THE ROLE OF GALANIN SYSTEM IN MODULATING DEPRESSION, ANXIETY, AND ADDICTION-LIKE BEHAVIORS AFTER CHRONIC RESTRAINT STRESS

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Abstract—There is high comorbidity between stress-related psychiatric disorders and addiction, suggesting they may share one or more common neurobiological mechanisms. Because of its role in both depressive and addictive behaviors, the galanin system is a strong candidate for such a mechanism. In this study, we tested if galanin and its receptors are involved in stress-associated behaviors and drug addiction. Mice were exposed to 21 days of chronic restraint stress (CRS); subsequently, mRNA levels of galanin, galanin receptors (GalRs), the rate-limiting enzymes for the synthesis of monoamines, and monoamine autoreceptors were measured in the nucleus accumbens by a quantitative real-time polymerase chain reaction. Moreover, we tested the effects of this stress on morphine-induced addictive behaviors. We found that CRS induced anxiety and depression-like behaviors, impaired the formation and facilitated the extinction process in morphine-induced conditioned place preference (CPP), and also blocked morphine-induced behavioral sensitization. These behavioral results were accompanied by a CRS-dependent increase in the mRNA expression of galanin, GalR1, tyrosine hydroxylase (TH), tryptophan hydroxylase 2, and 5-HT$_{1B}$ receptor. Interestingly, treatment with a commonly used antidepressant, fluoxetine, normalized the CRS-induced behavioral changes based on reversing the higher expression of galanin and TH while increasing the expression of GalR2 and α_{2A}-adrenceptor. These results indicate that activating the galanin system, with corresponding changes to noradrenergic systems, following chronic stress may modulate stress-associated behaviors and opiate addiction. Our findings suggest that galanin and GalRs are worthy of further exploration as potential therapeutic targets to treat stress-related disorders and drug addiction. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: galanin system, morphine, conditioned place preference, behavioral sensitization, monoamine neurotransmitters.

INTRODUCTION

Drug addiction is a chronically reoccurring disorder characterized by compulsive behaviors, the development of drug tolerance, and the appearance of a depressive state during withdrawal (Koob and Le Moal, 2005). Many attempts have been made to elucidate the mechanisms of these phenomena. The remarkably high comorbidity of stress-related psychiatric disorders (e.g., depression) and addiction (Koob, 2008), however, suggests shared neurobiology. More specifically, both clinical (Brown et al., 1990; Dewart et al., 2006; Ouimette et al., 2007; Bruijnzeel, 2012) and animal research (Shaham et al., 2000) demonstrate that stressful life experiences significantly predispose individuals to drug abuse and trigger relapse. Moreover, the negative affective symptoms and/or increases in stress-related corticotropic-releasing factor (CRF) associated with drug cessation may increase the risk of relapse (Bruijnzeel and Gold, 2005). Conversely, exposure to a severe acute stressor or chronic psychological stress attenuates dopaminergic content or tone (Willner et al., 1992; Cabib and Puglisi-Allegra, 1994, 1996; Moore et al., 2001; Miczek et al., 2011). Affective and reward pathways are thus highly intertwined, but a shared neurobiological mechanism is still not precisely known.

Many biochemical systems are engaged in both stress-related and drug-seeking behaviors. For example, the hypothalamo–pituitary–adrenal (HPA) axis, a system activated following stress, is associated with drug administration and withdrawal (Bruijnzeel and Gold, 2005; Sinha, 2008). Specifically, both cocaine administration and withdrawal increases CRF mRNA in the rat central nucleus of amygdala (Richter and Weiss, 1999; Maj et al., 2003); conversely, CRF antisera or receptor antagonists injected intracerebroventricularly (i.c.v.) blocks both cocaine-induced hyperactivity and cocaine withdrawal-induced anxiety-like behavior

(Sarnyai et al., 1992, 1995; Basso et al., 1999). The locus coeruleus (LC)–norepinephrine system is involved in a variety of behavioral effects of addictive drugs (Weinshenker and Schroeder, 2006; Sofuoglu and Sewell, 2008). Cocaine seeking is attenuated by antagonizing adrenergic receptors or suppressing noradrenergic neurotransmission but evoked by augmenting adrenergic neurotransmission through antagonism of α_2 adrenergic autoreceptors or the blockade of reuptake in reinstatement models (Mantsch et al., 2010). Many other neuropeptides (i.e., orexin, substance P, arginine vasopressin (AVP), neuropeptide Y (NPY), and nociceptin) play an important role in stress-mediated responses and are suggested to modulate drug addiction (Koob, 2008; Commons, 2009; See and Waters, 2011). In this study, however, we specifically test the role of a strongly implicated neuropeptide system, the galanin system, in stress-induced negative emotional states and morphine addiction.

Galanin is a 29 amino-acid peptide originally isolated from the porcine intestine (Tatemoto et al., 1983; Schmidt et al., 1991); the physiological actions of galanin are mediated through three G-protein-coupled receptors, all of which are encoded by distinct genes (Wang et al., 1997; Branchek et al., 2000). In the central nervous system, galanin receptor (GalR) 3 expression is less abundant and more restricted than GalR1 and GalR2 (Mennicken et al., 2002; Mitsukawa et al., 2008). Galanin is localized to the same brain regions as norepinephrine (LC), serotonin (dorsal raphe nucleus (DRN)), and dopamine (ventral-tegmental area (VTA)) (Holmes and Picciotto, 2006; Ögren et al., 2010). As such, galanin has been implicated in regulating anxiety, depression, and addiction-like behaviors (Charney, 2004; Lu et al., 2007; Kuteeva et al., 2008; Picciotto, 2008). An extensive amount of evidence has shown that galanin can block addictive behaviors (Zachariou et al., 1999; Hawes et al., 2007; Narasimhaiah et al., 2009), but there are more inconsistent findings in anxiety and depression-like behavior (Bing et al., 1993; Möller et al., 1999; Murck et al., 2004; Kuteeva et al., 2005). An explanation for these contradictory results is that the two main GalRs (i.e., GalR1 vs. GalR2) exhibit opposite effects on behavioral functions (Kuteeva et al., 2008).

Based on the aforementioned evidence, we sought to more directly test the role of galanin and its receptors in modulating anxiety, depression, and addiction-like behaviors. We hypothesize that the galanin system may act as a common mechanism to mediate stress-associated behaviors and drug addiction. Here, we employed 21 days of chronic restraint stress (CRS) to induce negative (e.g., depression-like) emotional states. Real-time polymerase chain reaction (RT-PCR) quantified mRNA expression of galanin and its receptors in the nucleus accumbens (Nac), a brain area implicated in both depression and addiction (Ikeda et al., 2012; Müller et al., 2012; Hikida et al., 2013). We also tested the effects of chronic restraint on morphine-induced conditioned place preference (CPP) and sensitization,

both with and without concurrent administration of fluoxetine (Flu), a commonly used antidepressant. Finally, to understand the effects of the galanin system on monoamine neurotransmitters such as dopamine, norepinephrine, and serotonin, which are all closely associated with stress-associated behaviors and drug addiction (Duman et al., 1997; Rao, 2006), the mRNA levels of the rate-limiting enzymes for their synthesis as well as their autoreceptors were also tested following chronic restraint.

