NEUROSCIENCE
RESEARCH ARTICLE

H. Ma et al. / Neuroscience 394 (2018) 164–176

Wnt7a in Mouse Insular Cortex Contributes to Anxiety-like Behavior During Protracted Abstinence from Morphine

Hui Ma, Na Wang, Xinjuan Wang, Meng Jia, Yijing Li and Cailian Cui*

Department of Neurobiology, School of Basic Medical Sciences, Neuroscience Research Institute, Key Laboratory for Neuroscience of the Ministry of Education/National Health and Family Planning Commission, Peking University, Beijing, China

Abstract—Anxiety is considered an important protracted abstinence symptom that can aggravate craving and relapse risk in opioid addicts. Although the insular cortex (IC) has been reported to be a key brain region in mediating emotional and motivational alterations induced by drug consumption and withdrawal, the role of IC in anxiety related to protracted abstinence remains elusive. In this study, we found that: (1) anxiety-like behavior in morphine-dependent mice became significant after 28 days of withdrawal, while their physical symptoms became undetectable. (2) Activated glutamatergic neurons in the medial IC, but not the anterior or posterior IC were significantly increased after 28 days of withdrawal. Bilateral lesion of the medial IC, but not the anterior or posterior IC with ibotenic acid (IBO) alleviated the anxiety-like behavior. (3) Expression of Wnt7a in the medial IC was significantly increased after 28 days of withdrawal, and specific down-regulation of Wnt7a with AAV-shWnt7a also alleviated the anxiety-like behavior. The findings reveal the medial IC is involved in mediating anxiety-like behavior related to morphine protracted abstinence, in which Wnt7a plays a critical role.

Key words: protracted abstinence, morphine, anxiety-like behavior, insular cortex, Wnt7a.

INTRODUCTION

Withdrawal from addictive drugs certainly produces debilitating physical symptoms and aversive emotional responses in addicts (Anraku et al., 2001). Although their physical symptoms are no longer detectable after prolonged abstinence, emotional responses gradually emerge or even worsen over time (Goeldner et al., 2011; Lee et al., 2014). For example, opioid addicts still display obvious anxiety symptom after 21 days of detoxification (Gossop et al., 1987). Morphine-dependent rodents also show more obvious anxiety-like behavior after 4 weeks of withdrawal than 1-week controls, that spending less time in the center of open field and entering less into the open arms of elevated plus-maze (Jia et al., 2013; Lee et al., 2014). Additionally, epidemiologic study reveals anxiety symptom in opioid addicts is positively associated with their craving score (Swift and Stout, 1992). Therefore, anxiety is an important potential risk for relapse after abstinence (Koob and Le Moal, 2008; Koob and Volkow, 2010).

Although most of previous studies on addiction-related brain structures focus on subcortical regions (Koob, 2009; Seif et al., 2013; Shen et al., 2016), the insular cortex (IC), as a critical neural substrate of interoceptive system, has attracted increasing attention in drug addiction studies (Hollander et al., 2008; Naqvi and Bechara, 2010; Naqvi et al., 2007; Scott and Hiroi, 2011). It has been proved that the IC encodes emotional alterations induced by drug consumption and withdrawal, and involves in incentive motivation and decision control behaviors (Naqvi et al., 2014). Patients with the IC damage experience less pleasure and satisfaction from smoking (Naqvi and Bechara, 2010), and are more likely to conquer addiction to smoking (Naqvi et al., 2007). Inactivation of the IC can block the expression and reconsolidation of morphine/amphetamine-induced conditioned place preference (CPP) (Contreras et al., 2012, 2007; Wu et al., 2014) and prevent the reinstatement of nicotine-seeking behavior in rodents (Forget et al., 2010; Pushparaj et al., 2013). Moreover, the IC receives inputs from the limbic areas such as the hypothalamus, nucleus accumbens and amygdala, all of which are not only involved in drug addiction (Naqvi and Bechara, 2009), but also regulate anxiety disorders (Adhikari, 2014). Previous fMRI

*Corresponding author. Address: Neuroscience Research Institute, Peking University, 38 Xueyuan Road, Beijing 100191, China. Fax: +86-10-82801120.
E-mail address: clcui@bjmu.edu.cn (C. Cui).

Abbreviations: AI, anterior insula; AIC, the anterior insula cortex; ANOVA, analysis of variance; CPP, conditioned place preference; EAAC1, excitatory amino acid carrier 1; GABA, gamma-aminobutyric acid; IC, insular cortex; MIC, the medial insular cortex; mRNA, messenger RNA; MW, morphine withdrawal; MW28d, 28 days of morphine withdrawal; PBS, phosphate-buffered saline; PI, posterior insula; PIC, the posterior insular cortex; shRNA, short hairpin RNA; SW, saline withdrawal; SW28d, 28 days of saline withdrawal.

https://doi.org/10.1016/j.neuroscience.2018.10.032
0306-4522/

studies have found that hyperactivity of human IC is related to various anxiety disorders (Alvarez et al., 2015; Hoehn-Saric et al., 2004; Kawaguchi et al., 2016). Recently, it has been reported that the Fos expression in the IC is up-regulated in mice displayed anxiety-like behavior after 4 weeks of morphine withdrawal, which suggests the IC may be involved in protracted abstinence-induced anxiety-like behavior (Becker et al., 2017).

Wnts are a family of secreted lipid-modified signaling proteins acting as important regulators in neuronal regeneration and synaptic plasticity (Ciani and Salinas, 2005; Packard et al., 2002), which can bind to cysteine-rich domain of Frizzled receptors and the coreceptors to activate intracellular signaling cascades. Wnt signaling pathways include the Wnt/β-catenin-dependent or canonical pathway and Wnt/Ca^{2+}, Wnt/planar cell polarity or non-canonical pathways (Oliva et al., 2013). It has been proved that inhibition of Wnt production in rat nucleus accumbens blocks the acquisition and expression of amphetamine-induced CPP (Islam et al., 2017), and inactivation of the canonical pathway in rat prefrontal cortex prevents cocaine-induced behavioral sensitization (Cuesta et al., 2017). Additionally, anxiety-like behavior in rats caused by chronic stress is accompanied with hyperactivation of the canonical pathway in hypothalamus, while Wnt signaling inhibitor can relieve this anxiety-like behavior (Ge et al., 2016). Obviously, Wnt signaling pathways in the central nervous system are involved in regulating not only drug addiction, but also emotion disorders (Maguschak and Ressler, 2012). So, whether Wnt signaling pathways in the IC can regulate anxiety during drug abstinence is obviously a question worth studying.

In this study, we firstly assessed alterations of physical symptoms and anxiety-like behavior from 1 to 28 days after morphine withdrawal in mice. Secondly, we investigated the role of the IC and Wnts in it in the abstinence-related anxiety-like behavior. Finally, we verified whether lesion of the specific subdivision of IC and inhibition of Wnt could alleviate the anxiety-like behavior.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6 mice (6–8 weeks of age, 25–30 g, Department of Laboratory Animal Sciences, Peking University Health Science Center) were housed in pathogen-free conditions, with a 12/12-h light/dark cycle and food and water available *ad libitum.* All experimental procedures were approved by the Animal Use Committee of Peking University Health Science Center.

Morphine withdrawal

Mice were injected (i.p.) with repeated pulses of morphine hydrochloride (First Pharmaceutical Factory of Qinghai, China) given in 11 escalating doses (20–100 mg/kg, 0.1 ml/mouse) or equal volume of normal saline every 12 h (9:00a.m. and 9:00p.m.) (Goeldner et al., 2011), then experienced spontaneous withdrawal in their home cages. After 1, 7, 14, 21 and 28 days of withdrawal (between 9:00a.m. and 11:00a.m.), mice were weighed before placing them into a clear beaker flask (5L) to score the physical symptoms (shakes and jumps) for 30 min, and the mice were weighed again. The number of shakes and jumps were manually recorded in a blind manner. Weight loss was measured as a percentage of the weight before physical symptoms' test.

Behavioral testing

Open-field test. Mice were placed in the center of a 40 × 40 × 40 cm box exposed to 30-lux illumination (between 11:00 a.m. and 1:00p.m.) after adapting to the environment for 30 min, and videotaped its activities for 10 min (Deng et al., 2015). The percentage of time spent in the center (20 × 20 cm) and total distance were measured with an automated video-tracking and analysis system (SMART Ver2.5.21, Panlab, Harvard Apparatus).

Elevated plus-maze test. The equipment was elevated 50 cm above the floor and consisted of two open arms (5 × 30 cm each), two closed arms (5 × 30 × 15 cm each) and a central platform (5 × 5 cm). Mice were placed onto the center platform exposed to 30-lux illumination (between 5:00p.m. and 7:00p.m.) heading toward the same open arm after adapting to the environment for 30 min, and observed its activities for 5 min (Zheng et al., 2017). The time spent in the open arms and the number of entries into the open arms and closed arms were recorded respectively.

