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Mu-opioid receptors in septum mediate the development of behavioral sensitization to a single morphine exposure in male rats

Running Title: Septum and Morphine Addiction

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Abstract

Behavioral sensitization (BS) is characterized by enhanced psychomotor responses to a dose of substance of abuse after prior repeated exposure. We previously reported BS can be induced by a single injection of morphine in rats, while septal nuclei are specifically involved in the development phase of BS. Here, we demonstrated intra-LS or -MS microinjections also incubated BS to a systemic morphine injection in a cross-sensitization fashion, while inactivation of either subdivision of septal nuclei (LS: lateral septum; MS: medial septum) can negate this ability of morphine. Then, non-selective (naloxone) and selective (μ-, δ- and κ-) opioid receptor antagonists were directly delivered into LS or MS, respectively, ahead of a morphine microinjection, while only μ-opioid receptors in both LS and MS play indispensable roles in mediating the BS development. Finally, there was a pronounced elevation in the levels of the monoamines (i.e., dopamine, homovanillic acid, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid) in the septum, 8 h after a morphine injection detected with a HPLC-ECD method, suggesting that dopaminergic and serotoninergic systems are

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implicated in the BS formation. Our studies demonstrated that septal nuclei critically participate in the BS development. Essentially, μ- instead of δ- or κ-opioid receptors in LS and MS mediate sensitization to opiates.

Keywords: morphine; behavioral sensitization; septum; cross-sensitization; μ-opioid receptor

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Introduction

Behavioral sensitization (BS) is a critical hallmark of abused substances, featured by augmented locomotor activity in response to a challenging dose after a drug-free period ensuing access to drugs.[1] The neurobiology underlying BS is implicated by modified neuronal transmission, signaling and plasticity in the central nervous system (CNS).[2, 3] To the best of our knowledge, most drug-induced BS studies focus on clarifying the adaptive changes within the mesolimbic system.[2, 4-7] However, the roles of septal nuclei in the development of the BS are still poorly elucidated, though septum was the first-identified rewarding area found in 1950s.[8, 9]

As reported, rodents can self-administer morphine and met-enkephalin via an intra-septal route, indicating that septal nuclei may participate in the reinforcement of opiates, supported by anatomic studies revealing that μ-, δ- and κ-opioid receptors are expressed throughout the septal region.[10, 11] We have previously shown that electrical ablation of septal nuclei can effectively attenuate the development, but not expression, of BS induced by a single morphine exposure, without affecting responses to aversive stimuli or reward seeking in rats.[1]

Thus, we hypothesize that the substructures and opioid receptors within septal nuclei may play specific roles in opiate-induced motor sensitization. To validate the hypothesis, a series of experiments were designed and performed here. First, BS induced by a single morphine administration (a systemic injection or a microinjection) was confirmed to evaluate the effectiveness of this BS paradigm. Second, the function of each septal subdivision (LS: lateral septum; MS: medial septum) was pharmacologically blocked with lidocaine in the development phase of BS to elucidate the roles of LS and MS in developing morphine-induced BS. Next, to clarify the

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function of septal opioid receptors, a microinjection of morphine into each area on Day 1 was then challenged with a systemic small-dose injection of morphine to develop BS on Day 8 with a cross-sensitization paradigm. Furthermore, non-selective and selective opioid (including μ-, δ- and κ-opioid) receptor antagonists were used to determine the specific subtypes of opioid receptors participating in the development of motor sensitization in LS and MS. Finally, the levels of monoamines in the septal region were measured under the same paradigm when BS was induced by a systemic morphine injection. In brief, the underlying mechanism of BS induced by a single dose of morphine in rats was, to some extent, elucidated in this study.

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Materials and Methods

Animals

Male Sprague-Dawley (SD) rats were 10 weeks old when they were formally tested (around 300-340 g), obtained from the Center of Laboratory Animal Science, Academy of Military Medical Sciences, Beijing, China. Experimental animals were housed in groups of four to five in clear plastic cages with free access to drinking water and food in a room with constant temperature (22 ± 2ºC) and humidity (50 ± 10%) on a 12/12 h light/dark cycle (lights on at 08:00 h). All experiments were conducted according to the *NIH Guide for the Care and Use of Laboratory Animals* (NIH publications no. 80-23, revised 1996), and the experimental procedures were approved by the local Committee on Animal Care and Use, Peking University Health Science Center.

Drugs

Morphine hydrochloride was bought from Qinghai Pharmaceutical Manufactory (Qinghai, China). Naloxone hydrochloride (Nal), β-funaltrexamine hydrochloride (FNA), naltrindole hydrochloride (NTI), norbinaltorphimine dihydrochloride (NBNI), lidocaine, sodium pentobarbital, penicillin and the chemical standards, including dopamine (DA), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), were purchased from Sigma Chemical Co. (St. Louis, USA). Drugs for injection were freshly dissolved in 0.9% saline before each experiment. The volume of a subcutaneous (s.c.) injection was 0.2 ml/100g for morphine and sodium pentobarbital.

Implantation and verification of microinfusion cannulae

To allow direct injections into septal nuclei, rats were fitted with the microinfusion

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cannulae overlying LS or MS. For surgery, rats were anaesthetized with sodium pentobarbital (50 mg/kg, s.c.) and placed in a stereotaxic instrument (RWD Life Science Co. Ltd, Shenzhen, China). Two cannulae (22 gauge, 3.5 mm diameter) for the intra-lateral septal implantation were respectively lowered into either side of the brain (0.7 mm anterior to bregma, ± 2.0 mm off the midline, -5.0 mm from the dural surface, angled 15° toward the midline). To point medial septum, one cannula was implanted right at the midline of brain (0.7 mm anterior to bregma and -6.0 mm from the dural surface). Three screws were set in the skull, and the assembly was secured with dental acrylic cement. Rats had 7 d to recover from the operation without any treatment in their homecage.[12] Post the operation, each rat was injected intramuscularly with 80,000 units of penicillin to prevent inflammation. During the intra-LS or -MS microinjection, rats were gently handled by an operator, and 30-gauge injectors that extended 0.5 mm below the tips of the microinfusion cannulae were used to deliver drug with a syringe pump (CMA 402, Carnegie Medicin AB, Stockholm, Sweden). The microinfusion speed was 0.6 μl per min, and the injector stayed untouched for 5 min to prevent backflow.

Experimental rats were killed at the end of tests, and then the brains were dissected and placed in 30% sucrose mixed with 10% buffered formalin for 7 d. Then brains were frozen and cut into 80-μm slices with a cryostat (CM1100, Leica Instruments). Every fifth slice was mounted on the slide glass, and then examined under a stereomicroscope (XTZ-02, Shanghai Optical Instrument Factory, Shanghai, China). Implantation placement was evaluated by two treatment-blind observers and reconstructed on the brain atlas modified from *The Rat Brain in Stereotaxic Coordinates* (Figure 1A and B).[13] If the implantation was misplaced, the corresponding data would be discarded.