EXPERIMENTAL PROCEDURES

Animals and drugs

C57BL/6J male mice (8 weeks old) weighing 20–25 g were obtained from Beijing Vital River Laboratories. They were housed in a group of four under constant temperature (23 ± 2 °C) and maintained on a 12-h light/dark cycle (lights on at 7 a.m.) with food and water available *ad libitum*. Prior to the start of the experiment, mice were allowed to adapt to the new environment for 1 week. All procedures were approved by the Institutional Animal Care Committee at Xi'an Jiaotong University.

Morphine hydrochloride (Mor, Qinghai Pharmaceutical Group, China) and Flu (Sigma Chemical, St. Louis, MO, USA) were dissolved in sterile 0.9% saline (Sal) and administered at 10 ml/kg.

CRS

To restrain mice, 50 ml conical centrifuge tubes with 60 evenly spaced 3-mm-diameter holes for ventilation were used. Mice were placed head first into these tubes, with only enough space for limited forward and backward movements. This restraint stress was delivered simultaneously to mice in their home cages for 6 h daily from 10 a.m. to 4 p.m. for 21 days in a separate colony room from non-stressed mice, thereafter, they were returned to the same room where non-stressed mice were housed and placed on different shelves.

Open field (OP)

A separate group of mice were placed into chambers measuring 43 × 43 × 43 cm by Panlab's Smart software (version 2.5, Barcelona, Spain) and allowed to move freely without any injection. The total distance (cm) traveled was recorded for 1 h.

Forced swim test (FST)

The FST was carried out according to published protocols (Porsolt et al., 1977). Briefly, mice were placed individually into plexiglas cylinders (height: 25 cm, diameter: 9 cm) filled to a height of 15 cm with water maintained at 23–25 °C; mice remained in the apparatus for 6 min. Their behavior was recorded. Immobility, defined as ceasing to move or making only those movements necessary to keep the animal's head above water, was measured during the last 4 min of the test.

White–black box shuttle (WBS)

The CPP apparatus described below was also used for the WBS task after the floor of the black chamber was replaced with the stainless-steel mesh used in the white chamber. Mice were placed in the black chamber facing the white chamber, the latter of which was illuminated by light of 50-lux intensity. The door between the chambers was open allowing for freedom of movement. During the 20-min test, the time spent in the white chamber and the number of full entries to the white chamber were quantified.

CPP

The CPP chambers consisted of two identical wooden compartments (white and black; $15 \times 15 \times 37$ cm) with different visual and tactile cues. White walls with a stainless-steel mesh floor and black walls with a stainless-steel bar floor (W and B compartments, respectively) were separated by a sliding wood panel with a 5 cm $\times$ 7 cm door in the center of the base. The door remained open during the test phase, but was shut during the training phase to prevent movement of mice between two compartments. Night vision-equipped cameras were used to digitally record the behavior of mice; time spent in the two compartments was measured by Shanghai Jiliang software. The CPP paradigm included two separate phases: formation and extinction.

CPP formation: First, mice were allowed to move freely among the two compartments for 15 min to determine any baseline preferences for the W or B compartment prior to morphine administration in the *pre-conditioning test* (pre-test). During the training period, mice were treated once daily for 6 consecutive days with three cycles of alternating i.p. injections of morphine in compartment W and then Sal in compartment B. Immediately following each injection, mice were confined for 40 min to either the W or B compartment for morphine or Sal injections, respectively. In the control group, Sal was given each training day, irrespective of the compartment. The *post-conditioning test* (post-test) was given 24 h following all six of the training cycles. Like the pre-test, the door separating the B and W compartments was left open in the post-test. The CPP score, an index of preference, was calculated as the difference between time spent in the drug-paired chamber and the time spent in the Sal-paired chamber during the test. A positive CPP effect is observed when the morphine treatment group exhibits significantly higher scores (e.g., preference for the drug-paired chamber) relative to the control group. All compartments were cleaned with 70% ethanol and wiped dry between animal runs.

CPP extinction: After the final post-test, morphine-conditioned mice and Sal controls were treated with Sal immediately before being placed into their previously conditioned compartment for 40 min. This procedure continued, identical to the training cycles of the control group in the CPP formation phase. After each extinction cycle, a post-test was given to detect whether the CPP

effect in the previous morphine group was still robust. Extinction was indicated by the absence of significantly different scores between the formation phase's morphine-conditioned mice and Sal control groups.

There were two different doses of morphine used in the CPP paradigm: low dose (3 mg/kg) and high dose (20 mg/kg). 3 mg/kg was used to test how CRS affects CPP formation with a post-test was administered after each of the three training cycles. We administered 20 mg/kg to induce robust CPP such that the hypothesis that CRS might influence the stability of the CPP effect (assessed by extinction training) could be tested.

Behavioral sensitization (SENS)

Locomotor activity was quantified in the OP chambers. Mice were first habituated to the chambers for 1 h for 2 consecutive days. Following this habituation period, mice received an injection of morphine (3 and 20 mg/kg, i.p.) immediately before being placed into the chambers once daily for 1 h for 7 days to induce sensitization. After a 7-day washout period during which animals were not exposed to the chambers, the same dose of morphine was again administered. Horizontal locomotor activity was then measured for 1 h. SENS was indicated by increased locomotor activity in response to morphine on the challenge day as compared to the first day of injection in the same animal.