Immunofluorescence staining

Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and perfused with 4% PFA (in 0.1 M PBS, PH 7.4) 90 min after anxiety-like behavioral tests. Brains were post-fixed and dehydrated in turn. 12 coronal brain sections (30-mm-thick, AP +2.22, +1.98, +1.70, +1.42, +1.10, +0.86, +0.50, +0.26, −0.22, −0.58, −0.82 and −1.22 mm) throughout the entire IC were sliced in each mouse using a cryostat microtome (model 1950, Leica). The sections were blocked with PBST (PBS with 0.1% Tween-20) containing 5% normal donkey serum for 1 h. Next, the sections were incubated with primary antibodies diluted in PBST with 1% normal donkey serum overnight at 4 °C: mouse anti-NeuN (1:800, MAB377, Millipore, Billerica, MA, USA), rabbit anti-c-Fos (1:500, sc-52, Santa Cruz Biotechnology, CA, USA), goat anti-EAAC1 (1:1000, AB1520, Millipore, Billerica, MA, USA), mouse anti-GAD67 (1:1000, MAB5406, Millipore, Billerica, MA, USA), rabbit anti-Wnt7a (1:50, ab100792, abcam, Cambrige, UK). The sections were then washed with PBST and incubated with secondary antibodies diluted in PBST with 1% normal donkey serum for 1.5 h at room temperature: Alexa Fluor 488 donkey anti-mouse IgG (for NeuN, 1:500, A21202, Invitrogen, Carlsbad, CA, USA), Alexa Fluor 555 donkey anti-rabbit IgG (for c-Fos, 1:500,

A31572, Invitrogen, Carlsbad, CA, USA), Cy™3-conjugated donkey anti-goat IgG (for EAAC1, 1:500, 305-165-003, Jackson, Lancaster, PA, USA), Alexa Fluor 555 donkey anti-mouse IgG (for GAD67, 1:500, A31570, Invitrogen, Carlsbad, CA, USA), Alexa Fluor 488 donkey anti-rabbit IgG (for Wnt7a, 1:500, A21206, Invitrogen, Carlsbad, CA, USA), Hoechst33258 (1:500, H3569, Invitrogen, Carlsbad, CA, USA). The images were taken by an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan) under 10×, 20× and 60× (with oil immersion) objectives, 3 frames. The number of c-Fos + NeuN double-positive cells in the IC was the sum of two hemisections for each mouse calculated separately at above-mentioned 12 sites. The double-positive (c-Fos + EAAC1/GAD67 and Wnt7a + EAAC1/ + GAD67) cells in the MIC were measured as a percentage of the number of c-Fos$^+$ cells averaged by 3 sections (AP: from +1.10 to −0.22 mm) for each mouse. Image quantifications were performed manually in a blinded manner.

Surgery

Mice were anesthetized and secured in a Kopf stereotaxic apparatus. A small burr hole was drilled through the skull bone to allow injection (Hamilton 1-μl syringe). Ibotenic acid (IBO, ab120041, abcam, Cambrige, UK; 10 μg/μl in 0.1 M PBS, pH 7.4; 0.3 μl/site, 0.05 μl/min) (Murray et al., 2015) was bilaterally microinjected into the IC (AIC: AP: +1.70 mm, ML: ±2.50 mm, DV: −2.70 mm; MIC: AP: +0.30 mm, ML: ±3.50 mm, DV: −3.50 mm; PIC: AP: −0.70 mm, ML: ±3.70 mm, DV: −3.50 mm) a week before anxiety-like behavioral tests. And a virus AAV2/9-CaMKII-shWnt7a-EGFP or AAV2/9-CaMKII-Scramble-EGFP (GL Biochem, Shanghai, China; 4×10^{12} V.G./ml; 0.3 μl/site, 0.05 μl/min) was also bilaterally microinjected into the MIC a week before behavioral tests. All stereotaxic target coordinates were based on Franklin and Paxinos' The Mouse Brain in Stereotaxic Coordinates Third Edition.

Quantitative RT-PCR

Mice were sacrificed immediately after anxiety-like behavioral tests respectively after 1 and 28 days of withdrawal. The bilateral IC tissues were quickly extracted. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was then synthesized using a first-strand synthesis kit (Tiangen, Beijing, China). Quantitative RT-PCR (qRT-PCR) was performed with a KAPA SYBR® FAST qPCR Kit (KAPA BIOSYSTEMS, Wilmington, MA, USA). The following primers were used: Gapdh (forward: TCCTGCGA CTTCAACAGCAA; reverse: AGTTGGGATAGGGCC TCTCTT), Wnt2 (forward: CTGCGAAGTTATGTGTT GTGGG; reverse: TGAGGGAAGAAAAGGCGAATA), Wnt3a (forward: TCAAGGTGCCGACAGAACG; reverse: CACAGTGGCATTTCTCCCTCC), Wnt4 (forward: TCCT CGTCTTCGCCGTGTT; reverse: CTCCGTGGCACCGT CAAA), Wnt5a (forward: AGGGAACGAATCCACG CTAA; reverse: ACTTCTCCTTGAGGGCATCG), Wnt7a (forward: GGGTGCGAGCATCATCTGT; reverse: CCACA GTCGCTCAGGTTGC), Wnt11 (forward: TTCGTGTATG CCCTGTCGG; reverse: ACCTTCATAGGAGCATCG GAAA). Gapdh quantification was used as an internal control for normalization. Relative mRNA levels were calculated using the $2-\Delta\Delta CT$ method.

Western blot

The bilateral IC tissues were quickly extracted. Total protein was isolated with RIPA buffer (R0278, Sigma–Aldrich, St. LOUIS, MO, USA) and determined by the BCA assay (Millipore, Billerica, MA, USA). Total protein (30 μg) were separated in 10% or 15% sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gels and transferred to polyvinylidene difluoride (PVDF, ISEQ00010, Millipore, Billerica, MA, USA) membranes. The blots were blocked in TBST (TBS with 0.1% Tween-20) containing 5% nonfat milk or 5% BSA for 1 h at room temperature and then incubated with primary antibody: mouse anti-β-actin (1:3000, A2228, Sigma-Aldrich, St. LOUIS, MO, USA) and rabbit anti-Wnt7a (1:300, ab100792, abcam, Cambridge, UK) diluted in TBST with 5% nonfat milk or 5% BSA overnight at 4 °C. The blots were then washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, ZB-2305, Zhongshan Biotechnology, Beijng, China) or goat anti-rabbit IgG (1:2000, ZB-2301 Zhongshan Biotechnology, Beijing, China) for 1 h at room temperature. Protein blots were detected using a chemiluminescence detection kit (WBKLS0500, Millipore, Billerica, MA, USA) and analyzed quantitatively by densitometry with the Quantity One® 1-D analysis software. β-actin quantification was used as an internal control for normalization.

Statistical analysis

Data were expressed as mean ± SEM and analyzed by software Graph Pad Prism 6.0 with a two-way ANOVA, followed by Sidak's post hoc test. In case of significant main effect or interactions following a two-way ANOVA, multiple group comparisons were performed (Lee et al., 2014). The results of the percentage of double-positive cells in Figs. 2 and 5 were analyzed using two-tailed unpaired t test. Statistical significance was set at $p < 0.05$.

RESULTS

Physical symptoms gradually decrease during morphine abstinence

The experimental timeline is depicted in Fig. 1A. Mice were divided into ten groups in a 2 (drugs: saline, morphine) × 5 (days after withdrawal: 1 d, 7 d, 14 d, 21 d, 28 d) factorial design and exposed to chronic morphine or saline before experienced spontaneous withdrawal. We first monitored the development of physical symptoms after withdrawal (Fig. 1B). The number of shakes and jumps and the percentage of weight loss were affected by morphine ($F_{1,\ 120} = 34.45$, $p < 0.0001$, $F_{1,\ 120} = 88.65$, $p < 0.0001$, $F_{1,\ 120} = 127.7$, $p < 0.0001$, respectively) and day ($F_{4,\ 120} =$

