
	Reverse	CGG ACA CGC ACA TCG TAG TG
mGlu3	Forward	GGA AGC AAG GCT ACG CAA CA
	Reverse	ACA ACT CGC GCG TTA GGT TT
mGlu4	Forward	ACG GCT CGG CTG AGT ACA AG
	Reverse	GCC ACT GCA TCC GCT CTA TT
mGlu5	Forward	GGG CAG TCC GTG AGC AGT AT
	Reverse	TGG GCA AGA GTG TGG GAT CT
mGlu6	Forward	TCA TGG TAG CCG AGC CTT GT
	Reverse	TTG CTC GAA AAT GCG GTA GA
mGlu7	Forward	AAC CTG CTG CCC AAC GTA AC
	Reverse	TAA GCG CCT GGA CGA AAG TG
mGlu8	Forward	AGC CAG GAG TAT GCG CAT TC
	Reverse	GTC TGT GGA TGC CCT TTT CC
NR1	Forward	GCA CAC AGG AGC GGG TAA AC
	Reverse	GCG CAC GCT CAT TGT TAA TG
NR2A	Forward	GGG TAC TCC AGC GCT GAA CA
	Reverse	TAA CGC CAC CAC GTT CAC AT
NR2B	Forward	TGG AAA GTG GGA CCC TCT CA
	Reverse	CCC CTT GCA GCA TTT TTT GA
NR2C	Forward	TGT AAG GCC TTC TGC ATC GA
	Reverse	ACC ATT CCA CAC ACC ACG AA
NR2D	Forward	GGA TCT GCC ACA ACG ACA AA
	Reverse	CGC AGT CGC CAG TAC ACA AG
GluR1	Forward	GTG GAC TGG AAG AGG CCA AA
	Reverse	CTC GCC GGG ATA TGT CAA TC
GluR2	Forward	CAA GAG GCG TCT ATG CGA TTT
	Reverse	ATG GAT GCG TGC CAT CTG TT
GluR3	Forward	GGA CCC TGG ACT CTG GTT CA
	Reverse	AAC TCG GGC TAC CCC ATC AG
GluR4	Forward	TGT TGG GAA GCA CGT CAA AG
	Reverse	TCG TCA CCA TGG GCG TAT TA
CREB	Forward	AGT GCC AAC CCC CAT TTA CC
	Reverse	ACC CCA TCC GTA CCA
D1R	Forward	GGA TGA CAA CTG TGA CAC GA
	Reverse	TAA TGG CTA CGG GGA TGT AA
D2R	Forward	CTG ACA GTC CTG CCA AAC CA
	Reverse	TGC GGC TCA TCG TCT TAA GG
Adora2a	Forward	CCC ACA GCA ATT CCG TTG TC
	Reverse	CGT GGG TTC GGA TGA TCT TC
Tac1	Forward	AAT CGA TGC CAA CGA TGA TCT
	Reverse	GGG CGA TTC TCT GCA GAA GA
PDYN	Forward	TTT GGC AAC GGA AAA GAA TC
	Reverse	CAT AGC GTT TGG CCT GTT TT
KOR	Forward	CCT TTT GGA GAT GTG CTA TGC A
	Reverse	TGT AGC GGT CCA CAC TCA TCA
DNMT3a	Forward	GCC AAT AAC CAT GAC CAG GAA
	Reverse	CAG GAG CCC TGT AGC AAT CC
GR	Forward	CAA GTG ATT GCC GCA GTG AA
	Reverse	GGC AAA TGC CAT GAG AAA CA
COMT	Forward	GAC GCA AAA GGC CAA ATC AT
	Reverse	CCA TTC GCA CGG CTG AGT A
OXTR	Forward	ACG CTC GCC GTC TAC ATT GT
	Reverse	GCC GTC TTG AGT CGC AGA TT
CRHR1	Forward	GAT CAG CAG TGT GAG AGC CT
	Reverse	TGT TGT AGC GGA CAC CGT AG
CRHR2	Forward	CAA GTA CAA CAC GAC CCG GA
	Reverse	CTG CTT GTC ATC CAA AAT GGG T
TH	Forward	TTC GAG GAG AGG GAT GGA AA
	Reverse	GGT GGA TTT TGG CTT CAA ATG
DAT	Forward	GCT GCT GGT GTC TGG AAG ATC
	Reverse	GTA GTG CAG TGC CCA TGC AA
OXR1	Forward	CCC CAC TGG GCC TCA TG
	Reverse	CCC CAG AGC TTG CGG AAT A
OXR2	Forward	TGC AAA GAC CAG AAG TAC AAC CA
	Reverse	CAG ATC CGA GCA CGA AGG AA
GAPDH	Forward	CAT GGC CTT CCG TGT TCC TA
	Reverse	GAT GCC TGC TTC ACC ACC TT