Quantitative RT-PCR (qRT-PCR)

Brains were rapidly harvested from decapitated mice, immediately frozen in liquid nitrogen, and stored at -80 °C. Nac samples were dissected on ice, placed into TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and homogenized at 4 °C by an Ultrasonic Processor (Sonics & Materials, Newtown, CT, USA). Thereafter, RNA was extracted and reverse transcribed into cDNA using PrimeScript 1st Strand cDNA synthesis kit system (TaKaRa, Ohtsu Shiga, Japan). A 12.5 μL amplification mixture contained cDNA (1 μL), 0.2 μM forward and reverse primers (0.5 μL), nuclease-free water (4.75 μL) and 6.25 μL SYBR Pre mix Ex Taq II (TaKaRa, Ohtsu Shiga, Japan) was used to perform RT-PCR with iQ5 optical system software (Bio-Rad Laboratories, Hercules, CA, USA). Relative gene expression normalized by β-actin was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Specific primers for detecting gene products as well as annealing temperatures are listed in Table 1. In contrast to the other genes studied, the dopamine D2 receptor has two alternatively spliced isoforms, named D2S (S = short) and D2L (L = long). The D2S gene lacks exon 6 of the D2L gene (87 bp). Moreover, D2S serves presynaptic autoreceptor functions while D2L acts mainly at postsynaptic sites (Usiello et al., 2000; De Mei et al., 2009). Thus, we designed two primer pairs: d2long (the binding regions are located in exon 6 and exon 7) and d2total (the binding regions are located in exon 2 and exon 3, and as such, the PCR product represents total transcript levels D2S and D2L genes). The relative expression of D2L normalized to the total gene expression of D2S and D2L (e.g., d2total replaces

Table 1. Primers used for real-time PCR

Gene of interest	Sequence	(5′–3′)	Size (bp)	Annealing temperature (°C)
Galanin	Forward	AGGCAAGAGGGAGTTACAACT	166	58
	Reverse	GGTGGCCAAGGGGATG		
Galanin receptor1[a]	Forward	GCCGCGATGTCTGTGGATCG	115	62
	Reverse	CGATGGACAGCGCCCAGATG		
Galanin receptor2[a]	Forward	GTGTGCCACCCAGCGTGGAG	111	64
	Reverse	TGGTGCGCGCATAGGTCAGG		
Galanin receptor3[a]	Forward	CCTGCCTCAACCCGCTCGTC	112	60
	Reverse	TGAAGGCGGTGGTGGTGGTG		
Tyrosine hydroxylase	Forward	TCAGTTCTGTGCGTCGGGTGT	135	62
	Reverse	CCAGGTCCAGGTCAGGGTCA		
D2L (specific)	Forward	ACAGGCGGAGAATGGATG	113	62
	Reverse	GTGGTGACTGGGAGGGAT		
D2S and D2L (total)	Forward	ATGCTGCTCACCCTCCTC	200	62
	Reverse	ATCCTGCTGAATTTCCAC		
α_{2A}-Adrenceptor	Forward	TCGGCGTGTTCGTGGTGT	167	62
	Reverse	GCGTCGGAAGTCGTGGTT		
Tryptophan hydroxylase 2	Forward	GGTCAACTGCGGGCGTAT	136	58
	Reverse	GAAAGGTGGTGATTAGGC		
5-HT$_{1B}$ receptor	Forward	GGAGGAGATGCTGGACTG	228	62
	Reverse	GAGGTGACCGAGGATGTG		
β-Actin[a]	Forward	GGCTACAGCTTCACCACCAC	414	60
	Reverse	TGCGCTCAGGAGGAGC		

[a] Cited from reference Hawes et al. (2005).

the primers of β-actin in real time PCR process) was used to reflect the expression of D2S. We also measured mRNA levels of the α_{2A}-adrenceptor because of its dominant expression and essential role in regulating neurotransmitter release; however, it should be noted that the adrenergic receptor subtype α_{2C}, which we did not quantify, also affects neurotransmitter release (Bücheler et al., 2002; Millan, 2004). Additionally, mRNA levels of 5-HT$_{1B}$ receptor, which is regarded as the terminal autoreceptor for 5-HT (Hoyer et al., 2002), were also measured.

Experiment design

Mice were first divided into four groups: Sal, Flu, Flu + CRS, and CRS. In the Flu + CRS group, mice were given 20 mg/kg Flu (i.p.) daily 30 min before receiving CRS. In the CRS group, Sal was injected instead of Flu for 21 days. The Flu and Sal groups received the same doses of Flu and Sal, respectively, but did not undergo CRS.

After 21 days of injections, some mice in each group were randomly selected and sacrificed (on Day 22) to detect the changes of specific gene expression via qRT-PCR. The additional mice were divided into two sets of experiments. In the first set, three behavioral tests (OP, WBS, FST) were performed to characterize depression-related behaviors. OP and WBS were tested on the first day and the interval between them was 8 h, while FST was tested on the second day. Each test was performed twice at different time points (OP + WBS: Day 22 and Day 40; FST: Day 23 and Day 41) to test if depressive behavior persisted for 19 days; however, distinct groups of mice were tested on these two time points to rule out the possibility that memory might participate in repeated exposure to the same test. These depression-related behavioral tests were performed from least invasive to most invasive (OP–WBS–FST) in order to decrease the influence of the prior test history (McIlwain et al., 2001). In the second set, mice were assigned to two morphine-induced, addiction-related behavior groups: CPP (Days 22–41) and SENS (Days 22–38). In each group, two doses of morphine (3 and 20 mg/kg) were used. CPP, SENS and sacrifice were carried out from 8 a.m. in every experimental day, while WBS and FST were from 4 p.m. Experimental groups and procedures are diagrammed in Fig. 1.

Statistical analysis

All data were expressed as mean ± SEM. Data obtained from SENS experiments were analyzed by three-way analysis of variance (ANOVA) for repeated measures [drug (Sal or Flu) × stress (Non CRS or CRS) × test day]. Data from CPP experiments were analyzed by two-way ANOVA for repeated measures (treatment × test day). Individual measures of SENS and CPP tests were compared among the groups using one-way ANOVA.

Data from other experiments were analyzed by two-way ANOVA [drug treatment (Sal or Flu) × stress (Non CRS or CRS)]. All post hoc comparisons used were Fischer's least significant difference (LSD). Statistical significance was considered $p < 0.05$.