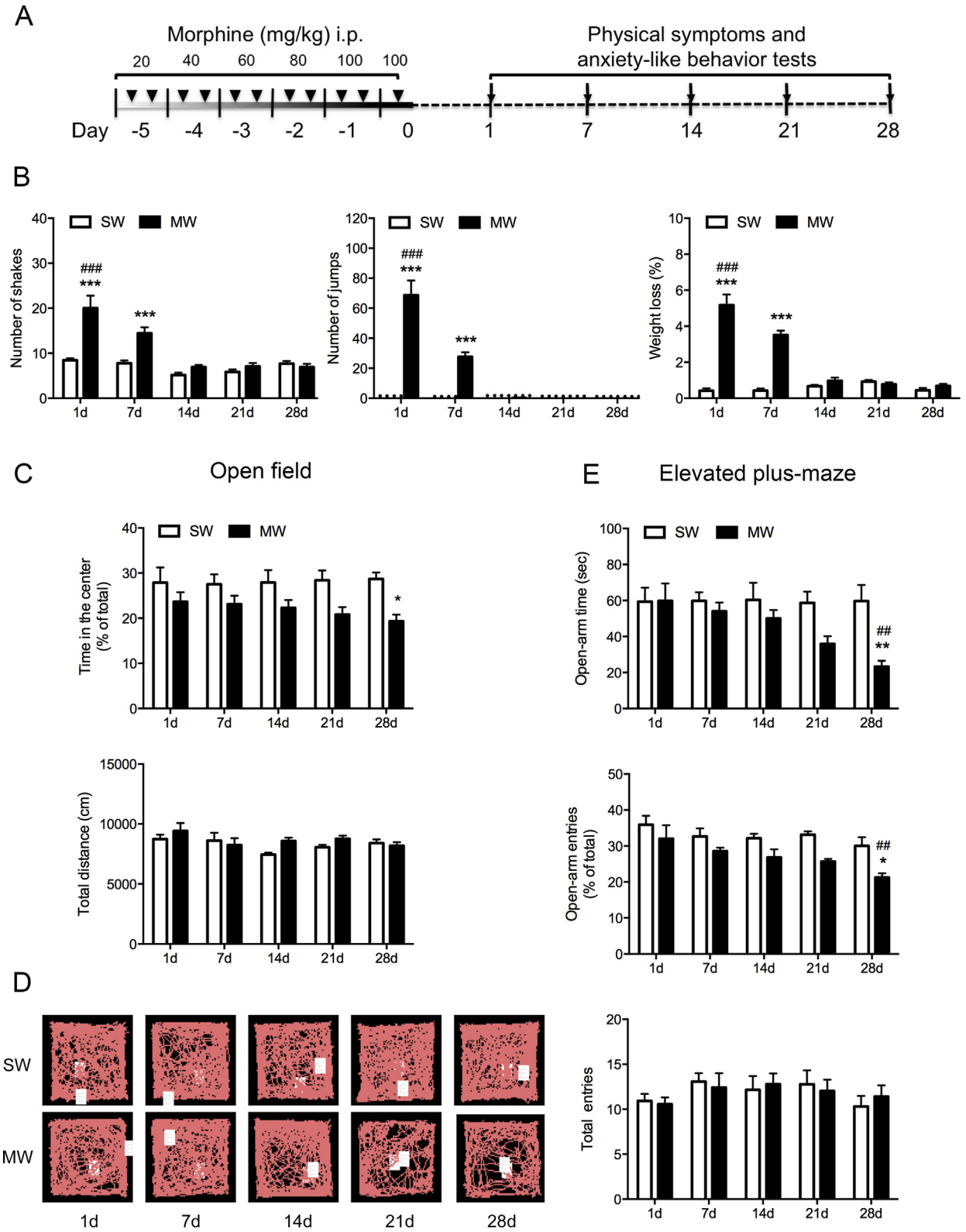

Fig. 1. Physical symptoms and anxiety-like behavior altered during protracted abstinence. (A) Experimental timeline. (B) Physical symptoms: shake (left), jump (middle) and weight loss (right) were decreased after 28 days of withdrawal. (C) The time spent (top) in the center of the open field was decreased after 28 days of withdrawal, and total distance (bottom) had no significant difference across groups. (D) Representative movement tracks (pink polylines) and the start and end points (white rectangles) in the open field. (E) The time spent (top) and percentage of entries (middle) into the open arms of the elevated plus-maze were decreased after 28 days of withdrawal, and total arm entries (bottom) had no significant difference across groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ MW vs. SW group; $^{\#\#}p < 0.01$. $^{\#\#\#}p < 0.001$ 28- vs. 1-day MW group, two-way ANOVA with Sidak's post hoc test, $n = 13$. SW: saline withdrawal; MW: morphine withdrawal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

20.54, $p < 0.0001$, $F_{4, 120} = 43.12$, $p < 0.0001$, $F_{4, 120} = 33.88$, $p < 0.0001$, respectively) as well as an interaction between these factors ($F_{4, 120} = 10.23$, $p < 0.0001$, $F_{4, 120} = 43.12$, $p < 0.0001$, $F_{4, 120} = 43.98$, $p < 0.0001$, respectively). Morphine-withdrawal mice shook, jumped and lost weight significantly more than saline controls after 1 and 7 days of withdrawal ($p < 0.001$), and all above differences were no longer significant after 14, 21 or 28 days. In addition, 28-day morphine-withdrawal mice exhibited significantly less physical symptoms than 1-day controls ($p < 0.001$). These results indicate that physical symptoms are most obvious in the period of acute abstinence and gradually decrease as abstinence unfolds.

Anxiety-like behavior becomes evident after protracted abstinence

We next investigated the alteration of anxiety-like behavior during morphine or saline abstinence, using the open-field test (Fig. 1C) and elevated plus-maze test (Fig. 1E). First, the percentage of time spent in the center of the field was affected by morphine ($F_{1, 120} = 21.27$, $p < 0.0001$), and was significantly decreased in 28-day morphine-withdrawal mice, compared with saline controls ($p < 0.05$). There was no significant difference in total distance across groups. Second, the time spent and percentage of entries into the open arms of the maze were affected by morphine ($F_{1, 120} = 12.25$, $p = 0.0007$, $F_{1, 120} = 20.86$, $p < 0.0001$, respectively) and day ($F_{4, 120} = 2.457$, $p = 0.0493$, $F_{4, 120} = 4.240$, $p = 0.0030$, respectively). In addition, 28-day morphine-withdrawal mice showed significantly less time spent and entries into the open arms, compared with 28-day saline controls ($p < 0.01$, $p < 0.05$, respectively) and with 1-day morphine-withdrawal mice ($p < 0.01$). There was no significant difference in total arms entries across groups. These results suggest that anxiety-like behavior become significant after a prolonged abstinence period.

Activated glutamatergic neurons in the medial IC are increased after morphine protracted-abstinence

In order to determine the role of the IC in abstinence-related anxiety-like behavior, we selected twelve levels of coronal brain sections and measured the number of c-Fos and NeuN double-positive cells of the IC in mice respectively undergoing acute (1 day) and protracted (28 days) withdrawal. At 28-day time point (Fig. 2A), the number of double-positive cells was affected by morphine ($F_{1, 48} = 128.9$, $p < 0.0001$) and level ($F_{11, 48} = 39.79$, $p < 0.0001$) as well as an interaction between these factors ($F_{11, 48} = 12.82$, $p < 0.0001$), and significantly increased in a specific subdivision (from +1.10 to −0.22 mm relative to bregma) in morphine-withdrawal mice, compared with saline controls ($p < 0.05$, $p < 0.001$). Therefore, we divided the mouse IC into three subdivisions in later studies: the anterior IC (AIC, AP: +2.46 to +1.10 mm), the medial IC (MIC, AP: +1.10 to −0.22 mm) and the posterior IC (PIC, AP: −0.22 to −1.22 mm). The double-positive cells of the medial IC were more abundant in mice undergoing 28-day morphine withdrawal than 1-day controls (Fig. 2B, $p < 0.001$). And the number in the entire IC was no difference between morphine- and saline-withdrawal mice at 1-day time point (the data isn't shown). Moreover, the c-Fos-positive cells were more co-labeled with glutamatergic neurons (EAAC1) (Lammel et al., 2012) than GABAergic neurons (GAD67) (Kaufman et al., 1986) (Fig. 2D, $t_4 = 9.791$, $p < 0.001$).

Bilateral lesion of the medial IC prevents the anxiety-like behavior

We then damaged the medial IC with IBO after 21 days of withdrawal and then examined anxiety-like behavior seven days later (Fig. 3A). First, both morphine ($F_{1, 25} = 6.169$, $p = 0.0201$) and IBO ($F_{1, 25} = 11.19$, $p = 0.0026$) influenced the time spent in the center of the field, but the interaction between these factors ($F_{1, 25} = 2.745$, $p = 0.1100$) was not significant (Fig. 3C). Consistent with our previous results, MW28d/PBS group significantly spent less time in the center than SW28d/PBS group ($p < 0.01$). IBO prevented this morphine withdrawal-induced deficit, because the time spent in the center of MW28d/IBO group was significantly increased compared with MW28d/PBS group ($p < 0.01$). There was no significant difference in total distance across groups. Second, both morphine ($F_{1, 25} = 4.441$, $p = 0.0453$, $F_{1, 25} = 6.645$, $p = 0.0162$, respectively) and IBO ($F_{1, 25} = 5.033$, $p = 0.0340$, $F_{1, 25} = 11.63$, $p = 0.0022$, respectively) modified the time spent and percentage of entries into the open arms of the maze, with a significant interaction between these factors ($F_{1, 25} = 16.63$, $p = 0.0004$, $F_{1, 25} = 10.84$, $p = 0.0030$, respectively) (Fig. 3E). MW28d/PBS group showed significantly less time spent and entries into the open arms than SW28d/PBS group ($p < 0.001$), and IBO prevented this deficit ($p < 0.001$). There was no significant difference in total arms' entries across groups. Interestingly, lesion of the anterior or posterior IC didn't affect the anxiety-like behavior (the date isn't shown). These results suggest that the medial IC is a crucial subdivision for the anxiety-like behavior associated with morphine protracted-abstinence.