experimental design.

RESULTS

1. Effect of the specific activation of sensory neurons by Gq-DREADD on the pain threshold

We first examined whether hyperalgesia could be induced by the conditional activation of sensory neurons. We created a way to artificially and specifically activate sensory neurons by injection of AAV6-Gq-coupled human muscarinic M3 (hM3Dq)-mCherry (Fig. 1A-i) or AAV-hSyn-EGFP (control, Fig. 1A-ii) into the sciatic nerve, and then examined the possible changes in the pain threshold after *i.p.* administration of clozapine *N*-oxide (CNO). We detected the expression of hM3Dq-mCherry co-localized with peripherin-expressing neurons in the DRG and not co-localized with FluoroMyelin in the sciatic nerve of AAV6-hM3Dq-injected mice 2 weeks after AAV injection (Fig. 1B–E), indicating that hM3Dq was preferentially expressed in unmyelinated-sensory neurons. In AAV6-hM3Dq-injected mice, the pain threshold was dramatically decreased by the *i.p.* administration of CNO (Fig. 1F, $^{***}p<0.001$ vs. control).

2. Induction of aversive behaviors by the conditional activation of sensory neurons by Gq-DREADD

Next, we evaluated whether aversive behaviors could be observed under the pain condition caused by conditional activation of sensory neurons. Two weeks after the injection of AAV6-hM3Dq into sciatic nerves, we first confirmed that hyperalgesia was induced by CNO ad-