RESULTS

CRS induced the anxiety-like and depression-like behaviors

As shown in Fig. 2A, 1 day after 21 days of Sal injection and CRS, mice exhibited a significant reduction in locomotor activity, as compared to animals that only
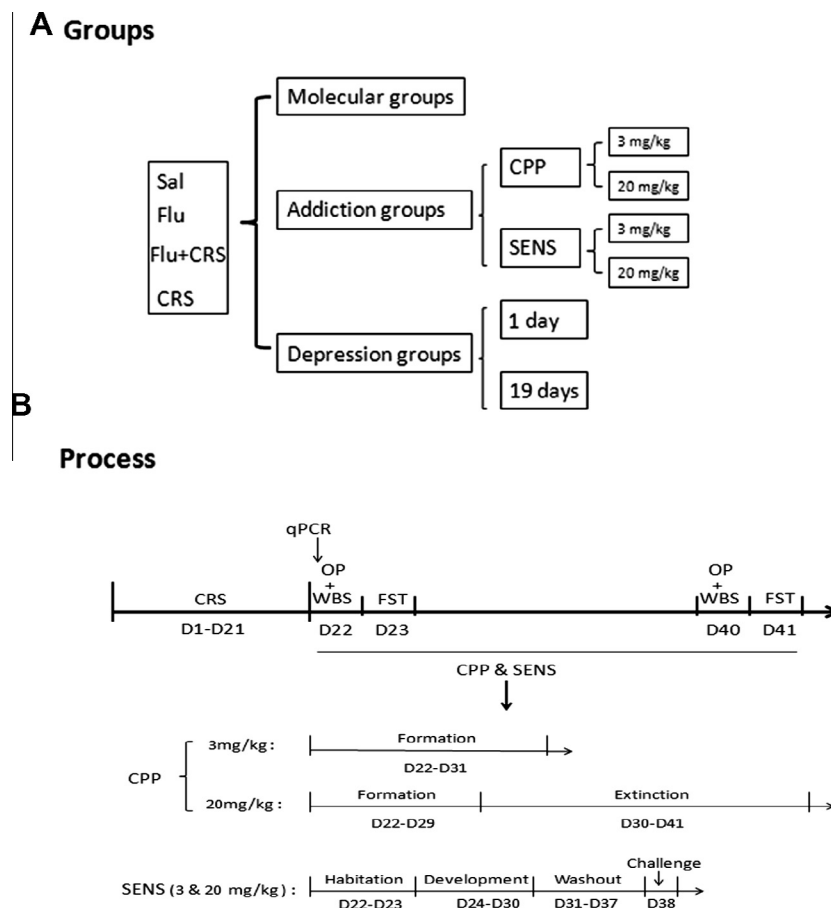
Fig. 1. Experimental design. (A) The assignment of groups. (B) Schematic of the experimental procedures in the present study. CPP, conditioned place preference; SENS, behavioral sensitization; CRS, chronic restraint stress; OP, open field; WBS, white–black box shuttle; FST, forced swim test; qPCR, quantitative PCR; D, day.

received Sal injection; this reduction could be reversed by Flu (drug × stress: $F_{(1,47)} = 6.336$, $p = 0.015$; post hoc analysis: CRS vs. Sal, $p = 0.018$; Flu + CRS vs. Sal, $p = 0.706$; $n = 12–14$ for each group). Decreased locomotion was no longer evident 19 days after the cessation of CRS (drug: $F_{(1,26)} = 0.011$, $p = 0.917$; stress: $F_{(1,26)} = 0.199$, $p = 0.659$; drug × stress: $F_{(1,26)} = 1.545$, $p = 0.255$; $n = 7–8$ for each group).

In the WBS test (Fig. 2B), mice that previously experienced Sal injection and CRS spent less time in the white chamber than mice that received Sal injections alone (drug × stress: $F_{(1,47)} = 4.709$, $p = 0.035$; post hoc analysis: CRS vs. Sal, $p = 0.008$). The number of visits to the white chamber ('shuttle times') was also lower in this group compared to control mice (drug × stress: $F_{(1,47)} = 7.008$, $p = 0.011$; post hoc analysis: CRS vs. Sal, $p = 0.020$). Nineteen days later, these anxiety-like behaviors persisted in CRS mice (time spent in the white chamber: drug × stress: $F_{(1,26)} = 5.834$, $p = 0.023$; post hoc analysis: CRS vs. Sal, $p = 0.008$) (shuttle times: drug × stress: $F_{(1,26)} = 5.545$, $p = 0.026$; post hoc analysis: CRS vs. Sal, $p = 0.003$). However, after Flu treatment, both the time spent in and the frequency of transitions to the aversive chamber increased in CRS mice, which was not significantly different from Sal-injected mice that did not

experience CRS (1 day, time spent in the white chamber and shuttle times: Flu + CRS vs. Sal, $p = 0.700, 0.135$; 19 days, time spent in the white chamber and shuttle times: Flu + CRS vs. Sal, $p = 0.948, 0.262$).

In the FST (Fig. 2C), the total time spent immobile was significantly higher in Sal-injected CRS mice, as compared to Sal-injected controls (drug × stress: $F_{(1,47)} = 5.393$, $p = 0.028$; post hoc analysis: CRS vs. Sal, $p = 0.007$). Flu normalized time spent immobile, as there was no significant difference between Flu + CRS mice and Sal mice (post hoc analysis: $p = 0.395$). Similar to the results of the WBS test, time immobile in the FST was still significantly elevated in CRS mice 19 days following restraint (drug × stress: $F_{(1,26)} = 5.309$, $p = 0.029$; post hoc analysis: CRS vs. Sal, $p = 0.008$), which also suggests that FST is more sensitive to detect depression-like behaviors than the OP test.