Expression of Wnt7a in the medial IC is increased after morphine protracted abstinence

Considering the important role of Wnt signaling pathways in drug addiction and anxiety, we examined the mRNA levels of major members of the Wnts in the IC (Fig. 4). We found that the level of *Wnt7a* in the medial IC, but not the anterior or posterior IC was affected by morphine ($F_{1, 20} = 5.894$, $p = 0.0248$) and day ($F_{1, 20} = 6.810$, $p = 0.0168$) as well as an interaction between these factors ($F_{1, 20} = 10.85$, $p = 0.0036$). The level of *Wnt3a* in the medial IC was affected by morphine ($F_{1, 20} = 6.688$, $p = 0.0176$) and day ($F_{1, 20} = 5.336$, $p = 0.0317$), rather than an interaction between these factors ($F_{1, 20} = 3.386$, $p = 0.0806$). Post hoc test showed that morphine withdrawal significantly increased the level of *Wnt7a* and *Wnt3a* in the medial IC at the 28-day time point only, compared

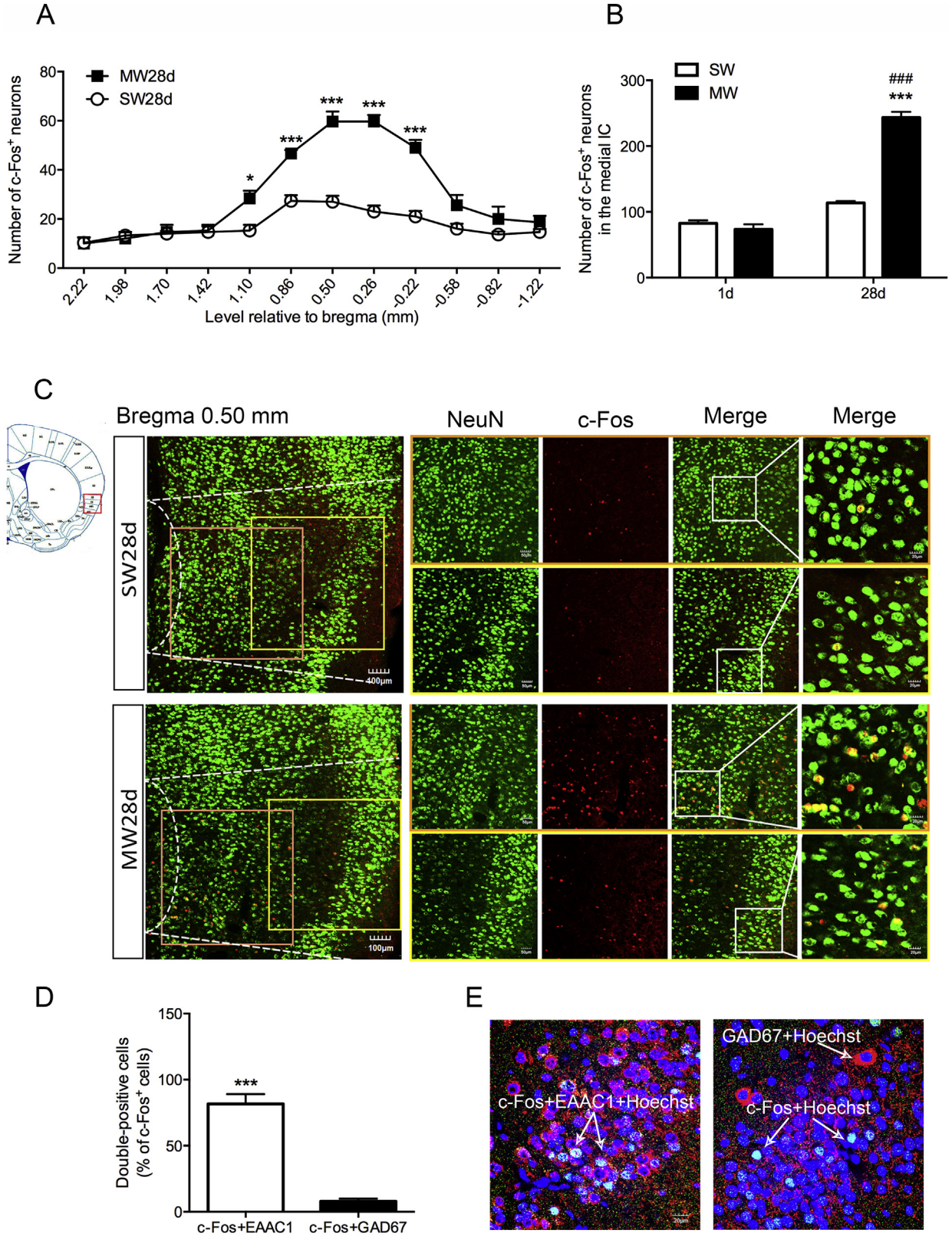

Fig. 2. Activated neurons in the IC measured after 28 days of withdrawal. (A, B) The number of c-Fos$^+$ neurons was increased in the medial IC, but not the anterior or posterior IC. $^{*}p < 0.05$, $^{***}p < 0.001$, MW28d vs. SW28d group; $^{\#\#\#}p < 0.001$ 28- vs. 1-day MW group, two-way ANOVA with Sidak's post hoc test, $n = 3$. (C) Representative immunofluorescence images (×10 left, ×20 middle, ×60 right) of c-Fos (red) and NeuN (green) labeling in the medial IC (bregma +0.5 mm). (D) Percentage of double-positive cells. $^{***}p < 0.001$, c-Fos + EAAC1 vs. c-Fos + GAD67 group, unpaired *t* tests, $n = 3$. (E) Representative immunofluorescence images (×60) of Hoechst (blue), c-Fos (green), EAAC1 or GAD67 (red). SW28d: 28 days of saline withdrawal; MW28d: 28 days of morphine withdrawal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

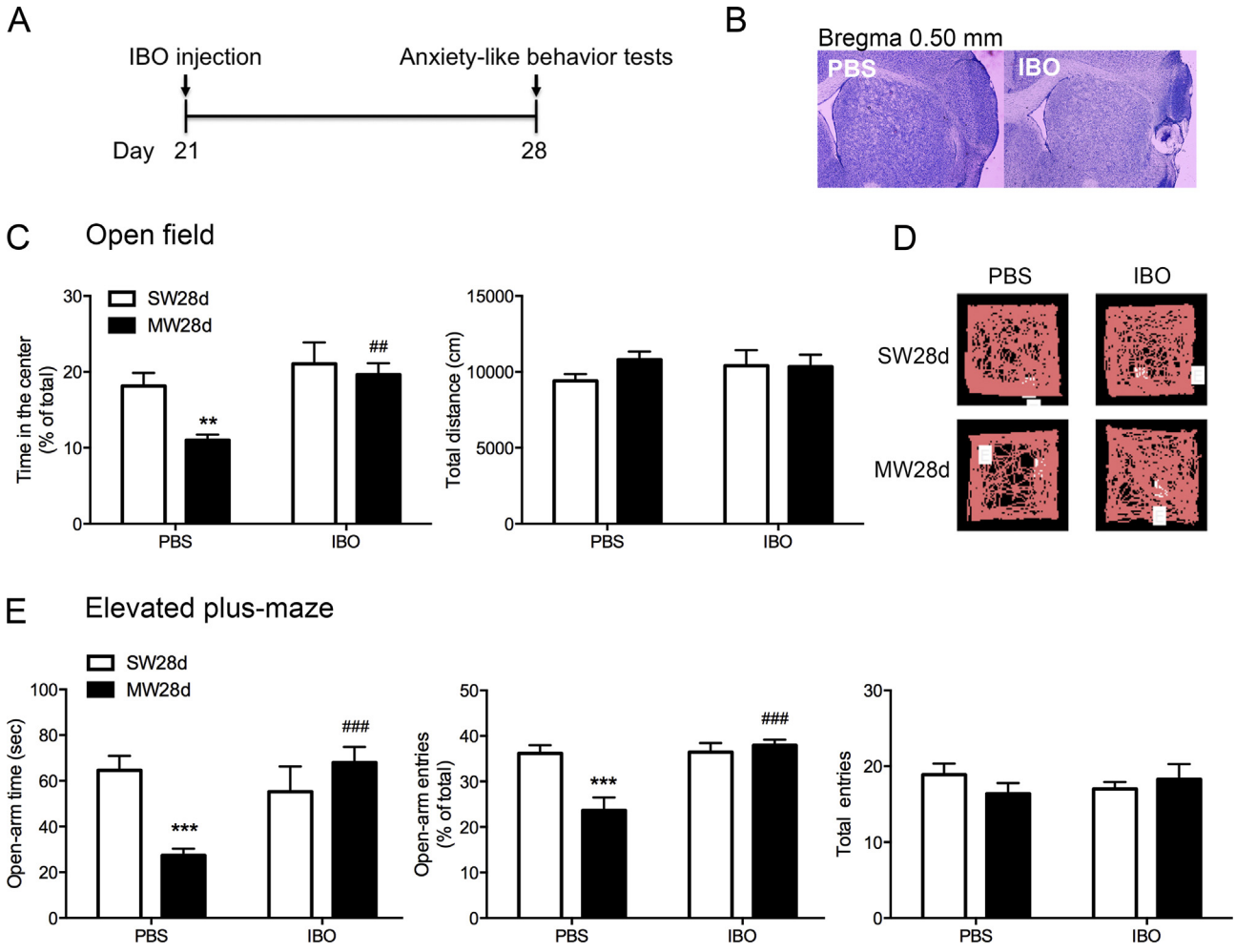

Fig. 3. Lesion of the medial IC alleviated the anxiety-like behavior. (A) Experimental timeline. (B) Representative photographs of the medial IC lesion (×1.25). (C) The time spent (left) in the center of the open field was decreased after 28 days of withdrawal with PBS treatment, which was reversed by IBO. Total distance (right) had no significant difference across groups. (D) Representative movement tracks (pink polylines) and the start and end points (white rectangles) in the open field. (E) The time spent (left) and percentage of entries (middle) into the open arms of the elevated plus-maze were decreased after 28 days of withdrawal with PBS treatment, which were reversed by IBO. Total arm entries (right) had no significant difference across groups. $^{**}p < 0.01$, $^{***}p < 0.001$ MW28d/PBS vs. SW28d/PBS group; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ MW28d/IBO vs. MW28d/PBS group, two-way ANOVA with Sidak's post hoc test, $n = 6–8$. SW28d: 28 days of saline withdrawal; MW28d: 28 days of morphine withdrawal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with saline controls ($p < 0.01$, $p < 0.05$, respectively) and with 1-day morphine-withdrawal mice ($p < 0.01$, $p < 0.05$, respectively), in which *Wnt7a* was more remarkable. Other members were slightly increased (*Wnt2*) or unchanged (*Wnt4, Wnt5a, Wnt11*). Western blot results also revealed the expression of Wnt7a protein in the medial IC, but not the anterior or posterior IC was significantly influenced by morphine ($F_{1, 20} = 5.990$, $p = 0.0237$), rather than day ($F_{1, 20} = 3.034$, $p = 0.0969$) and an interaction ($F_{1, 20} = 4.087$, $p = 0.0568$), which significantly increased in morphine-withdrawal mice only at the 28-day time point compared with saline controls (Fig. 5A, $p < 0.05$). The results suggest that Wnt7a expression in the medial IC is significantly increased after morphine protracted-abstinence. We then examined the distribution of Wnt7a protein (Fig. 5C), and it was co-localized greatly with glutamatergic neurons, but not with GABAergic neurons (Fig. 5B; $t_4 = 22.41$, $p < 0.001$).