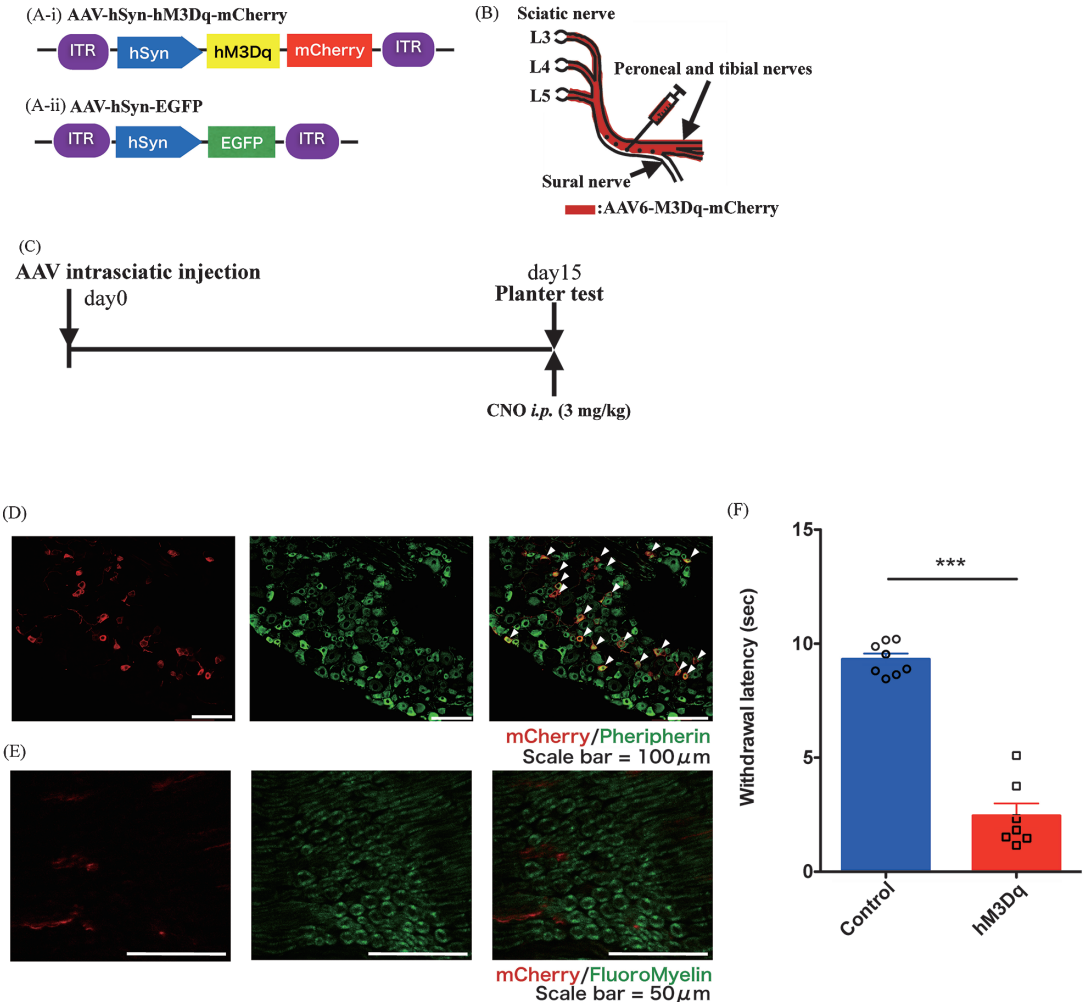

Fig. 1 Effect of specific activation of sensory neurons by Gq-DREADD on the pain threshold. (A) Schematic illustration of AAV-hSyn-hM3Dq-mCherry (A-i) and AAV-hSyn-EGFP (control) (A-ii). (B) Operative schematic. (C) Experimental timeline. (D) AAV-hSyn-hM3Dq-mCherry was injected into the sciatic nerve. Qualitative observation of mCherry fluorescence in histological sections suggested that hM3Dq-mCherry (red) was expressed in the lumbar DRG. Scale bars: 100 μm. Lumbar DRG section was stained with antibodies specific for a nociceptive marker (peripherin). hM3Dq: red, Peripherin: green. (E) Qualitative observation of mCherry fluorescence in histological sections suggested that hM3Dq-mCherry (red) was expressed in the sciatic nerve. Scale bars: 50 μm. Sciatic nerve section was stained with antibodies specific for a myelin marker (FluoroMyelin). hM3Dq: red, FluoroMyelin: green. (F) Changes in the pain threshold induced by the temporary activation of sensory neurons by the Gq-DREADD system, measured by a plantar test. Each column represents the mean with SEM (n = 7–8, $^{***}p < 0.001$ vs. control).

ministration (Fig. 2A). Under these conditions, we performed 12 conditioning sessions (6 for saline conditioning, 6 for CNO (3 mg/kg)-induced pain conditioning) according to the conditioned place aversion test (Fig. 2B). As we expected, there was no significant change in the time spent on the CNO-conditioning side in control mice (Fig. 2C-i), whereas there was a significant decrease in the time spent on the CNO-conditioning side in hM3Dq-injected mice (Fig. 2C-ii, $^{*}p<0.05$ vs. Pre-test).