The effect of CRS on morphine-induced CPP

Fig. 3A illustrates that following two training cycles of morphine, CPP scores were significantly higher in animals that received Sal pre-treatment only, but animals that experienced 21 days of CRS performed no differently than Sal-injected controls (post-test2:
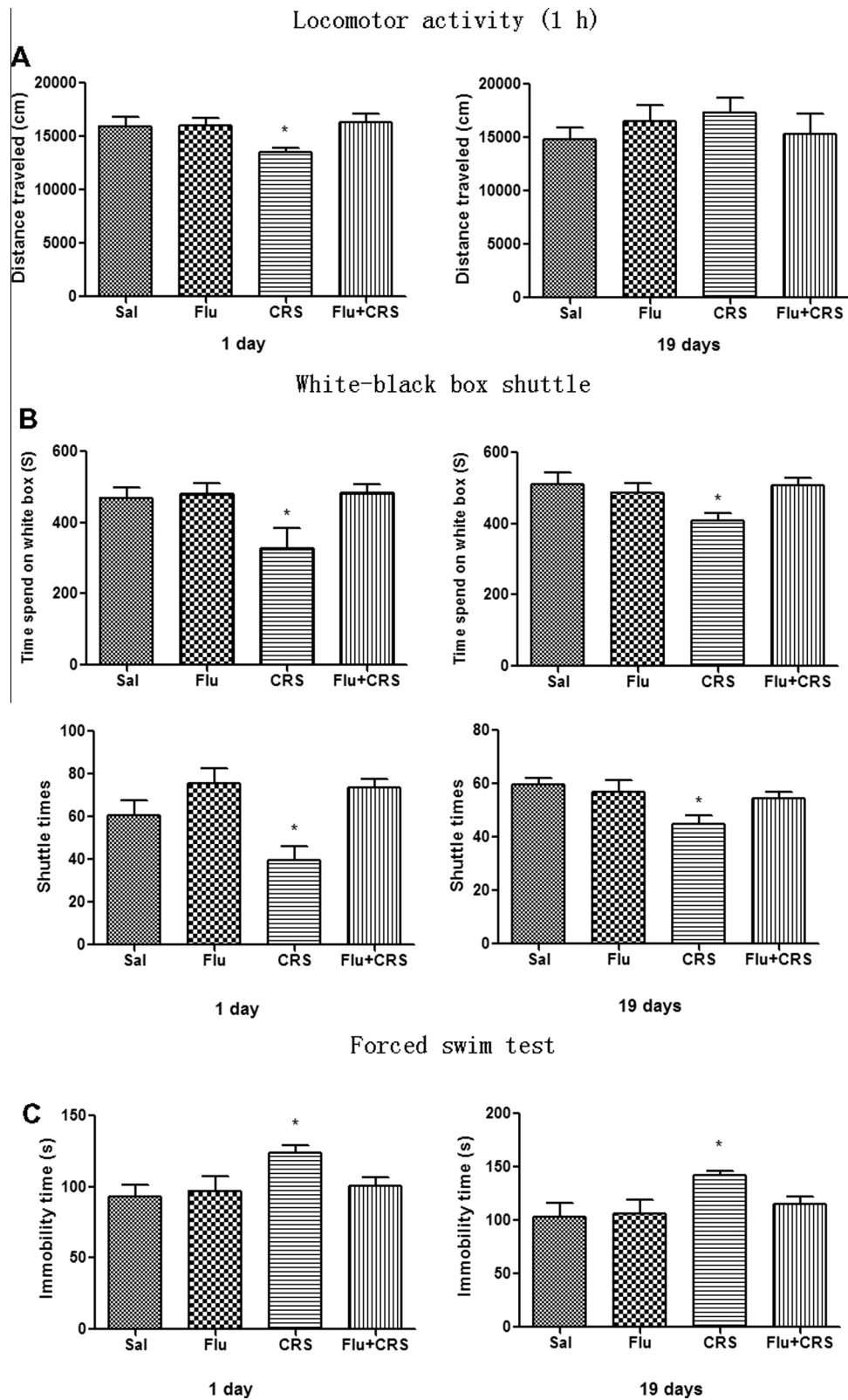
Fig. 2. CRS induced anxiety-like and depression-like behaviors. (A) One day after CRS locomotor activity was decreased, which recovered 19 days following restraint. (B) Anxiety-like behaviors were observed in the white–black box shuttle test. (C) CRS caused a significant increase in immobility time in the forced swim test. *$p < 0.05$ vs the other groups in each test.

$F_{(4,35)} = 5.319$, $p = 0.002$; Sal–Sal vs. Sal–M3, CRS–M3, $p = 0.001$, 0.651; $n = 8$ for each group). This absence of CPP in restrained mice was also evident following the third morphine training cycle (post-test3: $F_{(4,35)} = 4.555$, $p = 0.005$; Sal–Sal vs. CRS–M3, $p = 0.899$), suggesting that 21 days of restraint stress robustly interferes with the ability of mice to acquire the CPP effect of low dose morphine. Importantly, Flu administered before CRS completely restores morphine-induced CPP (post-test2: Sal–Sal vs. Flu + CRS–M3, $p = 0.034$ post-test3: Sal–Sal vs. Flu + CRS–M3, $p = 0.002$), without affecting expression of CPP alone (post-test2: Sal–Sal vs. Flu–M3, $p = 0.001$; post-test3: Sal–Sal vs. Flu–M3, $p = 0.035$).

We next tested whether chronic stress influences the stability of the CPP effect after CPP was successfully induced by higher doses of morphine. After one extinction cycle, the CPP effect, which was induced by 20 mg/kg morphine treatment, was readily extinguished in mice that had previously experienced CRS, but not in those that only received Sal pre-treatment (Exti-1: $F_{(4,40)} = 3.268$, $p = 0.021$; Sal–Sal vs. CRS–M20, Sal–M20, $p = 0.685$, 0.043; $n = 9$ for each group) (Fig. 3B). CPP was eliminated in the Sal-pretreated controls beginning at the completion of the third extinction training cycle (Exti-3: $F_{(4,40)} = 0.112$, $p = 0.977$; Sal–Sal vs. Sal–M20, $p = 0.578$; Exti-4: $F_{(4,40)} = 0.377$, $p = 0.823$; Sal–Sal vs. Sal–M20, $p = 0.677$). Flu pre-treatment also reversed the effect of CRS on CPP (Exti-

3: Sal–Sal vs. Flu + CRS–M20, $p = 0.871$; Exti-4: Sal–Sal vs. Flu + CRS–M20, $p = 0.792$) (Fig. 3B).

The effect of CRS on SENS

In the SENS test (Fig. 4), A three-way ANOVA showed a significant interaction of drug $\times$ stress $\times$ test day (3 mg/kg: $F_{(1,33)} = 6.350$, $p = 0.015$; 20 mg/kg: $F_{(1,32)} = 6.173$, $p = 0.018$). Post hoc analysis showed that there were no significant differences following the first morphine injection between any groups (3 mg/kg: $F_{(3,33)} = 0.371$, $p = 0.775$; 20 mg/kg: $F_{(3,32)} = 0.625$, $p = 0.587$; $n = 8–10$ for each group). However, following 20 mg/kg on the challenge day, the CRS group exhibited less overall locomotor activity as compared to the other groups (20 mg/kg: $F_{(3,32)} = 3.567$, $p = 0.025$; CRS–M20 vs. Sal–M20, Flu–M20, Flu + CRS–M20, $p = 0.01$, 0.049, 0.005) (Fig. 4B). After 7 consecutive days of injections, the mice that did not experience CRS exhibited SENS following both 3 and 20 mg/kg morphine, whereas this sensitization was not found in chronically restrained mice (1st vs. challenge: Sal–M3, $p < 0.001$; Flu–M3, $p = 0.001$; CRS–M3, $p = 0.861$; Sal–M20, $p < 0.001$; Flu–M20, $p < 0.001$; CRS–M20, $p = 0.508$). As in other studies, Flu pre-treatment completely restored SENS in mice previously restrained (1st vs. challenge: Flu + CRS–M3, $p = 0.005$; Flu + CRS–M20, $p < 0.001$).
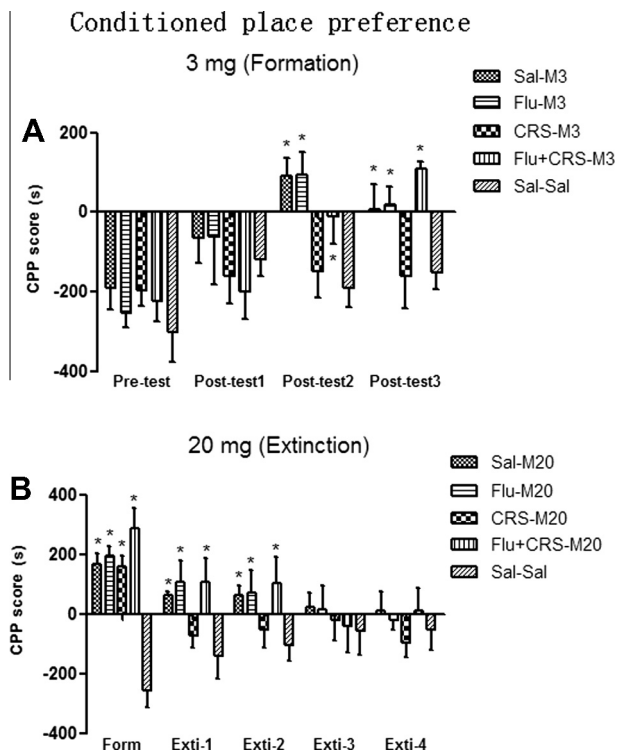