Down-regulation of Wnt7a alleviates the anxiety-like behavior

We next evaluated the specific role of Wnt7a in the anxiety-like behavior with AAV-shWnt7a microinjected into the medial IC seven days before behavioral tests (Fig. 6A), which could significantly down-regulate its expression (Fig. 6C). First, morphine ($F_{1, 48} = 10.62$, $p = 0.0021$) and AAV-shWnt7a ($F_{1, 48} = 4.247$, $p = 0.0447$) influenced the time spent in the center of the field, as well as an interaction between these factors ($F_{1, 48} = 5.290$, $p = 0.0258$) (Fig. 6D). MW28d/AAV-Scramble group significantly spent less time in the center of the field than SW28d/AAV-Scramble group ($p < 0.001$). AAV-shWnt7a prevented this morphine withdrawal-induced deficit, because time spent in the center of MW28d/AAV-shWnt7a group was significantly increased compared with MW28d/AAV-Scramble group ($p < 0.01$). There was no significant difference in total

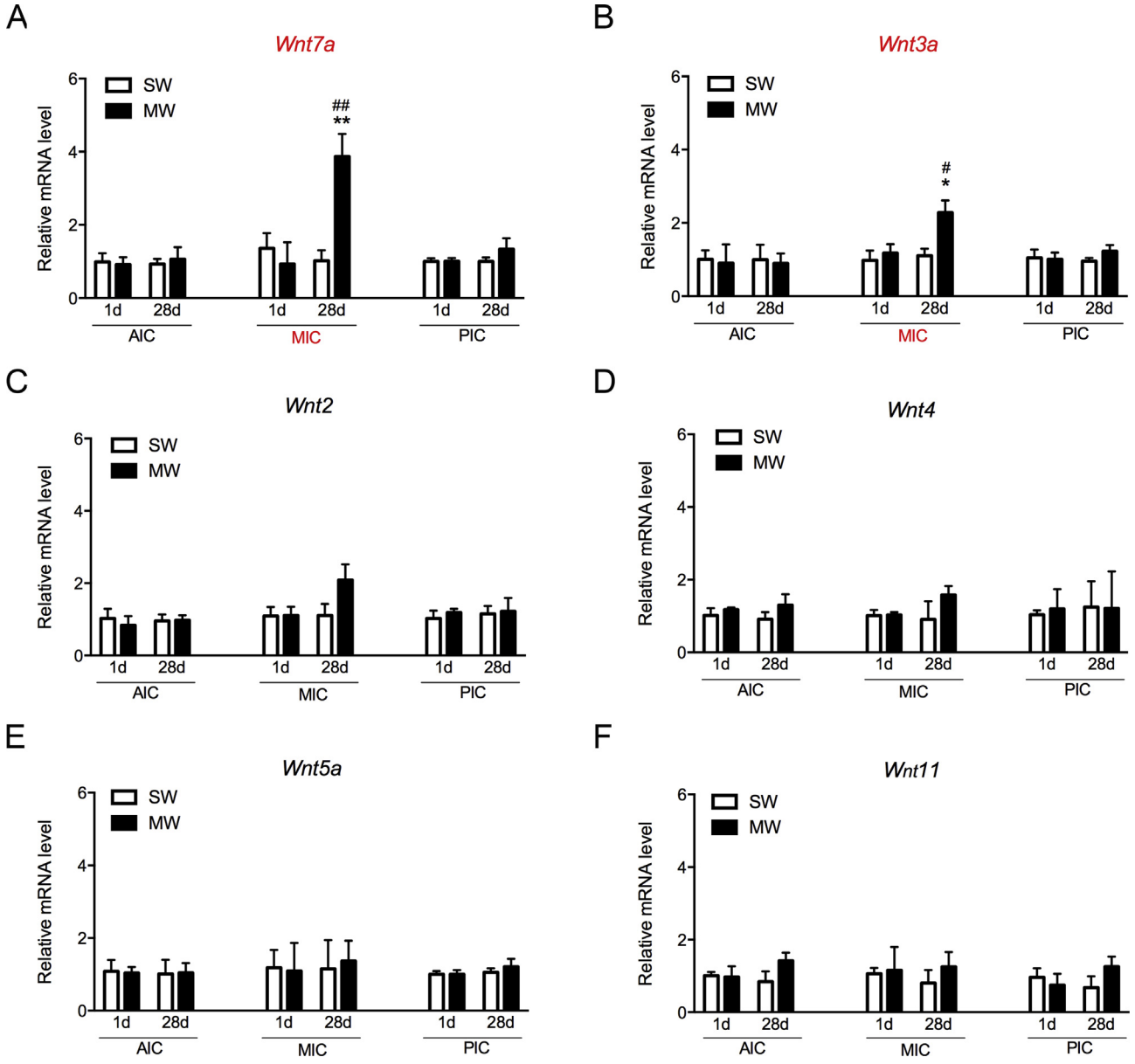

Fig. 4. Expression of Wnts mRNA in the IC measured after 1 and 28 days of withdrawal. (A, B) Wnt7a and Wnt3a mRNA were increased in the medial IC, but not the anterior or posterior IC after 28 days of withdrawal. (C–F) Wnt2, Wnt4, Wnt5a and Wnt11 mRNA were unchanged. $^{*}p < 0.05$, $^{**}p < 0.01$ MW vs. SW group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ 28- vs. 1-day MW group, two-way ANOVA with Sidak's post hoc test, $n = 6$. SW: saline withdrawal; MW: morphine withdrawal. AIC: the anterior IC, MIC: the medial IC, PIC: the posterior IC.

distance across groups. Second, morphine ($F_{1,\ 48} = 7.564$, $p = 0.0084$, $F_{1,\ 48} = 6.561$, $p = 0.0136$, respectively) and AAV-shWnt7a ($F_{1,\ 48} = 6.309$, $p = 0.0154$, $F_{1,\ 48} = 4.608$, $p = 0.0369$, respectively) influenced the time spent and percentage of entries into the open arms of the maze, as well as an interaction between these factors ($F_{1,\ 48} = 6.229$, $p = 0.0161$, $F_{1,\ 48} = 8.318$, $p = 0.0059$, respectively) (Fig. 6F). MW28d/AAV-Scramble group showed significantly less time spent and entries into the open arms than SW28d/AAV-Scramble group ($p < 0.01$; $p < 0.001$, respectively) and AAV-shWnt7a prevented this deficit ($p < 0.01$). And there was no significant difference in total arms' entries across groups. These results suggest that specific down-regulation of Wnt7a in the medial IC alleviates anxiety-like behavior associated with morphine protracted abstinence.

DISCUSSION

The present study demonstrates that the medial IC is a critical subdivision for anxiety-like behavior related to morphine abstinence in mice. Meanwhile, Wnt7a in the medial IC specifically involves in mediating the anxiety-like behavior.