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Experimental procedures

Experimental rats were treated with a single subcutaneous dose (0, 1, 3, 10 and 30 mg/kg) or a microinfusion (30 μg/rat) of morphine on Day 1, and then challenged with a subcutaneous dose of morphine at 3 mg/kg on Day 8. A syringe pump (CMA 402) was utilized when morphine or saline was microinfused into the septal substructures. Locomotion was measured using the Digbehv activity monitor system (Shanghai Jiliang Software Technology Co. Ltd., Shanghai, China) (Figure 1C). The system was equipped with 4 identical monitoring chambers (49 cm × 49 cm × 59 cm, without ceiling) held in soundproof cabinets to record horizontal locomotion traveled for 200 min immediately after the treatment (Figure 2 - 4).

In Experiment 1, total 35 rats divided to 5 groups on average were treated with a single dose (n = 7; 0, 1, 3, 10 and 30 mg/kg, s.c.) of morphine on Day 1 (Figure 2A). On Day 8, a challenge test was conducted with a dose of morphine at 3 mg/kg in all the groups (Figure 2B). Locomotion in both phases was monitored to evaluate the responses induced by an acute morphine injection and the BS effect triggered by a sub-effective dose without the capacity to result in BS. Next, the paradigm of cross sensitization was used to explore the roles of the septal substructures in the development of BS. Total 30 rats were used, 16 for LS and 14 for MS morphine microinjection (LS: n = 8; MS: n = 7). Rats were bilaterally microinjected by a dose of morphine injected into LS or MS (0 or 30 μg/rat, 2 μl/rat, 1 μl/site) and challenged on Day 8 as clarified (Figure 2C and D).

In Experiment 2, the roles of the septal substructures in morphine-induced BS were re-confirmed through deactivating LS or MS by lidocaine, a potent local anaesthetic. Total 48 rats separated into 6 groups (n = 8 /group) received lidocaine (0 or 10 μg/rat, 1 μl/rat,

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0.5 μl/site) into LS or MS (3 groups each) 10 min before a subcutaneous morphine injection on Day 1, while a BS effect was assessed after being challenged on Day 8 (Figure 3A and B).

In Experiment 3, similar protocols were utilized to test whether opioid receptors in septal nuclei took an active part in the development of BS induced by a single microinjection of morphine. Total 38 rats separated into 6 groups (n = 6-7 /group) received a non-selective opioid receptor antagonist naloxone (0 or 1.5 μg/rat, 2 μl/rat, 1 μl/site) into LS or MS (3 groups each) 10 min before a morphine treatment on Day 1, while a BS effect was assessed after being challenged on Day 8 (Figure 3C and D).

In Experiment 4, we sought to clarify the subtype of opioid receptor specifically mediating morphine-induced BS in rats. The protocols used in Experiment 3 were adopted and followed here. On Day 1, Total 103 rats, among them 52 rats separated into 8 groups for the LS microinjection (n = 6-8 /group) and the other 51 rats for the MS microinjection (n = 6-7 /group), were microinjected with selective opioid receptor antagonists, i.e., a μ-receptor antagonist FNA (0 or 2 μg/rat, 1 μl/rat, 0.5 μl/site), a δ-receptor antagonist NTI (0 or 4 μg/rat, 2 μl/rat, 1 μl/site) and a κ-receptor antagonist NBNI (0 or 6 μg/rat, 1 μl/rat, 0.5 μl/site), respectively, into LS or MS 10 min prior to the intra-LS or -MS microinfusion of morphine (0 or 30 μg/rat, 2 μl/rat, 1 μl/site). The doses of opioid antagonists were chosen based on the published results. [14, 15] On Day 8, locomotion was measured after a rat has been challenged with a subcutaneous injection of morphine (Figure 4A and B).

In Experiment 5, the roles of μ-opioid receptors in BS were re-validated. A systemic injection of 30 mg/kg morphine was used, following the microinfusion of FNA (0 or 2

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μg/rat, 1 μl/rat, 0.5 μl/site) into LS or MS on Day 1. Each rat was challenged with a subcutaneous injection of 3 mg/kg morphine on Day 8. Here, total 56 male rats were used, 31 rats separated into 4 groups for the LS microinfusion (n = 7-8 /group), and 25 rats into 4 groups for the MS microinfusion (n = 6-7 /group) (Figure 4C and D).

In Experiment 6, morphine-triggered alterations in biogenic amine levels, including DA, HVA, 5-HT and 5-HIAA in septal nuclei, were measured to indicate the potential mechanism underlying BS induced by morphine. Experimental rats were given a single injection of morphine (0 or 30 mg/kg) and decapitated at different time points (0, 2, 4, 8 and 16 h after the injection), respectively. Total 50 male rats were used and equally separated into 10 groups (n = 5 /group). Brains were rapidly extracted and dissected. Coronal slices (2 mm thick) containing the septal nuclei (approximately 0 to 2 mm anterior to the optic chiasma) from bilateral dissections were prepared using a rat brain mould (McCormick Scientific, USA) on ice for HPLC-ECD analysis. Subsequently, septal nuclei were quickly immersed into 120 μl of 1.1 M perchloric acid, extracted with mechanical trituration and high-speed centrifugation (twice, 16,000 g for 20 min). High Performance Liquid Chromatography-Electrochemical Detector (HPLC-ECD) was used to monitor the levels of DA, HVA, 5-HT, and 5-HIAA in the samples (Figure 5). The HPLC system consisted of a microbore reverse-phase column (particle size 5 μm, 150 mm × 4.6 mm, Model C-18; DIKMA Technologies Ltd., Beijing, China), an Agilent 1100 pump (flow rate 1.0 ml/min; Agilent Technologies, Palo Alto, CA, USA) and a DECADE II electrochemical detector (Antec Leyden BV, NV Zoeterwoude, Netherlands) with VT03 flow cell glassy carbon working electrode set at +700 mV (with respect to an Ag/AgCl reference electrode). The mobile phase contained 85 mM citrate, 100 mM sodium acetate, 0.9 mM octyl-sodium sulfate, 0.2 mM EDTA, 200 ml (10%) methanol, and 1800 ml ultrapure water (pH 3.4). External standard curves were used to quantify DA, HVA, 5-HT, and 5-HIAA in each sample based on the area under

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curve (AUC). The injection volume was 50 µl, and the detection limit of the assay was 20 pg/sample.

Statistics

The time course of locomotion (Figure 2) and the monoamine levels (Figure 5) were analyzed by two-factor repeated measures analysis of variance (RM-ANOVA) with time as a repeated measure (time × treatment). A greenhouse geisser correction method was used to adjust the degrees of freedom and p values, if violations of sphericity occur. One-way ANOVA and unpaired t tests were carried out to detect the effect of drug treatments (Figure 2 - 4). All data were expressed as the mean ± SEM. The level of significance was set as $p < 0.05$.