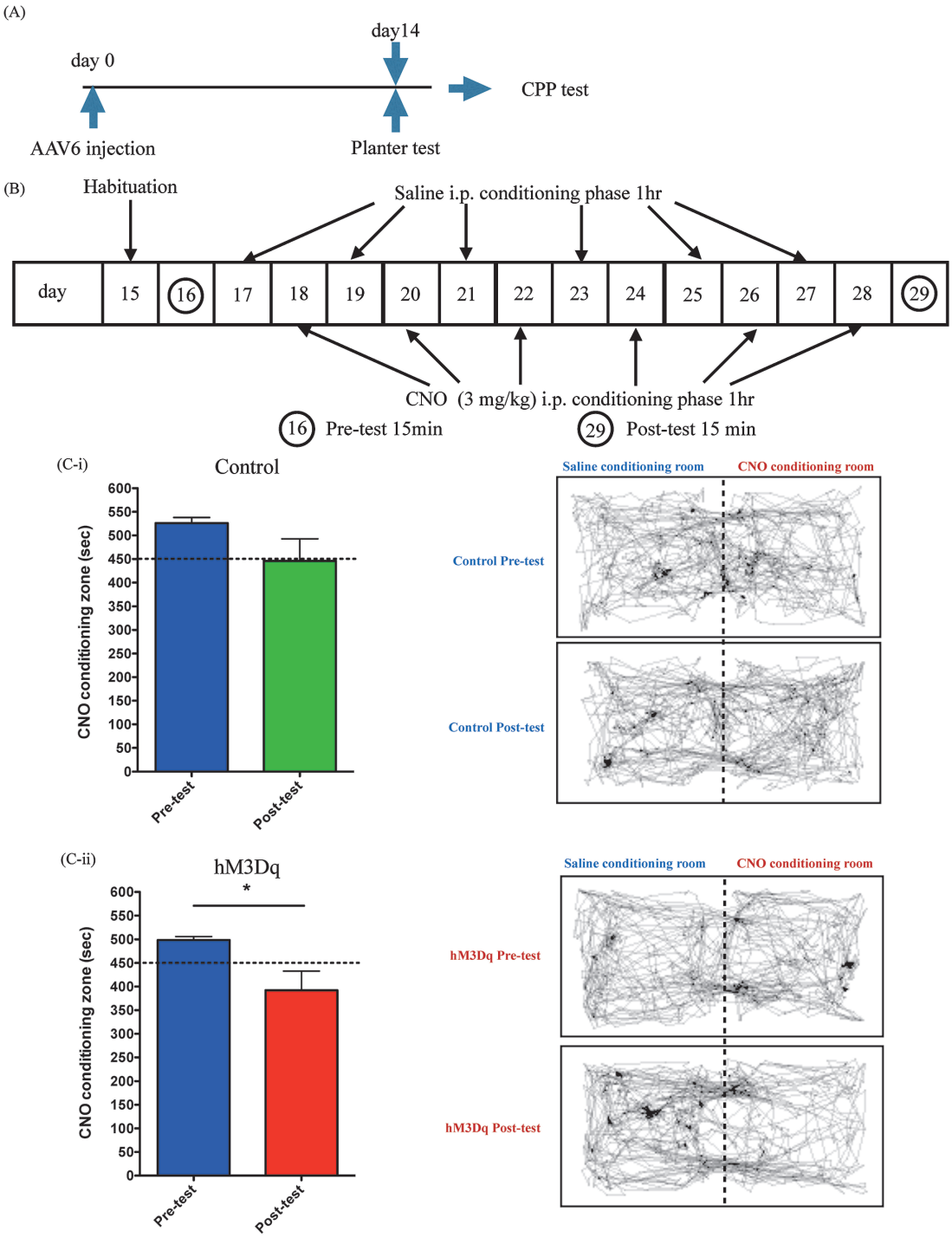

Fig. 2 Induction of conditioned place aversion by the artificial activation of sensory neurons by Gq-DREADD. (A) Experimental timeline. (B) CPP conditioning and test schedule. (C) Changes in the place preference induced by the temporary activation of sensory neurons by the Gq-DREADD system using a CPP test in control (C-i, left: CPP test results, right: Traces) and hM3Dq (C-ii, left: CPP test results, right: Traces). Each column represents the mean with SEM ($n = 5$–6, $^{*}p < 0.05$ vs. Pre-test).