Opioid addicts usually display some emotion-related disorders such as anxiety and depression (Nunes et al., 2004; Peles et al., 2007), whereas these emotion disor-

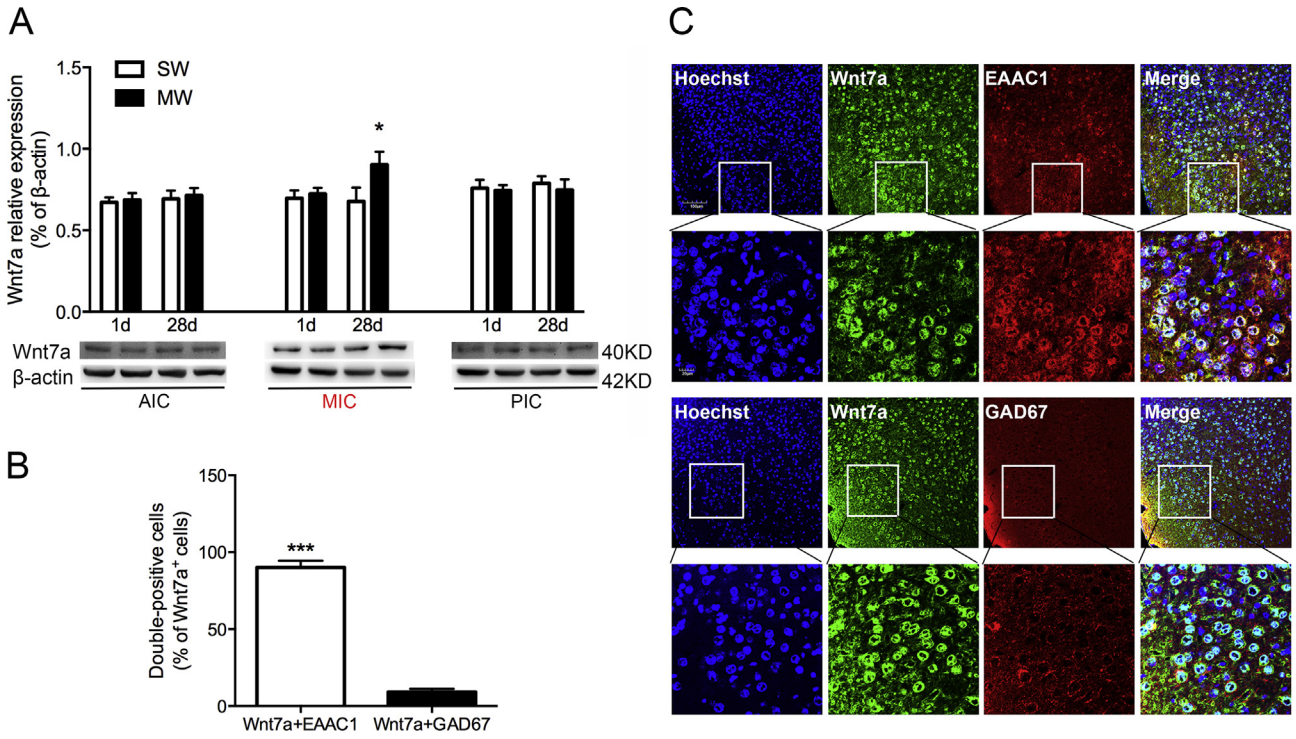

Fig. 5. Expression of Wnt7a protein in the IC measured after 1 and 28 days of withdrawal. (A) Wnt7a protein was increased in the medial IC, but not the anterior or posterior IC after 28 days of withdrawal. $^{*}p < 0.05$ MW vs. SW group, two-way ANOVA with Sidak's post hoc test, $n = 6$. (B) Percentage of double-positive cells. $^{***}p < 0.001$ Wnt7a + EAAC1 vs. Wnt7a + GAD67 group, unpaired t tests, $n = 3$. (C) Representative immunofluorescence images (×20 top, ×60 bottom) of Hoechst (blue), Wnt7a (green), EAAC1 or GAD67 (red). SW: saline withdrawal; MW: morphine withdrawal. AIC: the anterior IC, MIC: the medial IC, PIC: the posterior IC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ders have been reported to aggravate their craving and even relapse after abstinence (Negus, 2006). In this study, morphine withdrawal mice develop anxiety-like deficits assessed by the open-field test and elevated plus-maze test, which gradually increase with the duration of abstinence, while the physical symptoms obviously attenuate, as described previously in other studies (Jia et al., 2013; Lee et al., 2014). It should be noted that although no statistical difference, there is still a tendency of anxiety-like deficits in the time prior to 28 days of morphine abstinence. These results reveal the comorbidity between opioid addiction and anxiety (Brooner et al., 1997; Darke et al., 2009; Teesson et al., 2005), which maybe concealed by the serious physical symptoms at early period of abstinence. However, Goeldner et al. fail to reveal the anxiogenic-like effect of morphine protracted-withdrawal by open-field and light–dark tests (Goeldner et al., 2011). Although Becker et al. detect increased anxiety in marble burying and novelty-suppressed feeding tests in mice after 4 weeks of morphine withdrawal, a paradoxical increase in open-arm exploration in elevated plus-maze test is also detected in morphine abstinent animals (Becker et al., 2017). These discrepancies likely result from methodological issues, for example, different light conditions: 150 lux in the open-field test (Goeldner et al., 2011) and 15 lux in the elevated plus-maze test (Becker et al., 2017) compared to 30 lux in our two tests. It has been proved that rat anxiety responses are decreased with decreasing light intensity in elevated plus-maze test (Cosquer et al., 2005). Therefore, under our experimental condition, anxiety-like behavior is more significant during protracted abstinence from morphine.

According to "the somatic marker theory of addiction", there should be a potential link between emotional alterations in drug abusers and their impaired decision-making (Verdejo-Garcia and Bechara, 2009). It has been proved that the IC, as an important neural substrate of the interoception system, involves in translating various visceral sensations into subjective emotions/feelings in drug abusers such as "liking" and "wanting" following drug use or "anxiety" and "irritability" following drug abstinence, and thus influences their motivation and decision (Naqvi et al., 2014; Verdejo-Garcia and Bechara, 2009). The IC is generally divided into two subdivisions. The anterior insula (AI) may be a high-order interoceptive area, connecting with the posterior insula (PI) and the limbic regions such as the hypothalamus, nucleus accumbens and amygdala (Ohara et al., 2003). On the other hand, the PI receives visceral information from the parabrachial nuclei and thalamus (Allen et al., 1991; Cechetto, 1987), indicating the role of a primary interoceptive area. Previous studies have shown that inhibition of protein synthesis in the AI abolishes the retrieval of amphetamine-induced CPP but that has no effect on the PI (Contreras et al., 2012). Inactivation of the PI, but not the AI, disrupts