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Results

Behavioral sensitization was induced by a single subcutaneous injection or an intra-septal microinfusion of morphine

Here, an acute treatment of morphine subcutaneously injected at 0, 1, 3, 10 or 30 mg/kg produced a significant difference in a 20-min time course of locomotion [$F_{morphine\ 4,\ 30}$ = 8.218, p = 0.000; $F_{time\ 4.534,\ 270}$ = 15.505, p = 0.000] accompanied by a significant dose × time interaction effect [$F_{morphine\times time\ 18.135,\ 270}$ = 11.819, p = 0.000], analyzed with RM-ANOVA (Figure 2A) on Day 1. The inset of Figure 2A shows a typical inverted U-shaped dose-effect relationship between accumulated locomotion over 200 min, and the morphine doses ranging from 1 to 30 mg/kg under the paradigm of initial acute morphine administration [$F_{morphine\ 4,\ 30}$ = 8.218, p = 0.000], analyzed with One-way ANOVA. On Day 8, the rats challenged with a systemic morphine injection at 3 mg/kg had a robust effect of morphine pretreatment on locomotion [$F_{morphine\ 4,\ 30}$ = 3.858, p = 0.012; $F_{time\ 5.165,\ 270}$ = 22.512, p = 0.000], without a dose × time interaction effect [$F_{morphine\times time\ \ 20.66,\ 270}$ = 1.222, p = 0.241] as shown in Figure 2B. Interestingly, locomotor responses triggered by a challenge were significantly enhanced depending on the doses of morphine pretreatment [$F_{morphine\ 4,\ 30}$ = 3.858, p = 0.012], as shown in the inset of Figure 2B, while *post-hoc* analysis displayed that rats pretreated with 30 mg/kg morphine had highly increased locomotor activity ($p < 0.05$). Consequently, a dose of 30 mg/kg was chosen to induce BS on Day 1.

To determine whether activation of opioid receptors in either lateral or medial septum was able to develop BS, we designed and conducted a cross-sensitization experiment, that is, a pretreatment with a single intra-LS or MS microinfusion of morphine (0 or 30 μg/rat) on Day 1 and a challenge with a dose of 3 mg/kg morphine on Day 8 (an intra-septal microinfusion × a challenge). Figure 2C and D show that the rats had a clearly

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enhanced time course of locomotion within 200 min in the intra-LS morphine group [$F_{morphine\ 1,\ 14} = 5.503$, $p = 0.034$; $F_{time\ 3.04,\ 126} = 5.026$, $p = 0.004$] without a pronounced dose × time interaction effect [$F_{morphine\times time\ 9,\ 126} = 2.742$, $p = 0.54$], and in the intra-MS morphine group [$F_{morphine\ 1,\ 12} = 17.011$, $p = 0.001$; $F_{time\ 2.653,\ 108} = 6.015$, $p = 0.003$] without a dose × time effect [$F_{morphine\times time\ 2.653,\ 108} = 2.685$, $p = 0.069$], either. In particular, the intra-LS and intra-MS morphine groups displayed a significant increase in accumulated locomotion (LS: $t_{14} = -2.32$, $p = 0.036$; MS: $t_{9.012} = -4.059$, $p = 0.002$), approximately 63% and 71% higher than that in the corresponding control group, respectively, as shown in the insets of Figure 2C and D.

Intra-septal lidocaine abolished the development of BS induced by an intra-septal microinfusion of morphine

To investigate the effects of blocking the lateral or medial septal region on the BS formation, rats were randomly allocated into 3 groups respectively, that is, intra-LS/MS saline plus s.c. saline [SS], intra-LS/MS saline plus s.c. morphine (30 mg/kg) [SM], and intra-LS/MS lidocaine (10 μg/rat) plus s.c. morphine (30 mg/kg) [LM] on Day 1. Invalidation of LS or MS by lidocaine abolished BS [SM vs LM, LS: $t_{10.369} = 2.612$, $p = 0.025$; MS: $t_{11} = 2.219$, $p = 0.048$] on Day 8, while the effectiveness of the BS paradigm was validated through that locomotion in SM group was significantly higher than that in SS group [LS: $t_{14} = -4.805$, $p = 0.000$; MS: $t_{6.713} = -2.659$, $p = 0.025$] in Figure 3A and B.

Intra-septal naloxone abolished the development of BS induced by a systemic injection of morphine

Naloxone, a non-selective opioid receptor antagonist, was chosen to examine the role

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of opioid receptors in lateral or medial septal nuclei in the development of locomotor sensitization to a single injection of morphine. In Figure 3C, an intra-LS naloxone pretreatment significantly suppressed BS [t_{11} = -2.214, p = 0.049]. Unpaired *t* tests showed that motor sensitization was only detected between the saline-saline and saline-morphine groups [t_{13} = -2.546, p = 0.024]. In Figure 3D, an intra-MS naloxone pretreatment inhibited the sensitized motor response [t_{11} = -4.285, p = 0.001] with the successfully-established BS model [t_{12} = -4.059, p = 0.002], when comparing the saline-morphine to the saline-saline group.

Intra-septal FNA abolished the development of BS induced by an intra-septal microinfusion of morphine

We then identified the specific subtype of opioid receptors involved in morphine-induced BS. Here, FNA (μ-opioid receptor antagonist), NTI (δ-opioid receptor antagonist) or NBNI (κ-opioid receptor antagonist) was singly injected into the LS 10 min ahead of the intra-LS delivery of morphine on Day 1. On Day 8, FNA was the only one that attenuated the BS phenotype [t_{10} = -0.775, p = 0.456], while NTI or NBNI did not pose a significant impact on motor sensitization [NTI: t_{11} = -2.338, p = 0.039; NBNI: t_{10} = -3.044, p = 0.012] induced by an intra-LS morphine injection compared to the respective control group (antagonist + saline group) in Figure 4A.

Similar results emerged when FNA, NTI or NBNI was pre-injected into the MS on Day 1. On Day 8, FNA remained the only chemical to reduce BS [t_{10} = -1.21, p = 0.254], while NTI or NBNI did not affect locomotion in rats [NTI: t_{11} = -2.73, p = 0.020; NBNI: t_{10} = -2.748, p = 0.021], compared to the respective control group in Figure 4B.

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Intra-septal FNA abolished the development of the BS induced by a systemic injection of morphine

The results demonstrated that μ-opioid receptors seem to mediate BS induced by an intra-septal morphine injection. To consolidate the conclusion, the paradigm of BS induced by a systemic injection was used here. Figures 5C and D show that intra-septal antagonism of μ-opioid receptors (FNA, 2 μg/rat) abolished BS induced by a systemic injection of morphine. No sensitized responses were found in either intra-LS FNA [t_{13} = -0.199, p = 0.845] or intra-MS groups [t_{10} = 0.841 p = 0.42], compared to the control group (FNA + saline), respectively. The BS model was effective based on the comparisons between saline-saline vs saline-morphine groups (data not shown here).