Fig. 3. The effect of CRS on different stages of CPP. (A) Formation: CRS inhibited low dose (3 mg/kg) morphine-induced CPP. (B) Extinction: after only one cycle of extinction training, CPP was eliminated in mice that previously experienced CRS. It should be noted that CPP was still successfully induced by high dose (20 mg/kg) of morphine after CRS. $^*p < 0.05$ vs. Sal–Sal control group in the same day test.
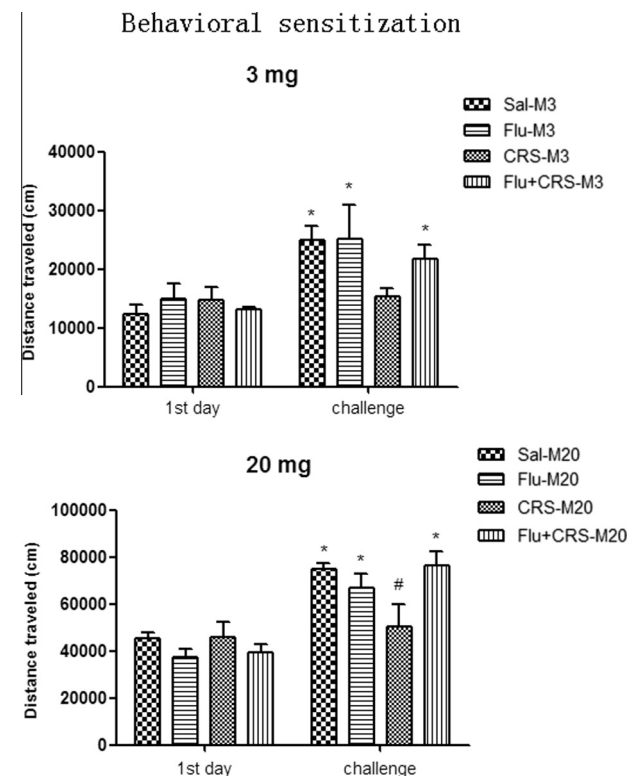

Fig. 4. The effect of CRS on SENS. (A) CRS blocked low dose (3 mg/kg) morphine-induced SENS. (B) CRS significantly decreased locomotor activity on the challenge day in mice treated with high dose (20 mg/kg) morphine, which resulted in SENS not being elicited. $^*p < 0.05$ 1st day vs. challenge day in the same group, $^\#p < 0.05$ vs. the other groups in challenge day test.

The expression of galanin and GalRs following CRS in Nac

As shown in Fig. 5, quantitative RT-PCR demonstrated that the expression of galanin in the Nac of CRS group was significantly greater than that of the other groups (drug × stress: $F_{(1,41)} = 6.849$, $p = 0.013$; post hoc analysis: CRS vs. Sal, Flu, Flu + CRS, $p = 0.003$, 0.010, 0.009; $n = 9–12$ each group). GalR1, GalR2, and GalR3 were also measured to determine whether the galanin binding sites were changed by CRS in Nac. After 21 days of restraint stress, the level of GalR1 mRNA increased, and Flu did not prevent this stress-induced increase (stress: $F_{(1,38)} = 20.442$, $p < 0.001$; drug × stress: $F_{(1,38)} = 0.594$, $p = 0.447$; post hoc analysis: Sal vs. CRS, Flu + CRS, $p = 0.013$, 0.001). With regard to GalR2, neither CRS nor Flu treatment alone affected its expression. However, when Flu was given prior to CRS, significantly higher expression of GalR2 was detected as compared to vehicle-treated mice (drug × stress:

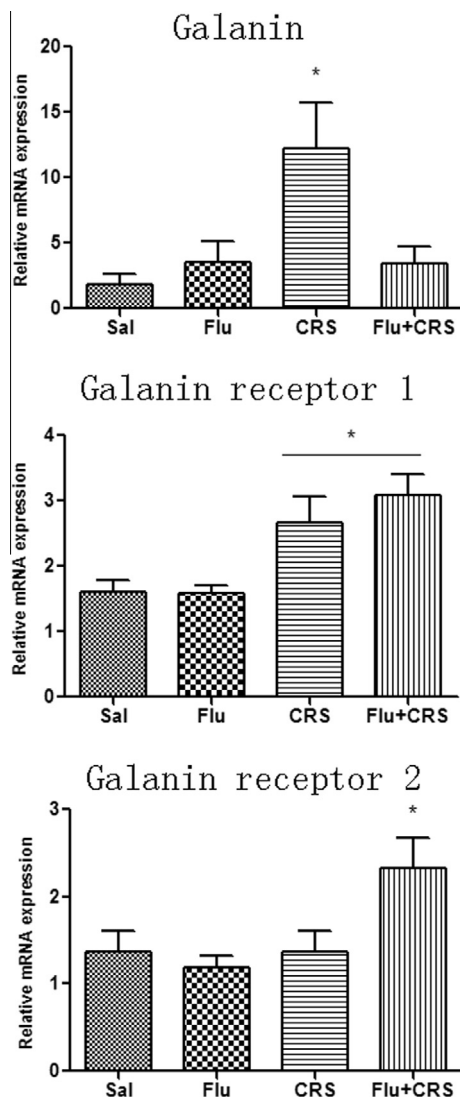$F_{(1,38)} = 5.114$, $p = 0.030$; post hoc analysis: Sal vs. Flu, CRS, Flu + CRS, $p = 0.990$, 0.620, 0.011). Using primers which had been successfully employed (Hawes et al., 2005), we could not detect the mRNA of GalR3 (sometimes > 15 cycles difference from reference gene). This may be attributed to its very low expression in the Nac.