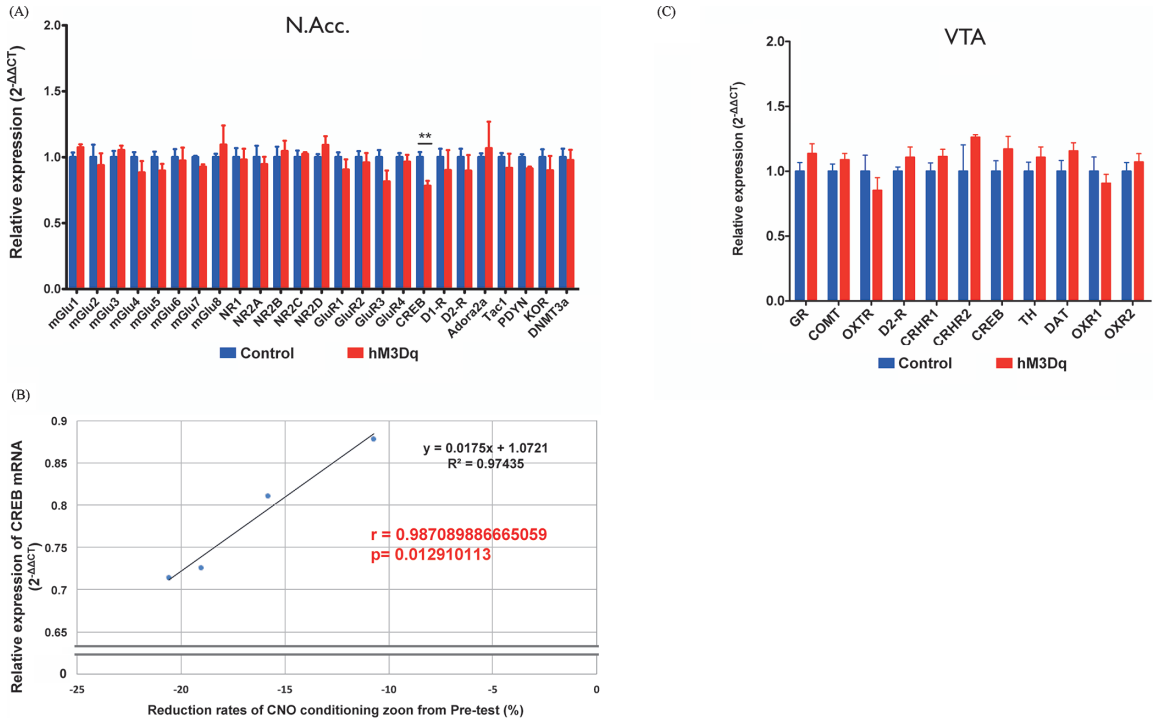

Fig. 3 Changes in mRNAs of neural markers in the N.Acc. under the aversive condition induced by activating sensory neurons via Gq-DREADD. (A) Changes in mRNA levels of metabotropic glutamate receptors (mGlu1, mGlu2, mGlu3, mGlu4, mGlu5, mGlu6, mGlu7, mGlu8), *N*-methyl-*d*-aspartate receptors (NR1, NR2A, NR2B, NR2C, NR2D), DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR; GluR1, GluR2, GluR3, GluR4), cAMP response element binding protein (CREB), dopamine receptors (D1-R, D2-R), adrenoceptor alpha 2A (Adra2a), tachykinin 1 (Tac1), prodynorphin (PDYN), kappa opioid receptor (KOR) and DNA methyltransferase 3a (DNMT3a) in the N.Acc. of the control and hM3Dq groups. Each column represents the mean with SEM ($n = 4$–5, $^{**}p < 0.01$ vs. control). (B) Relationship between the aversive state and CREB mRNA expressions levels. The data were subjected to a comparative analysis by testing the null hypothesis for the Pearson product moment correlation. (C) Changes in mRNA levels of glucocorticoid receptor (GR), catechol-*O*-methyltransferase (COMT), oxytocin receptor (OXTR), D2-R, corticotropin-releasing hormone receptors (CRHR1, CRHR2), CREB, tyrosine hydroxylase (TH), dopamine transporter (DAT) and orexin receptors (OXR1, OXR2) in the VTA of the control and hM3Dq groups.