Levels of monoamines in septal nuclei were altered in the development phase of BS induced by a systemic injection of morphine

To elucidate the underlying mechanism of BS at the level of neurotransmitters, we delineated the time course of the concentrations of certain monoamines at 0, 2, 4, 8 and 16 h after one subcutaneous injection at 30 mg/kg, the dose capable to induce BS in rats. In this study, it could significantly increase the concentrations of DA and its metabolite HVA in the septum [DA: $F_{morphine\ 1,\ 8}$ = 17.664, p = 0.003; $F_{time\ 4,\ 32}$ = 9.339, p = 0.000; $F_{morphine\times time\ 4,\ 32}$ = 7.692, p = 0.000; HVA: $F_{morphine\ 1,\ 8}$ = 40.498, p = 0.000; $F_{time\ 4,\ 32}$ = 2.309, p = 0.079; $F_{morphine\times time\ 4,\ 32}$ = 0.64, p = 0.638] in Figure 5A and B. Similarly, Figure 5C and D show that both 5-HT and its metabolite 5-HIAA in septal nuclei changed over time after a morphine treatment [5-HT: $F_{morphine\ 1,\ 8}$ = 10.633, p = 0.0012; $F_{time\ 4,\ 32}$ = 8.128, p = 0.000; $F_{morphine\times time\ 4,\ 32}$ = 4.189, p = 0.008; 5-HIAA: $F_{morphine\ 1,\ 8}$ = 32.209, p = 0.000; $F_{time\ 4,\ 32}$ = 15.326, p = 0.000; $F_{morphine\times time\ 4,\ 32}$ = 4.848, p = 0.000]. Unpaired *t* test revealed that an enhancement of DA ($t_{4.415}$ = 4.205, p = 0.011), HVA (t_{8} = 8.345, p = 0.002), 5-HT (t_{8} = 8.028, p = 0.000) and 5-HIAA (t_{8} =

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6.527, p = 0.000) in morphine-treated groups, and the highest level detected in septal region 8 h after a morphine treatment was approximately 50%- 500% higher than that in the corresponding control group (Figure 5).

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Discussion

Previously, we reported that septal nuclei specifically mediate BS induced by a single injection of 30 mg/kg morphine in a phase-specific (in the phase of development but not expression) manner.[1] In that study, we also found that electric ablation of the septum did not affect the responses to noxious stimuli or reward cognition.[1, 16] To step forward, we extended the investigation on three levels: (1) to examine roles of the substructures in the septum, (2) to clarify the function of opioid receptors and (3) the altered concentrations of major neurotransmitters, during the development of BS in this study.

Here, the animal model used for studying BS is a well-established with high construct validity. Behavioral sensitization as the enduring and progressive augmentation of certain behaviors after drug use is highly related to drug craving and relapse-like phenotypes, which shares overlapping circuitry and neurotransmitter systems with drug addiction.[17-19] Moreover, behavioral sensitization has close connections with the context, which can also, to some extent, mimic the transition from controllable drug use to obsessive drug seeking. It was successfully established by a systemic administration or an intra-septal microinfusion of morphine in rats, confirming that opioid receptors in the septum participate in the development of BS (Figure 2).[5] In particular, direct and restricted activation of opioid receptors in either LS or MS by morphine in the development phase of BS was sufficient to trigger BS (Figure 2C and D), suggesting a critical role of septal opioid receptors in morphine-induced BS. In this study, a systemic injection of morphine at 30 mg/kg was taken to establish the BS model, of which the dose was selected to induce the most pronounced BS phenotype in rats based on our previously studies.[1,3] Here to establish the BS model at the level of specific brain area, morphine at 30 μg/rat were bilaterally microinfused into LS or MS at the 2 μl/rat, that is, at a volume of 1 μl/site. One concern is raised that whether this volume may cause a

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diffusion between LS and MS, which took the responsibility for the conclusion. However, our pilot study proved that the volume of 1 ul did not induce an over-range diffusion when 1ul dye infused into LS or MS showed a restricted diffusion in LS or MS. A volume of 1 ul was also usually taken in the previous studies.[20, 21]

The septal region, a cluster of nuclei located along the midline in the vertical limb of the diagonal band of Broca and surrounded by the lateral ventricle, is involved in emotion control and reward seeking.[6, 22, 23] Septum can be anatomically divided into lateral, medial and posterior parts, the former two comprising the largest proportion of the nuclei.[8] In general, the lateral and medial parts may play distinct roles in physiological activities, though they have distinct patterns of neural connection and functions.[22] In the context of drug use, LS is strongly connected to mesolimbic circuits e.g., NAc and amygdala, to mediate drug seeking behavior.[3, 24, 25] Medial septal nuclei are implicated with the context learning and memory, which are important elements in the transition to addiction, when drug addiction is considered as a pathological learning process.[26, 27] Furthermore, via lidocaine-induced inhibition of septal nuclei, the BS induced by an intra-LS or intra-MS morphine was eliminated (Figure 3). Then we conclude that both lateral and medial septum play a vital role in mediation of BS.

To draw a solid conclusion, lidocaine was used to diminish the function of LS or MS in the development phase of BS. Lidocaine, a potent local anesthetic, can block the fast voltage gated sodium (Na^+) channels in the neuronal cell membrane that are responsible for signal propagation. Lidocaine is widely employed to study the function of many targeted brain nuclei, e.g., the VTA regarding morphine reward, NAc regarding DA efflux, hippocampus regarding memory and rostral ventromedial medulla regarding nociception.[10, 28-33] In our study, lidocaine was microinjected into the LS or

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MS 10 min before the first injection of morphine. An earlier report has demonstrated that the critical time window for an effective intervention in a single morphine sensitization is within 30 min before the first injection.[34] From these findings, we conclude that both lateral and medial septal nuclei essentially mediate the development of BS, though lidocaine can produce a complete anesthesia to the whole area, including local neurons and the passing fibers, may be taken as a shortcoming, which was compensated with the following experiments.[35]

To verify the precise subtype of opioid receptors mediating morphine-induced BS, we further evaluated the effects of non-selective (naloxone) and selective μ-, δ- and κ-opioid receptor antagonists (FNA, NTI and NBNI) on the development of motor sensitization in rats. We found that naloxone pre-injection could completely attenuate the hyper-locomotion responding to a morphine challenge. More importantly, FNA was singled out as the only subtype-selective antagonist able to modulate the development of BS (Figure 4).