The expression of tyrosine hydroxylase (TH), tryptophan hydroxylase 2 (TRH2), D2S, α_{2A}-adrenceptor, and 5-HT$_{1B}$ receptor after CRS in Nac

In Fig. 6, consistent with the changes seen with galanin following CRS, the mRNA expression of TH, the rate-limiting enzyme in dopamine and norepinephrine synthesis, significantly increased following 21 days stress, which was normalized by Flu (drug × stress: $F_{(1,38)} = 7.242$, $p = 0.012$; post hoc analysis: Sal vs. CRS, Flu + CRS, $p = 0.003$, 0.845; $n = 9–10$ each group including Flu). CRS also increased the expression of TRH2, and Flu did not affect this increase (stress: $F_{(1,38)} = 13.582$, $p = 0.001$; drug × stress: $F_{(1,38)} = 0.014$, $p = 0.905$; post hoc analysis: Sal vs. CRS, Flu + CRS, $p = 0.015$, 0.039). Levels of 5-HT$_{1B}$ receptor mRNA followed a similar pattern as those seen with TRH2 mRNA (stress: $F_{(1,38)} = 21.706$, $p < 0.001$; drug × stress: $F_{(1,38)} = 0.021$, $p = 0.887$; post hoc analysis: Sal vs. CRS, Flu + CRS, $p = 0.003$, < 0.001). However, similar to what was seen with GalR2, combining both chronic stress and Flu induced a significant increase of α_{2A}-adrenceptor, but neither alone affected its expression (drug × stress: $F_{(1,38)} = 6.361$, $p = 0.016$; post hoc analysis: Sal vs. CRS, Flu, Flu + CRS, $p = 0.611$, 0.701, < 0.001). Lastly, when comparing the expression of D2L with total gene levels (D2S and D2L), we found that the threshold cycles between these two were too close ($\leqslant 1$ cycle difference), indicating that the expression of D2S might be too low to be analyzed.

DISCUSSION

While confirming that chronic restraint induces long-lasting anxiety and depression-like behaviors, these data also indicate that CRS is intricately involved in drug addiction. Namely, CRS raised the threshold of morphine-induced CPP, facilitated the extinction process after CPP formation, and eliminated the sensitization effect following a week of morphine administration. These behavioral results were accompanied by a CRS-dependent increase in the mRNA expression of galanin, GalR1, TH, TRH2, and 5-HT$_{1B}$ receptor. Moreover, a commonly prescribed antidepressant, Flu, normalized all of the CRS-induced behavioral changes while also reversing the higher expression of galanin and TH and increasing the expression of GalR2 and α_{2A}-adrenceptor. These results indicate that long-term restraint stress activates the galanin and norepinephrine systems while affecting many stress-associated and addiction-like behaviors.
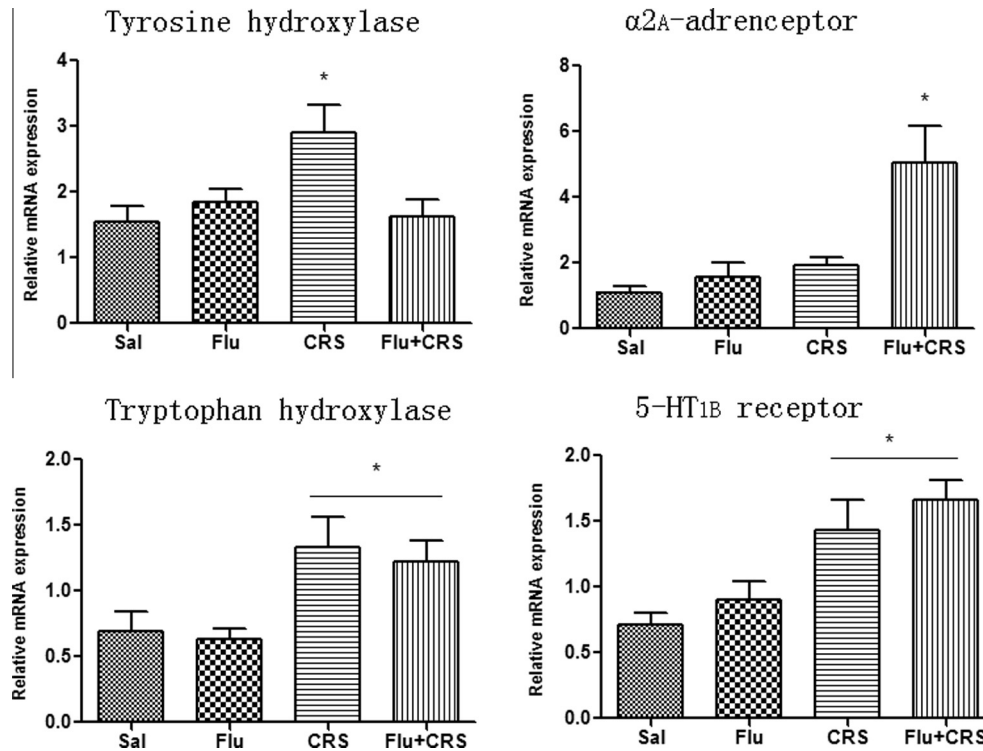

Fig. 5. The effect of CRS on the expression of galanin and galanin receptors in Nac. *$p < 0.05$ vs. the other groups.

Fig. 6. The effect of CRS on the expression of tyrosine hydroxylase, tryptophan hydroxylase 2, $\alpha 2_A$-adrenceptor and 5-HT$_{1B}$ receptor in Nac. $^*p < 0.05$ vs. the other groups.

Stress and addiction

In previous studies, animals exposed acutely to a stressor – such as tail pinch, foot shock, or restraint – exhibited increased self-administration of opiates, alcohol, and psychostimulant, whereas continuous social stress or chronic mild stress markedly suppressed the response of dopaminergic neurons to psychostimulant drugs (Willner et al., 1992; Sinha, 2008; Miczek et al., 2011; Shimamoto et al., 2011). In our experiments, CRS increased the threshold dose of morphine in CPP formation and promoted extinguishment of the CPP effect, which also indicates that the dopamine-based response is blunted by chronic stress. Previous studies have indicated that the direction of dopamine-based response (excitatory or inhibitory) depends on the types of stressors used. Our CRS may decrease spontaneous activity of a subpopulation of DA neurons which locate in the laterodorsal VTA (also referred to as medial and central VTA) (Lammel et al., 2012; Valenti et al., 2012), whereas short-term stress or other different chronic stressors (e.g., repeated intermittent stress) (Shaham et al., 2000, 2003) may increase activity of these cells (Moore et al., 2001; Valenti et al., 2011, 2012). In addition, these reward-mediating DA neurons project mainly to the ventral striatum (Nac) (Lammel et al., 2012).