3. Changes in mRNAs of neural markers in the N.Acc. under the aversive condition induced by activating sensory neurons via Gq-DREADD

To clarify the mechanisms that underlie aversive behaviors induced by the hyperactivity of sensory neurons, we semi-comprehensively analyzed the changes in the expression levels of neural markers in the N.Acc. of mice, which had established conditioned place aversion by the selective stimulation of sensory nerves. Among 25 neuronal markers, including metabotropic glutamate receptors (mGlu1, mGlu2, mGlu3, mGlu4, mGlu5, mGlu6, mGlu7, mGlu8), *N*-methyl-*d*-aspartate receptors (NR1, NR2A, NR2B, NR2C, NR2D), DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR; GluR1, GluR2, GluR3, GluR4), cAMP response element binding protein (CREB), dopamine receptors (D1-R, D2-R), adrenoceptor alpha 2A (Adra2a), tachykinin 1 (Tac1), prodynorphin (PDYN), kappa opioid receptor (KOR), and DNA methyltransferase 3a (DNMT3a), the DREADD-mediated activation of sensory nerves only and significantly decreased the mRNA level of CREB in hM3Dq-transfected mice (Fig. 3A). As shown in Fig. 3B, the changes in the mRNA level of CREB in the N.Acc. were significantly correlated with the production of aversive behaviors. On the other hand, mRNA levels of glucocorticoid receptor (GR), catechol-*O*-methyltransferase (COMT), oxytocin receptor (OXTR), D2-R, corticotropin-releasing hormone receptors (CRHR1, CRHR2), CREB, tyrosine hydroxylase (TH), dopamine transporter (DAT) and orexin receptors (OXR1, OXR2) in the VTA were not changed under this pain state with aversion (Fig. 3C).

DISCUSSION

It has been well-recognized that pain is a complex experience composed of both sensory-discriminative and emotional-affective components, and negative emotion leads to an enhanced response to noxious stimuli. The artificial activation of sensory neurons decreases dopamine production, storage and release in the N.Acc., which belongs to the basal ganglia and is involved in processing of aversion and reward[4], whereas tonic activation of mesolimbic dopaminergic neurons terminating at the N.Acc. inhibits hyperalgesia[9]. Consistently, negative and positive activations of N.Acc. have been ob-

served at pain onset and offset, respectively, in a human functional-MRI study with a prolonged painful stimulus[12]. It has been widely accepted that negative emotion or aversive behavior is, at least in part, accompanied by decreased dopamine transmission in the mesolimbic pathway[13–19]. Taken together, these findings suggest that the mesolimbic dopaminergic network, which plays a crucial role in pain threshold regulation as well as emotional control, could be damaged under sustained pain. Despite recent progress in understanding the dysfunction of mesolimbic dopaminergic transmission under consistent pain, its mechanism is still poorly understood.

In the present study, we used a small-molecule ligand CNO with the sciatic nerve-injection of AAV6-Gq-coupled hM3Dq to directly and conditionally activate peripheral sensory neurons, and observed both hyperalgesia and conditioned place aversion. In the N.Acc. of these pained and aversive mice, we successfully detected a significant decrease in the mRNA level of the neuron-specific transcriptional factor CREB, which often serves as a prototype for calcium-dependent regulators of transcription, but not of mGlu1, mGlu2, mGlu3, mGlu4, mGlu5, mGlu6, mGlu7, mGlu8, NR1, NR2A, NR2B, NR2C, NR2D, GluR1, GluR2, GluR3, GluR4, D1-R, D2-R, Adra2a, Tac1, PDYN, KOR or DNMT3a. In contrast, no change in the expression levels of GR, COMT, OXTR, D2-R, CRHR1, CRHR2, CREB, TH, DAT, OXR1 or OXR2 was observed in the VTA. More interestingly, we performed a correlation analysis and concluded that there was a strongly positive correlation between pain-induced aversive behaviors and the decreased level of CREB mRNA in the N.Acc. A growing body of evidence suggests that CREB is a main downstream molecule of D1-R-containing median spiny neurons in the N.Acc. that regulates diverse cellular responses, including cell excitation, synaptic plasticity, and survival[20–24]. Furthermore, it has been reported that D1-R activation plays a crucial role in the induction of preference behaviors[25]. Thus, we propose here that negative emotion with a decreased pain threshold may be regulated by the concomitant attenuation of neural activity in the N.Acc.

In conclusion, we demonstrated that repeated activation of sensory nerves using a Gq-DREADD system induced conditioned place aversion in AAV6-Gq-coupled hM3Dq-expressing mice, which exhibited a specific decrease in the mRNA level of the neuron-specific transcriptional factor CREB in the N.Acc.

Conflict of interest: The authors declare no conflict of interest associated with this manuscript.

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