It is well acknowledged that BS to morphine is characterized by increased motor activity and the alterations to neural systems involved in rewarding.[2, 36] Previous studies have shown that the administration of drugs of abuse, such as morphine and cocaine, can produce widespread and long-lasting sensitization of dopaminergic, noradrenergic and serotonergic systems in the brain to alter monoamine release in the CNS.[6, 17] Pharmacological function of morphine is directly related to activation of μ-, δ-, or κ-opioid receptors.[11, 36] Studies have revealed that the three subtypes of opioid receptors are all recruited in response to drugs of abuse, and modified as addiction develops.[9] It is also clear that they play different roles in the same brain region for modulating certain functions. For instance, a direct comparison of mice lacking each of the three opioid-

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receptor genes reveals that μ- and δ-opioid receptors act oppositely in regulating emotional reactivity.[18, 37]

As defined, BS to morphine is characterized by increased motor activity and alterations to neural systems.[2, 36] To delineate the mechanism of morphine-induced BS at the level of neurotransmission, we measured the concentrations of DA, HAV, 5-HT and 5-HIAA in septum, highly related to the transition to addiction with the HPLC-ECD method. Here, we discovered the increased levels of DA and 5-HT as well as the derivatives HAV and 5-HIAA in septal nuclei 8 hours after a single morphine exposure, as the critical time point within the development phase of sensitization (Figure 5).[7-38] Combined with the behavioral evidence, we would draw a simplified mechanism underlying the morphine-induced BS, that is, μ-opioid receptors in both lateral and medial septum directly mediate the development of BS induced by a single injection of morphine in rats.

To conclude, our studies show that μ-opioid receptors in both the lateral and medial septum are direct and major targets to mediate BS induced by a single dose of morphine. The dopaminergic and serotonergic systems appear to be implicated in neural adaptation in BS formation in rats.

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Disclosure

The authors declare no conflict of interest.

Statement of Contribution

YLL, SW and QL as the first authors participated in designing experiments, acquiring data and writing the manuscript. QG, QJZ, TGZ and ZY contributed to experimental design and the interpretation of data. SW and AJL made a critical contribution in improving the English writing. FC and AJL provided really helpful suggestions in the conception and the interpretation of data. JHL participated in all the processes including design, data gathering, analysis and manuscript writing.

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References

1. Liu Q, Zhang M, Qin WJ, et al. Septal nuclei critically mediate the development of behavioral sensitization to a single morphine injection in rats. *Brain Res*. 2012;1454:90-99.

2. Mickiewicz AL, Dallimore JE, Napier TC. The ventral pallidum is critically involved in the development and expression of morphine-induced sensitization. *Neuropsychopharmacology*. 2009;34(4):874-886.

3. Luo J, Jing L, Qin WJ, et al. Transcription and protein synthesis inhibitors reduce the induction of behavioural sensitization to a single morphine exposure and regulate Hsp70 expression in the mouse nucleus accumbens. *Int J Neuropsychopharmacol*. 2011;14(1):107-121.

4. Vanderschuren LJ, Kalivas PW. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berl)*. 2000;151(2-3):99-120.

5. Vanderschuren LJ, Tjon GH, Nestby P, et al. Morphine-induced long-term sensitization to the locomotor effects of morphine and amphetamine depends on the temporal pattern of the pretreatment regimen. *Psychopharmacology (Berl)*. 1997;131(2):115-122.

6. Thomas MJ, Beurrier C, Bonci A, Malenka RC. Long-term depression in the nucleus accumbens: a neural correlate of behavioral sensitization to cocaine. *Nat Neurosci*. 2001;4(12):1217-1223.

7. Bhatti AS, Hall P, Ma Z, Tao R, Isgor C. Hippocampus modulates the behaviorally-sensitizing effects of nicotine in a rat model of novelty-seeking: potential role for mossy fibers. *Hippocampus*. 2007;17(10):922-933.

8. Olds J, Milner P. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J Comp Physiol Psychol*. 1954;47(6):419-427.

9. Le Merrer J, Becker JA, Befort K, Kieffer BL. Reward processing by the opioid system in the brain. *Physiol Rev*. 2009;89(4):1379-1412.

10. Stein L, Belluzzi JD. Brain endorphins: possible role in reward and memory formation. *Fed Proc*. 1979;38(11):2468-2472.

11. Mansour A, Fox CA, Akil H, Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci*. 1995;18(1):22-29.

12. Aliota MT, Bassit L, Bradrick SS, et al. Zika in the Americas, year 2: What have we learned? What gaps remain? A report from the Global Virus Network. *Antiviral Res*. 2017;144:223-246.

13. Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates, 6th Edition. San Diego: Academic Press. 1998.

14. Bodnar RJ, Lamonte N, Israel Y, et al. Reciprocal opioid-opioid interactions between the ventral tegmental area and nucleus accumbens regions in mediating mu agonist-induced feeding in rats. *Peptides*. 2005;26(4):621-629.

15. Miner P, Shimonova L, Khaimov A, et al. General, kappa, delta and mu opioid receptor antagonists mediate feeding elicited by the GABA-B agonist baclofen in the ventral tegmental area and nucleus accumbens shell in rats: reciprocal and regional interactions. *Brain Res*. 2012;1443:34-51.

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16. Wei S, Li YL, Gong Q, et al. Brucine N-oxide reduces ethanol intake and preference in alcohol-preferring male Fawn-Hooded rats. Alcohol Clin Exp Res. 2020;44(6):1321-1328.

17. Lewitus GM, Konefal SC, Greenhalgh AD, et al. Microglial TNF-α Suppresses Cocaine- Induced Plasticity and Behavioral Sensitization. Neuron 2016; 90(3):483-91.

18. Steketee JD, Kalivas PW. Drug wanting: behavioral sensitization and relapse to drug-seeking behavior. Pharmacol Rev. 2011;63(2):348-65.

19. Richtand NM. Behavioral sensitization, alternative splicing, and d3 dopamine receptor-mediated inhibitory function. Neuropsychopharmacol. 2006;31(11):2368-75.

20. Leroy F, Park J, Asok A, et al. A circuit from hippocampal CA2 to lateral septum disinhibits social aggression. Nature 2018;564(7735):213-218.

21. Oddie SD, Stefanek W, Kirk IJ, et al. Intraseptal procaine abolishes hypothalamic stimulation-induced wheel-running and hippocampal theta field activity in rats. J Neurosci. 1996;16(5):1948-56.

22. Talishinsky A, Rosen GD. Systems genetics of the lateral septal nucleus in mouse: heritability, genetic control, and covariation with behavioral and morphological traits. *PLoS One* 2012;7(8):e44236.

23. Traynor JR, Elliott J. delta-Opioid receptor subtypes and cross-talk with mu-receptors. *Trends Pharmacol Sci*. 1993;14(3):84-86.

24. Luo AH, Tahsili-Fahadan P, Wise RA, Lupica CR, Aston-Jones G. Linking context with reward: a functional circuit from hippocampal CA3 to ventral tegmental area. *Science*. 2011;333(6040):353-357.

25. Ryabinin AE, Yoneyama N, Tanchuck MA, Mark GP, Finn DA. Urocortin 1 microinjection into the mouse lateral septum regulates the acquisition and expression of alcohol consumption. *Neuroscience*. 2008;151(3):780-790.

26. Chrobak JJ, Stackman RW, Walsh TJ. Intraseptal administration of muscimol produces dose-dependent memory impairments in the rat. *Behav Neural Biol*. 1989;52(3):357-369.