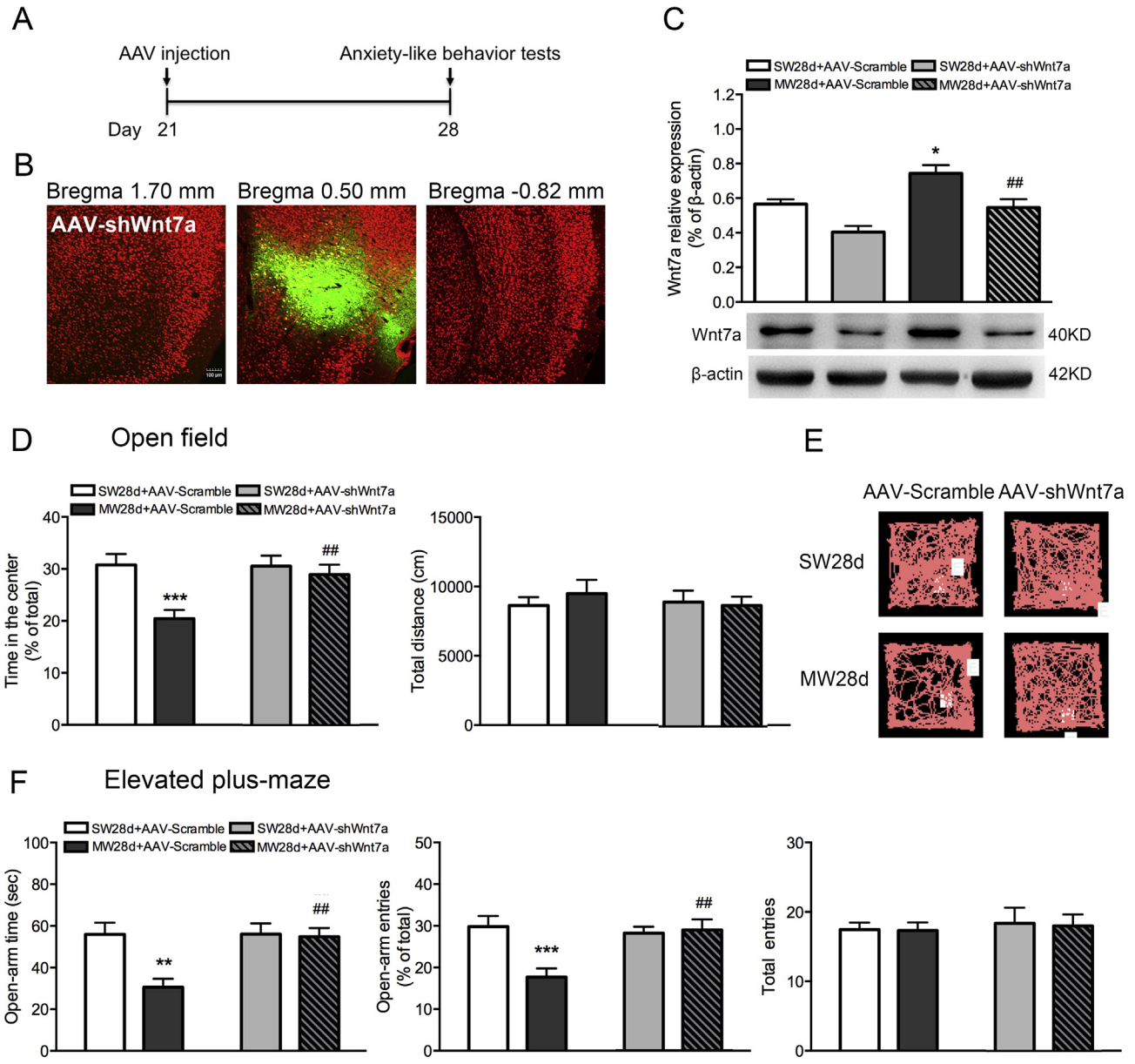

Fig. 6. Down-regulation of Wnt7a in the medial IC alleviated the anxiety-like behavior. (A) Experimental timeline. (B) Representative immunofluorescence images (×10) of AAV-shWnt7a (green) injection site. (C) Wnt7a expression in the medial IC was down-regulated by AAV-shWnt7a. $^{*}p < 0.05$ MW28d/AAV-Scramble vs SW28d/AAV-Scramble group; $^{\#\#}p < 0.01$ MW28d/AAV-shWnt7a vs MW28d/AAV-Scramble group, two-way ANOVA with Sidak's post hoc test, $n = 6$. (D) The time spent (left) in the center of the open field was decreased after 28 days withdrawal with AAV-Scramble treatment, which was reversed by AAV-shWnt7a. Total distance (right) had no significant difference across groups. (E) Representative movement tracks (pink polylines) and the start and end points (white rectangles) in the open field. (F) The time spent (left) and percentage of entries (middle) into the open arms of the elevated plus-maze were decreased after 28 days of withdrawal with AAV-Scramble treatment, which were reversed by AAV-shWnt7a. Total arm entries (right) had no significant difference across groups. $^{**}p < 0.01$, $^{***}p < 0.001$, MW28d/AAV-Scramble vs SW28d/AAV-Scramble group; $^{\#\#}p < 0.01$ MW28d/AAV-shWnt7a vs MW28d/AAV-Scramble group, two-way ANOVA with Sidak's post hoc test, $n = 13$. SW28d: 28 days of saline withdrawal; MW28d: 28 days of morphine withdrawal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the acquisition of morphine-induced CPP (Li et al., 2013). These data imply that different subdivisions of the IC may contribute to different phases of drug addiction. However, the specifically subdivision of the IC-regulating emotional alterations after drug abstinence is still unclear. Interestingly, some other researches document that just the medial part is a region of IC obviously responding to visceral stimulation (Mesulam and Mufson, 1982; Schier et al., 2016), and our results also found glutamatergic neurons in mouse medial rather than anterior or posterior IC were significantly activated after 28 days of morphine withdrawal. Moreover, selectively lesion of the medial IC significantly alleviates abstinence-related anxiety-like behavior. These results confirm that the medial IC is a

critical subdivision in anxiety-like deficits after drug abstinence.

Protracted abstinence-related anxiety symptom cannot be reversed by opioid maintenance therapy (Yin et al., 2015), suggesting a long-term adaptive process develops outside the opioid system. Wnt signaling pathways, which play an important role in developmental processes of the nervous system, may function in synaptic plasticity in adult brain (Chen et al., 2006; Wayman et al., 2006). Recently, Wnt signaling pathways also have been proved to involve in drug addiction and anxiety-like symptom (Cuesta et al., 2017; Wang et al., 2017; Wang et al., 2018). So, we firstly examined the mRNA levels of Wnts. Wnts are divided into two sub-classes: the proteins such as Wnt2, Wnt3a and Wnt7a, involved in the canonical pathway (He et al., 2015) and the proteins such as Wnt4, Wnt5a and Wnt11, involved in the non-canonical pathway (Willert and Nusse, 2012). It reveals that *Wnt7a* and *Wnt3a* in the medial IC, but not anterior or posterior IC are significant increased after 28 days of morphine withdrawal and *Wnt7a* increased more remarkably. In contrast, others are slightly increased (*Wnt2*) or unchanged (*Wnt4, Wnt5a* and *Wnt11*). These results suggest that Wnt7a and Wnt3a up-regulation may be related to morphine abstinence-induced anxiety.

Chronic (10 days) but not acute (12 hours) morphine withdrawal elicits not only a presynaptic potentiation but also a postsynaptic potentiation of glutamate synaptic transmission at MSN glutamatergic synapses in rat nucleus accumbens (Wu et al., 2012). Interestingly, both Wnt7a and Wnt3a can bind to Frizzled receptors and activate the canonical pathway in presynaptic terminal. Such activation can promote the trafficking of neurotransmitter vesicles, which increases synaptic transmission and receptor recycling (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Oliva et al., 2013). These ligands also increase the clustering of presynaptic proteins and the number of presynaptic puncta, suggesting the role in the presynaptic assembly (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008). Although Wnt7a has been designated as a ‘‘canonical” Wnt, it also activates the non-canonical pathway and specifically regulates the postsynaptic compartment of excitatory synapses rather than inhibitory synapses by increasing the density and maturity of dendritic spines (Ciani et al., 2011). In this study, we found Wnt7a protein, co-localized with glutamatergic neurons of the medial IC, was significantly increased after 28 days of morphine withdrawal; specific down-regulation of the Wnt7a expression alleviated the abstinence-related anxiety-like behavior. Moreover, we also found that one of the putative mechanisms for anxiolytic-like effect of Wnt7a may be to prevent the increased density of dendritic spines in the medial IC after morphine protracted-abstinence. These results indicate that Wnt7a is an important regulator for morphine abstinence-related anxiety-like behavior.

In conclusion, this study for the first time reveals Wnt7a in the medial IC plays a critical role in the anxiety-like behavior during morphine protracted-abstinence. And the finding provides a novel molecular clue for intervening morphine withdrawal symptoms.

ACKNOWLEDGMENTS

This work is supported by grants from the National Natural Science Foundation of China (grant number 81471353), the National Basic Research Program of China (grant number 2015CB553500) and Science Fund for Creative Research Groups from the National Natural Science Foundation (81521063) of China to CL Cui.

AUTHORS CONTRIBUTION

Cailian Cui and Hui Ma conceived and designed the research; Hui Ma performed all experiments and drafted the manuscript; Na Wang, Xinjuan Wang, Meng Jia and Yijing Li provided technical support; Cailian Cui and Hui Ma edited and revised the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Adhikari A (2014) Distributed circuits underlying anxiety. Front Behav Neurosci 8:112.

Ahmad-Annuar A, Ciani L, Simeonidis I, Herreros J, Fredj NB, Rosso SB, Hall A, Brickley S, Salinas PC (2006) Signaling across the synapse: a role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. J Cell Biol 174:127–139.

Allen GV, Saper CB, Hurley KM, Cechetto DF (1991) Organization of visceral and limbic connections in the insular cortex of the rat. J Comp Neurol 311:1–16.

Alvarez RP, Kirlic N, Misaki M, Bodurka J, Rhudy JL, Paulus MP, Drevets WC (2015) Increased anterior insula activity in anxious individuals is linked to diminished perceived control. Transl Psychiatry 5 e591.

Anraku T, Ikegaya Y, Matsuki N, Nishiyama N (2001) Withdrawal from chronic morphine administration causes prolonged enhancement of immobility in rat forced swimming test. Psychopharmacology (Berl) 157:217–220.

Becker JAJ, Kieffer BL, Le Merrer J (2017) Differential behavioral and molecular alterations upon protracted abstinence from cocaine versus morphine, nicotine, THC and alcohol. Addict Biol 22:1205–1217.

Brooner RK, King VL, Kidorf M, Schmidt CJ, Bigelow GE (1997) Psychiatric and substance use comorbidity among treatment-seeking opioid abusers. Arch Gen Psychiatry 54:71–80.

Cechetto DF (1987) Central representation of visceral function. Fed Proc 46:17–23.

Cerpa W, Godoy JA, Alfaro I, Farias GG, Metcalfe MJ, Fuentealba R, Bonansco C, Inestrosa NC (2008) Wnt7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. J Biol Chem 283:5918–5927.

Chen J, Park CS, Tang SJ (2006) Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. Journal of Biological Chemistry 281:11910–11916.

Ciani L, Boyle KA, Dickins E, Sahores M, Anane D, Lopes DM, Gibb AJ, Salinas PC (2011) Wnt7a signaling promotes dendritic spine growth and synaptic strength through Ca^{2+}/Calmodulin-dependent protein kinase II. Proc Natl Acad Sci U S A 108:10732–10737.

Ciani L, Salinas PC (2005) WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat Rev Neurosci 6:351–362.

Contreras M, Billeke P, Vicencio S, Madrid C, Perdomo G, Gonzalez M, Torrealba F (2012) A role for the insular cortex in long-term memory for context-evoked drug craving in rats. Neuropsychopharmacology 37:2101–2108.