In the SENS test, compared to other groups, stress-exposed mice showed less locomotor activity on the challenge day, suggesting that CRS may dampen DA release following morphine in Nac and thus reflect attenuated dopaminergic neuronal activity. This is supported by evidence that the degree of locomotion following administration of rewarding drugs correlates with extracellular DA levels in Nac (Miczek et al., 2011; Shimamoto et al., 2011; Valenti et al., 2012). Moreover, the observed increases in TH mRNA expression as well as the elevated anxiety observed in the WBS test following CRS suggest that Nac noradrenergic activity increases after stress. That NE cells in the LC inhibit DA neurons (Grenhoff et al., 1993; Paladini and Williams, 2004) further supports the notion that CRS increases noradrenergic while decreasing dopaminergic activity.

In order to rule out the possibility that immobility in FST may yield inconclusive results on its own, the results of the FST and OP experiments are discussed together. In our studies, the emergence of decreased locomotor activity in an OP and more immobile time in the FST suggest that CRS successfully induced a depression-like state. However, it should be noted that other forms of chronic restraint ($\leqslant$14 days and $\leqslant$2 h/day) do not affect locomotion in the same manner as we observed (Araujo et al., 2003; Marin et al., 2007), which may reflect a habituated response (Grissom and Bhatnagar, 2009; Koolhaas et al., 2011). This discrepancy may be because our protocol (6 h of restraint per day lasting 21 days) is too intense for animals to properly manage, manifesting as decreased exploratory activity in the OP test and more immobile time in FST. Supporting this notion are other studies that also use strong restraint protocols and show results consistent with our own (Conrad et al., 1999; Bowman et al., 2002; Kim and Han, 2006; Christiansen et al., 2011).

Galanin system in depression and addiction

A number of studies have shown that stress can change the expression of galanin (Sweerts et al., 2000; Khoshbouei et al., 2002; Christiansen et al., 2011). There is also abundant research providing evidence that galanin can modulate anxiety and depression-like behaviors. An increase in the immobility during the FST was observed both in galanin-overexpressing mice and in mice in which galanin was infused either intraventricularly or into the VTA (Kuteeva et al., 2005, 2007; Weiss et al., 2006). Also, the Flinders Sensitive Line (FSL), which is highly immobile in the FST, shows an up-regulation of the galanin-binding sites in the DRN (Bellido et al., 2002). Nevertheless, contrary to these findings, i.p. injection of galmic and galnon, agonists of GalR1 and GalR2, exhibit anti-depressant effects (Bartfai et al., 2004; Lu et al., 2005). Similar results are also observed with galanin administration itself (Murck et al., 2004; Klenerova et al., 2011). More work is clearly needed to characterize the circumstances when galanin acts as either as a pro- or anti-depressant. With respect to anxiety-like behavior, there are also many inconsistent results. For example, i.c.v. administration of galanin is anxiolytic in the Vogel conflict test (Bing et al., 1993) but not in the light–dark box (Karlsson et al., 2005); opposite effects are observed when galanin is injected into the amygdala or bed nucleus of stria terminalis (Möller et al., 1999; Khoshbouei et al., 2002).

These discrepancies may be attributed to the different physiological roles of the GalR subtypes. For example, GalR1 and GalR3 activate inhibitory G proteins Gi/Go, while GalR2 couples to Gq/G11 to mediate excitatory signaling (Wang et al., 1997; Branchek et al., 2000). Furthermore, since galanin and its receptors are co-localized with noradrenergic and serotonergic systems, slight variability in the modulation of these circuits and the transmitters involved may elicit the seemingly contradictory results described above. Selective activation of the GalR2 increases 5-HT release after i.c.v. or DRN infusion (Mazarati et al., 2005; Kuteeva et al., 2008). In agreement with previous work done in DRN (Lu et al., 2005), we found that Flu following chronic stress elevates GalR2 mRNA in the Nac. Moreover, that Flu has no effect on the expression of TRH2 and $5-HT_{1B}$ receptor suggests that GalR2 may have little impact on the 5-TH system in the Nac (Lu et al., 2005; Kuteeva et al., 2008). As of yet, there is little to no direct evidence showing the influence of GalR2 on NE release. Nonetheless, our results suggest a candidate mechanism that following Flu treatment, GalR2 may enhance α_{2A}-adrenceptor gene expression.

The inhibitory effect of galanin on 5-HT transmission, at least in the DRN, is largely due to the activation of GalR1 and GalR3 receptors (Mazarati et al., 2005); moreover, in the LC, GalR1 also mediates the effects of heightened NE activity (Zachariou et al., 2003). In our experiment, the mRNA levels of galanin, GalR1, and TH were enhanced following stress, suggesting activation of the galanin and NE systems in Nac. Indeed, there is evidence that galanin is simultaneously elevated in areas with activated NE neurons following stress (Holmes et al., 1995); the inhibitory effects of GalR1 may be an important mechanism in offsetting the increase in NE release following stress. In addition, the blunting effect of Flu on GalR1, THP2, and $5-HT_{1B}$ receptor expression indicates they are not the key targets of antidepressants in Nac.

In addictive behavior, i.c.v. injection of galanin attenuates morphine place preference, whereas galanin knock-out mice show enhanced CPP (Zachariou et al., 1999; Hawes et al., 2007). The DA system is critical for the rewarding and motivational effects of drugs of abuse (Koob, 1992), and galanin infusion into the VTA is suggested to inhibit DA neurons (Ericson and Ahlenius, 1999). Following addictive drug administration, phosphorylation of ERK1/2 and CREB increases in the VTA and NAc, and this increase can be reversed by galnon in galanin knock-out mice, thereby implicating inhibition of galanin on DA systems (Hawes et al., 2007; Narasimhaiah et al., 2009). Further, galanin's inhibitory actions are suspected to act through GalR1 (Hawes et al., 2005; Picciotto, 2008). It is thus plausible that the increased expression of galanin and GalR1 following CRS, and the resultant effects on the dopaminergic systems, may be associated with the observed impairments in sensitization to and rewarding effects of morphine following chronic stress in our present study.

CONCLUSION

The current studies provide additional evidence that the galanin system interacts with monoamines, an important mechanism to mediate stress-induced behavioral changes and addictive behavior. Galanin and GalRs will be valuable targets for further studies examining therapeutic targets to treat stress-related disorders and drug addiction.

We acknowledge there are several limitations in our experiments: first, given that the present study focuses on the effects of CRS on galanin and stress-linked behaviors, the housing of both stressed and non-stress mice in the same colony room may induce a little social stress on no-stressed mice, though they were placed on different shelves; second, significant sex-differences exist in HPA-function, as well as in prevalence of anxiety, depression and addiction, but we did not explore the differences between male and female mice in the present study. Thus, more research is needed to confirm this conclusion.

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