27. Hyman SE. Addiction: a disease of learning and memory. *Am J Psychiatry*. 2005;162(8):1414-1422.

28. Knowlton BJ, Thompson RF. Microinjections of local anesthetic into the pontine nuclei reduce the amplitude of the classically conditioned eyelid response. *Physiol Behav*. 1988;43(6):855-857.

29. Sarihi A, Fathollahi Y, Motamedi F, Naghdi N, Rashidy-Pour A. Effects of lidocaine reversible inactivation of the median raphe nucleus on long-term potentiation and recurrent inhibition in the dentate gyrus of rat hippocampus. *Brain Res*. 2003;962(1-2):159-168.

30. Sarihi A, Motamedi F, Naghdi N, Rashidy-Pour A. Lidocaine reversible inactivation of the median raphe nucleus has no effect on reference memory but enhances working memory versions of the Morris water maze task. *Behav Brain Res*. 2000;114(1-2):1-9.

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Author Manuscript

31. Howland JG, Taepavarapruk P, Phillips AG. Glutamate receptor-dependent modulation of dopamine efflux in the nucleus accumbens by basolateral, but not central, nucleus of the amygdala in rats. *J Neurosci*. 2002;22(3):1137-1145.

32. Yang ZL, Gao YJ, Wu GC, Zhang YQ. The rostral ventromedial medulla mediates the facilitatory effect of microinjected orphanin FQ in the periaqueductal gray on spinal nociceptive transmission in rats. *Neuropharmacology*. 2003;45(5):612-622.

33. Moaddab M, Haghparast A, Hassanpour-Ezatti M. Effects of reversible inactivation of the ventral tegmental area on the acquisition and expression of morphine-induced conditioned place preference in the rat. *Behav Brain Res*. 2009;198(2):466-471.

34. Qin WJ, Wang YT, Zhang M, et al. Molecular chaperone heat shock protein 70 participates in the labile phase of the development of behavioural sensitization induced by a single morphine exposure in mice. *Int J Neuropsychopharmacol*. 2013;16(3):647-659.

35. Catterall WA. Molecular mechanisms of gating and drug block of sodium channels. *Novartis Found Symp*. 2002;241:206-218; discussion 218-232.

36. Vigano D, Rubino T, Di Chiara G, et al. Mu opioid receptor signaling in morphine sensitization. *Neuroscience*. 2003;117(4):921-929.

37. Pascoe JE, Williams KL, Mukhopadhyay P, et al. Effects of mu, kappa, and delta opioid receptor agonists on the function of hypothalamic-pituitary-adrenal axis in monkeys. *Psychoneuroendocrinology*. 2008;33(4):478-486.

38. Li YL, Liu Q, Gong Q, et al. Brucine suppresses ethanol intake and preference in alcohol-preferring Fawn-Hooded rats. *Acta Pharmacol Sin*. 2014;35(7):853-861.

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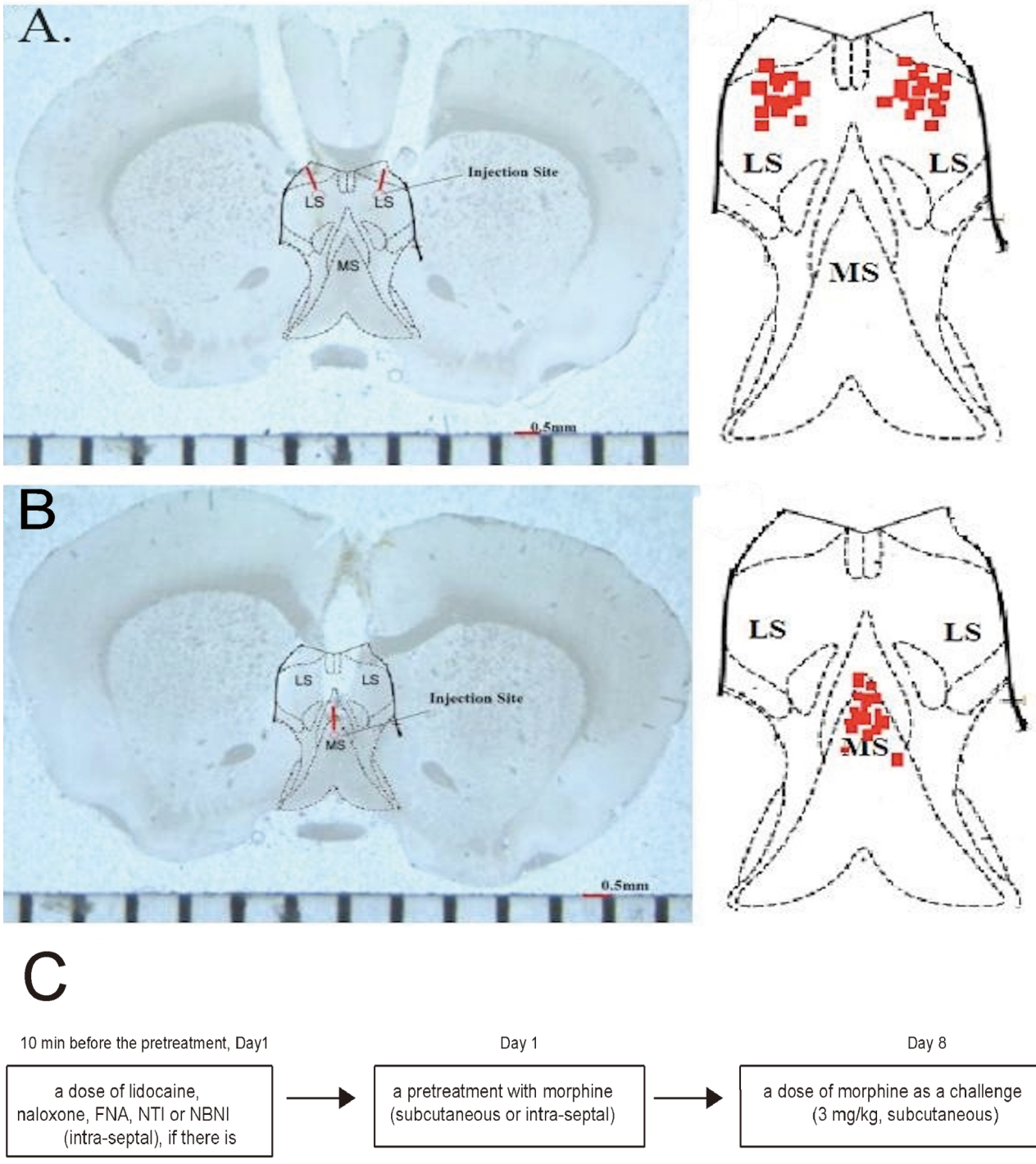