Contreras M, Ceric F, Torrealba F (2007) Inactivation of the interoceptive insula disrupts drug craving and malaise induced by lithium. Science 318:655–658.
Cosquer B, Galani R, Kuster N, Cassel JC (2005) Whole-body exposure to 2.45 GHz electromagnetic fields does not alter anxiety responses in rats: a plus-maze study including test validation. Behav Brain Res 156:65–74.
Cuesta S, Severin MJ, Batuecas J, Rosso SB, Pacchioni AM (2017) Wnt/β-catenin pathway in the prefrontal cortex is required for cocaine-induced neuroadaptations. Addict Biol 22:933–945.
Darke S, Mills K, Teesson M, Ross J, Williamson A, Havard A (2009) Patterns of major depression and drug-related problems amongst heroin users across 36 months. Psychiatry Res 166:7–14.
Davis EK, Zou Y, Ghosh A (2008) Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. Neural Dev 3:32.
Deng S, Zhang L, Zhu T, Liu YM, Zhang H, Shen Y, Li WG, Li F (2015) A behavioral defect of temporal association memory in mice that partly lack dopamine reuptake transporter. Sci Rep 5:17461.
Forget B, Pushparaj A, Le Foll B (2010) Granular insular cortex inactivation as a novel therapeutic strategy for nicotine addiction. Biol Psychiatry 68:265–271.
Ge JF, Xu YY, Qin G, Cheng JQ, Chen FH (2016) Resveratrol ameliorates the anxiety- and depression-like behavior of subclinical hypothyroidism rat: possible involvement of the HPT axis, HPA axis, and Wnt/β-catenin pathway. Front Endocrinol 7:44.
Goeldner C, Lutz PE, Darcq E, Halter T, Clesse D, Ouagazzal AM, Kieffer BL (2011) Impaired emotional-like behavior and serotonergic function during protracted abstinence from chronic morphine. Biol Psychiatry 69:236–244.
Gossop M, Bradley B, Phillips GT (1987) An investigation of withdrawal symptoms shown by opiate addicts during and subsequent to a 21-day inpatient methadone detoxification procedure. Addict Behav 12:1–6.
He S, Lu Y, Liu X, Huang X, Keller ET, Qian CN, Zhang J (2015) Wnt3a: functions and implications in cancer. Chin J Cancer 34:554–562.
Hoehn-Saric R, Schlund MW, Wong SH (2004) Effects of citalopram on worry and brain activation in patients with generalized anxiety disorder. Psychiatry Res 131:11–21.
Hollander JA, Lu Q, Cameron MD, Kamenecka TM, Kenny PJ (2008) Insular hypocretin transmission regulates nicotine reward. Proc Natl Acad Sci U S A 105:19480–19485.
Islam F, Xu K, Beninger RJ (2017) Inhibition of Wnt signalling dose-dependently impairs the acquisition and expression of amphetamine-induced conditioned place preference. Behav Brain Res 326:217–225.
Jia W, Liu R, Shi J, Wu B, Dang W, Du Y, Zhou Q, Wang J, Zhang R (2013) Differential regulation of MAPK phosphorylation in the dorsal hippocampus in response to prolonged morphine withdrawal-induced depressive-like symptoms in mice. PLoS One 8 e66111.
Kawaguchi A, Nemoto K, Nakaaki S, Kawaguchi T, Kan H, Arai N, Shiraishi N, Hashimoto N, Akechi T (2016) Insular volume reduction in patients with social anxiety disorder. Front Psychiatry 7:3.
Koob GF (2009) Brain stress systems in the amygdala and addiction. Brain Res 1293:61–75.
Koob GF, Le Moal M (2008) Addiction and the brain antireward system. Annu Rev Psychol 59:29–53.
Koob GF, Volkow ND (2010) Neurocircuitry of addiction. Neuropsychopharmacology 35:217–238.
Lammel S, Lim BK, Ran C, Huang KW, Betley MJ, Tye KM, Deisseroth K, Malenka RC (2012) Input-specific control of reward and aversion in the ventral tegmental area. Nature 491:212–217.
Lee B, Sur BJ, Shim I, Hahm DH, Lee H (2014) Acupuncture stimulation attenuates impaired emotional-like behaviors and activation of the noradrenergic system during protracted abstinence following chronic morphine exposure in rats. Evid Complement Alternat Med 2014 216503.
Li CL, Zhu N, Meng XL, Li YH, Sui N (2013) Effects of inactivating the agranular or granular insular cortex on the acquisition of the morphine-induced conditioned place preference and naloxone-precipitated conditioned place aversion in rats. J Psychopharmacol 27:837–844.
Maguschak KA, Ressler KJ (2012) A role for WNT/β-catenin signaling in the neural mechanisms of behavior. J Neuroimmune Pharmacol 7:763–773.
Mesulam MM, Mufson EJ (1982) Insula of the old world monkey. III: efferent cortical output and comments on function. J Comp Neurol 212:38–52.
Murray JE, Belin-Rauscent A, Simon M, Giuliano C, Benoit-Marand M, Everitt BJ, Belin D (2015) Basolateral and central amygdala differentially recruit and maintain dorsolateral striatum-dependent cocaine-seeking habits. Nat Commun 6:10088.
Naqvi NH, Bechara A (2009) The hidden island of addiction: the insula. Trends Neurosci 32:56–67.
Naqvi NH, Bechara A (2010) The insula and drug addiction: an interoceptive view of pleasure, urges, and decision-making. Brain Struct Funct 214:435–450.
Naqvi NH, Gaznick N, Tranel D, Bechara A (2014) The insula: a critical neural substrate for craving and drug seeking under conflict and risk. Ann N Y Acad Sci 1316:53–70.
Naqvi NH, Rudrauf D, Damasio H, Bechara A (2007) Damage to the insula disrupts addiction to cigarette smoking. Science 315:531–534.
Negus SS (2006) Choice between heroin and food in nondependent and heroin-dependent rhesus monkeys: effects of naloxone, buprenorphine, and methadone. J Pharmacol Exp Ther 317:711–723.
Nunes EV, Sullivan MA, Levin FR (2004) Treatment of depression in patients with opiate dependence. Biol Psychiatry 56:793–802.
Ohara PT, Granato A, Moallem TM, Wang BR, Tillet Y, Jasmin L (2003) Dopaminergic input to GABAergic neurons in the rostral agranular insular cortex of the rat. J Neurocytol 32:131–141.
Oliva CA, Vargas JY, Inestrosa NC (2013) Wnt signaling: role in LTP, neural networks and memory. Ageing Res Rev 12:786–800.
Packard M, Koo ES, Gorczyca M, Sharpe J, Cumberledge S, Budnik V (2002) The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell 111:319–330.
Peles E, Schreiber S, Naumovsky Y, Adelson M (2007) Depression in methadone maintenance treatment patients: rate and risk factors. J Affect Disord 99:213–220.
Pushparaj A, Hamani C, Yu W, Shin DS, Kang B, Nobrega JN, Le Foll B (2013) Electrical stimulation of the insular region attenuates nicotine-taking and nicotine-seeking behaviors. Neuropsychopharmacology 38:690–698.
Schier LA, Blonde GD, Spector AC (2016) Bilateral lesions in a specific subregion of posterior insular cortex impair conditioned taste aversion expression in rats. J Comp Neurol 524:54–73.
Scott D, Hiroi N (2011) Deconstructing craving: dissociable cortical control of cue reactivity in nicotine addiction. Biol Psychiatry 69:1052–1059.
Seif T, Chang SJ, Simms JA, Gibb SL, Dadgar J, Chen BT, Harvey BK, Ron D, Messing RO, Bonci A, Hopf FW (2013) Cortical activation of accumbens hyperpolarization-active NMDARs mediates aversion-resistant alcohol intake. Nat Neurosci 16:1094–1100.
Shen F, Wang XW, Ge FF, Li YJ, Cui CL (2016) Essential role of the NO signaling pathway in the hippocampal CA1 in morphine-associated memory depends on glutaminergic receptors. Neuropharmacology 102:216–228.
Swift RM, Stout RL (1992) The relationship between craving, anxiety, and other symptoms in opioid withdrawal. J Subst Abuse 4:19–26.
Teesson M, Havard A, Fairbairn S, Ross J, Lynskey M, Darke S (2005) Depression among entrants to treatment for heroin dependence in the Australian Treatment Outcome Study (ATOS): prevalence, correlates and treatment seeking. Drug Alcohol Depend 78:309–315.
Verdejo-Garcia A, Bechara A (2009) A somatic marker theory of addiction. Neuropharmacology 56(Suppl 1):48–62.

Wang J, Zhu G, Huang L, Nie T, Tao K, Li Y, Gao G (2017) Morphine administration induces change in anxiety-related behavior via Wnt/β-catenin signaling. Neurosci Lett 639:199–206.

Wang X, Zhao X, Yi Z, Ma B, Wang H, Pu Y, Wang J, Wang S (2018) WNT5A promotes migration and invasion of human osteosarcoma cells via SRC/ERK/MMP-14 pathway. Cell Biol Int 42:598–607.

Wayman GA, Impey S, Marks D, Saneyoshi T, Grant WF, Derkach V, Soderling TR (2006) Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. Neuron 50:897–909.

Willert K, Nusse R (2012) Wnt proteins. Cold Spring Harb Perspect Biol 4 a7864.

Wu W, Li H, Liu Y, Huang X, Chen L, Zhai H (2014) Involvement of insular muscarinic cholinergic receptors in morphine-induced conditioned place preference in rats. Psychopharmacology (Berl) 231:4109–4118.

Wu X, Shi M, Wei C, Yang M, Liu Y, Liu Z, Zhang X, Ren W (2012) Potentiation of synaptic strength and intrinsic excitability in the nucleus accumbens after 10 days of morphine withdrawal. J Neurosci Res 90:1270–1283.

Yin W, Pang L, Cao X, McGoogan JM, Liu M, Zhang C, Li Z, Li J, Rou K (2015) Factors associated with depression and anxiety among patients attending community-based methadone maintenance treatment in China. Addiction 110(Suppl 1):51–60.

Zheng J, Jiang YY, Xu LC, Ma LY, Liu FY, Cui S, Cai J, Liao FF, Wan Y, Yi M (2017) Adult hippocampal neurogenesis along the dorsoventral axis contributes differentially to environmental enrichment combined with voluntary exercise in alleviating chronic inflammatory pain in mice. J Neurosci 37:4145–4157.

(Received 29 June 2018, Accepted 16 October 2018)
(Available online 24 October 2018)