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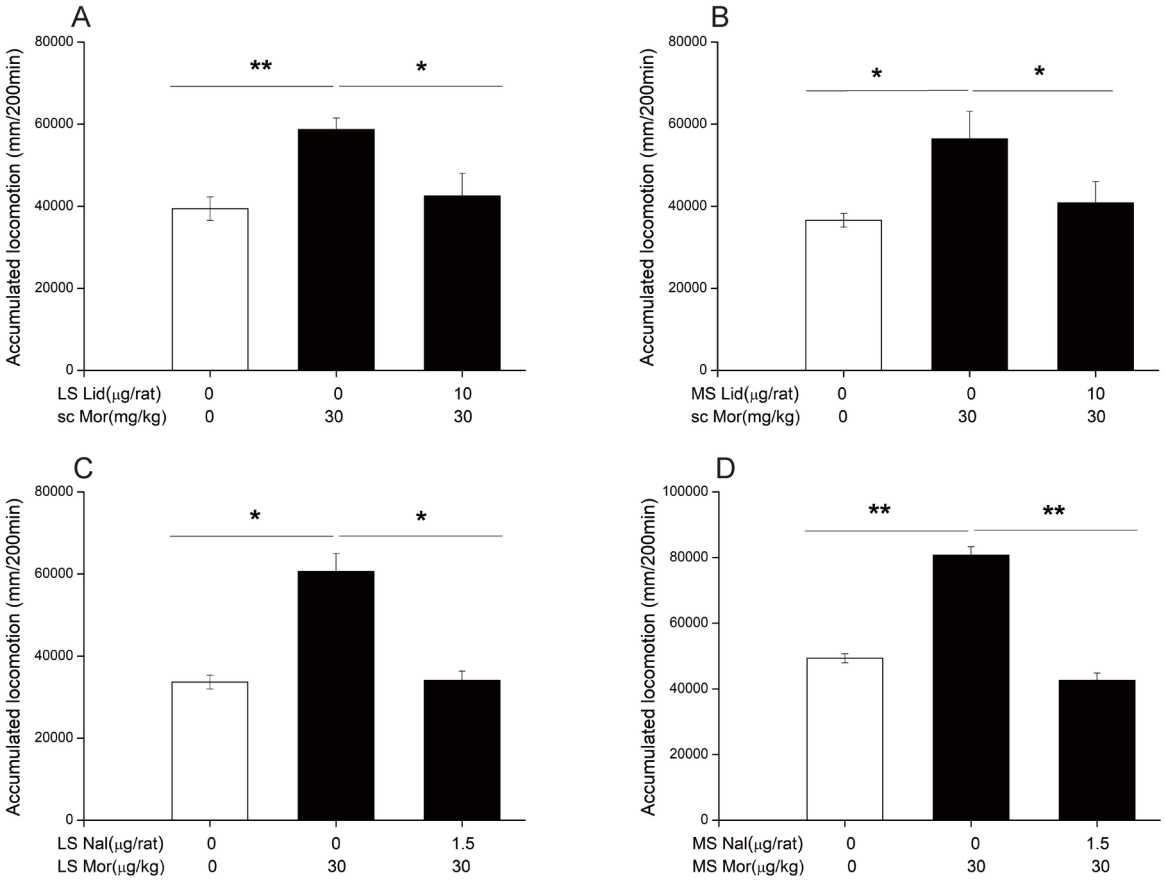

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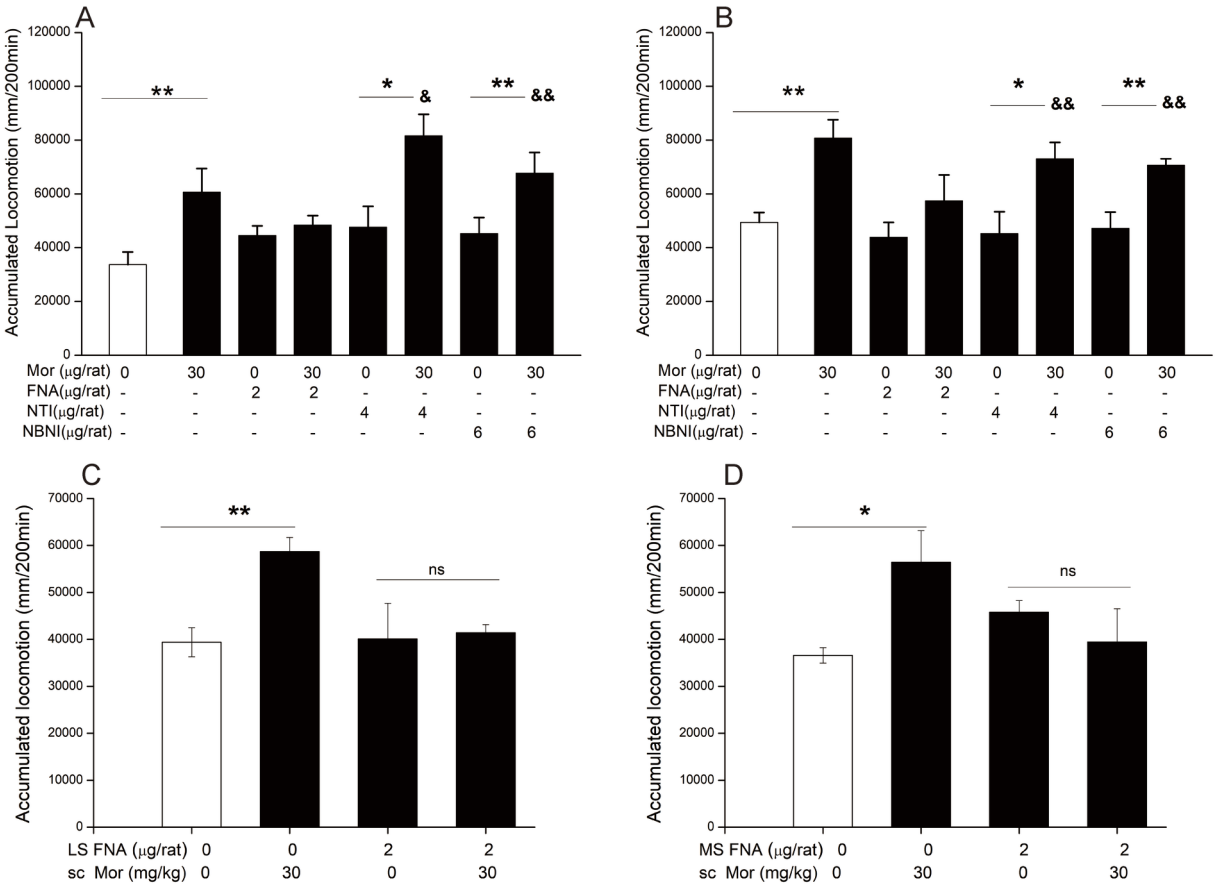

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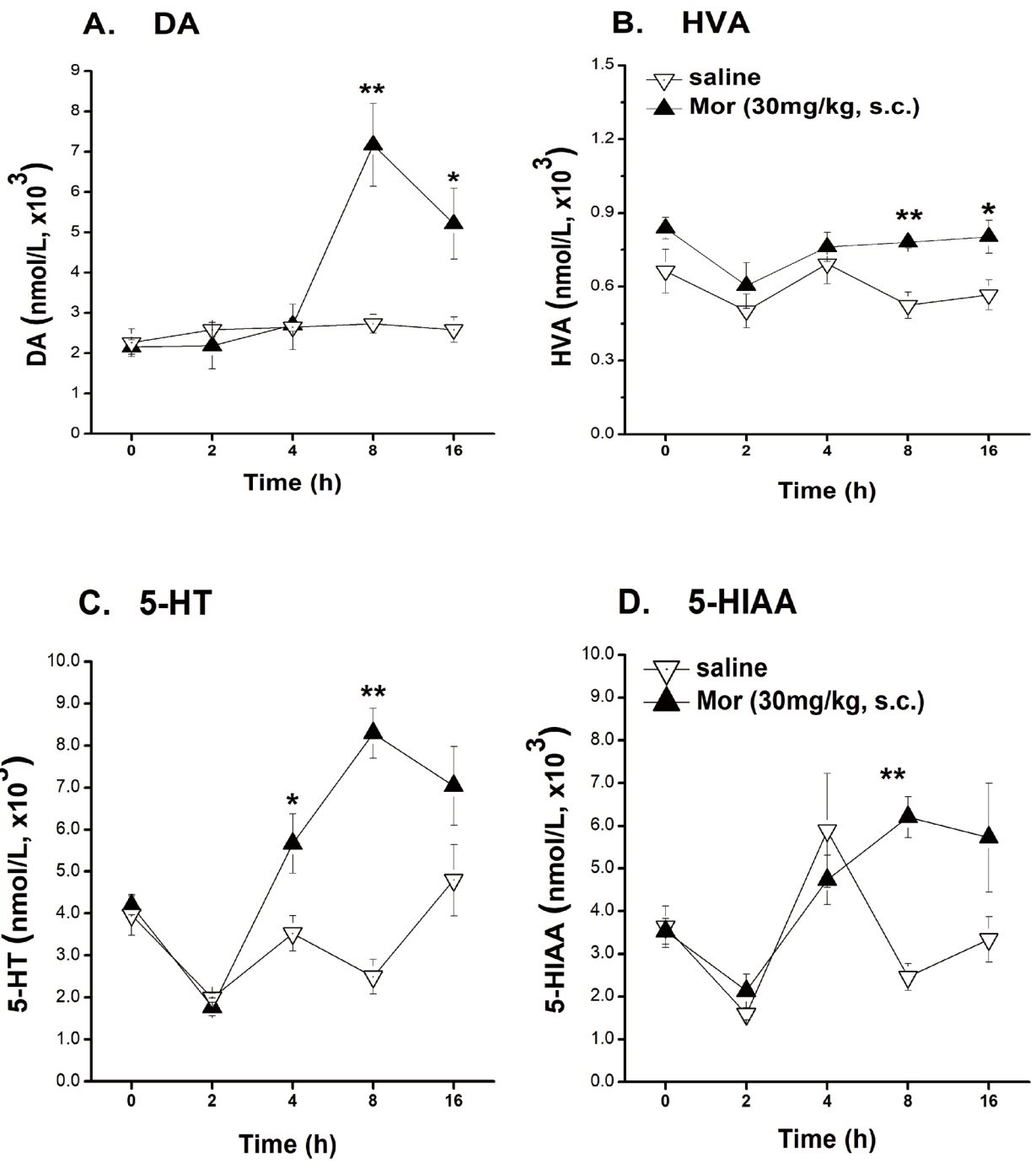

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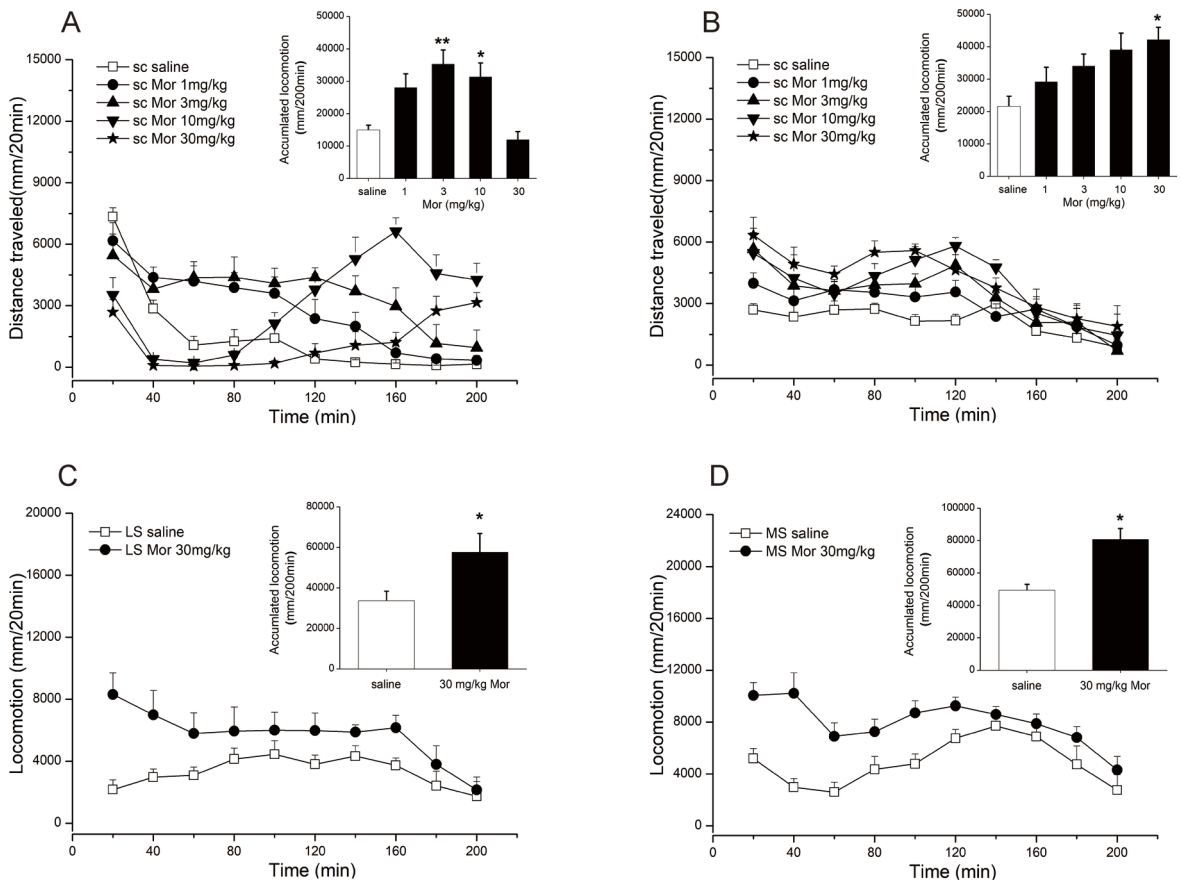

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