Original Research

Cognitive Enhancer Donepezil Attenuates Heroin-Seeking Behavior Induced by Cues in Rats

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Abstract

Purpose: Opioid use disorder is a significant global problem. Chronic heroin use is associated with impairment of cognitive function and conscious control ability. The cholinergic system can be disrupted following heroin administration, indicating that activation of the cholinergic system may prevent chronic heroin misuse. Donepezil as an inhibitor of cholinesterase has been reported to clinically improve cognition and attention. In this study, the inhibition of heroin self-administration and heroin-seeking behaviours by donepezil were evaluated in rats. **Methods**: Rats were trained to self-administer heroin every four hours for 14 consecutive days under a fixed ratio 1 (FR1) reinforcement schedule, then underwent withdrawal for two weeks. A progressive ratio schedule was then used to evaluate the relative motivational value of heroin reinforcement. After withdrawal, a conditioned cue was introduced for the reinstatement of heroin-seeking behaviour. Donepezil (0.3–3 mg/kg, i.p.) was used during both the FR1 heroin self-administration and progressive ratio schedules. Immunohistochemistry was used to investigate the mechanism of action of donepezil in the rat brain. **Results**: Pre-treatment with high dose donepezil (3 mg/kg) but not low doses (0.3–1 mg/kg) significantly inhibited heroin self-administration under the FR1 schedule. Donepezil decreased motivation values under the progressive ratio schedule in a dose-dependent manner. All doses of donepezil (1–3 mg/kg) decreased the reinstatement of heroin seeking induced by cues. Correlation analysis indicated that the inhibition of donepezil on heroin-seeking behaviour was positively correlated with an increased expression of dopamine receptor 1 (D1R) and dopamine receptor 2 (D2R) in the nucleus accumbens (NAc) and increased expression of choline acetyltransferase (ChAT) in the ventral tegmental area (VTA). **Conclusions**: The present study demonstrated that donepezil could inhibit heroin intake and heroin-seeking behaviour. Further, donepezil could regulate dopamine receptors in the NAc via an increase of acetylcholine. These results suggested that donepezil could be developed as a potential approach for the treatment of heroin misuse.

Keywords: drug use disorder; cholinesterase inhibitor; donepezil; opiate; addiction

1. Introduction

Opioid use disorder presents a significant global challenge. Besides its immediate impact on physical and mental health, it is also a burden on public safety and social economics [1]. The United Nations Office on Crimes and Drugs estimated that 70 % of negative health impacts related to drug use could be attributed to the misuse of opioids [2], while heroin is one of the most misused opioids. The 2020 Drug Situation in China estimated that near 40.8% of illegal drug users who were officially registered were dependent on opioids. Heroin addiction is a chronic relapsing disorder, characterized by a compulsion to use heroin and the emergence of a negative emotional state after withdrawal [3]. Long-term use of heroin disrupts the functions of consciousness and decreases consciousness control [4]. Additionally, the experience of opioid withdrawal has effects on memory that contribute to the persistence of addictive behaviour [2]. Cognitive impairments are the most fre-

quently reported deleterious effect associated with heroin addiction. Chronic exposure to heroin leads to cognitive deficits, as shown by poor performances on cognitive behaviour tests [4,5]. Furthermore, heroin withdrawal results in impairment of attentional accuracy, omissions, and latencies in correct response [6]. Consequently, cognitive enhancers might be usefully in the treatment of heroin addiction.

The cholinergic system plays an important role in the regulation of attention, memory, processing speed, and sensory gating [7]. Moreover, the cholinergic system can be disrupted by administration of heroin [8]. Additionally, immunotoxin-mediated ablation of cholinergic neurons in nucleus accumbens (NAc) enhances not only morphine sensitivity in conditioned place preference but also negatively reinforces morphine withdrawal in conditioned place aversion [9]. Previous studies have shown that the enhancement of cholinergic transmission in different brain regions differ-

entially regulates heroin-seeking behaviour, indicating that cholinergic system dysfunction might be involved in heroin relapse and that a cholinergic enhancer might be useful for the treatment of heroin-seeking behaviour [10].

Donepezil is a rapid, reversible acetylcholinesterase (AChE) inhibitor, and has been approved for treating mild to moderate Alzheimer disease. The maximum recommended dose of donepezil is about 23 mg/day [11]. Donepezil is generally well tolerated, with most adverse events mild and transient [12]. It is reported that cocaine dependent patients had few somatic-dysphoric effects when receiving donepezil, indicating that it is safe for the treatment of drug misuse disorders [13]. This study investigated the effect of donepezil on heroin intake and motivation for heroin reinforcement in self-administration and the reinstatement of heroin-seeking behaviour induced by cues after a prolonged withdrawal.

2. Materials and Methods

2.1 Animals and Drugs

Male Sprague-Dawley rats initially weighing 260–300 g (Zhejiang Experimental Animal Center, Hangzhou, China) were individually housed in a temperature-controlled, ventilated colony room with food and water available ad libitum. To mimic natural activity, all experiments were conducted during the dark phase of the light-dark cycle (light: 6:30 AM to 6:30 PM). Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition). The protocols were approved by the Animal Care and Use Committee of Ningbo University (SYXK-2019-0005).

Heroin (diacetylmorphine HCl, National Institute of Forensic Science, Beijing, China), donepezil hydrochloride (HY-B0034, MCE, Monmouth, NJ, USA), sodium pentobarbital (30 mg/mL, P3761, Sigma-Aldrich, Wuxi, China), heparin sodium (H32022088, Qianhong Bioscience, Changzhou, China), and benzylpenicillin sodium (H13021632, Shiyao Bioscience, Shijiazhuang, China) were dissolved in sterile saline.

2.2 Surgery

Rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and implanted with chronically indwelling jugular catheters as previously described [10,14]. The catheter was flushed daily with 0.3 mL saline containing heparin (5 units) and benzylpenicillin sodium (20,000 units) to prevent bacterial infection and maintain catheter patency. Following the surgery, rats recovered for seven days.

2.3 Intravenous Self-Administration Training

All rats were trained to self-administer heroin (50 μg/kg per infusion) in operant chambers for 4 h/d for 10 consecutive days as previously described [10,14]. Each operant chamber was equipped with two nose-poke holes lo-

cated 5 cm above the floor of the chamber. One was an active nose-poke hole which injected one infusion of heroin into the jugular vein on activation by a rat nose-poke. The other nose-poke hole was inactive. A blue light was located within and a house light was situated on the wall above each nose-poke hole. Each session was started when the blue light inside the active nose-poke hole was switched on. The rat was subjected to the fixed ratio (FR) 1 schedule which means one active nose-poke received a single infusion. Each infusion was paired with 5 s illumination of the house light which, in combination with the noise of the infusion pump, served as a discrete conditioned stimulus (CS) paired with the drug infusion. A timeout period was imposed for 20 s, during which the nose-poke produced no programmed consequences but was still recorded. Illumination of the blue light in the active nose-poke signalled the end of the 20 s timeout period. Another session then started as described previously [15,16]. The daily training session ended after 4 h.

2.4 The Effect of Donepezil on Heroin Self-Administration Behaviour

The heroin self-administration trained rats were divided into four groups (n = 7 per group). Each group was i.p. treated with vehicle or donepezil (0.3, 1 or 3 mg/kg) 30 min prior to the FR1 training session on day 11. The amount of heroin self-administered over 4 h was recorded. After donepezil testing, heroin self-administration continued without drug treatment during days 12–13. On day 14 each group was treated with vehicle or donepezil (0.3, 1 or 3 mg/kg) 30 min prior to the progressive ratio (PR) schedule test.

2.5 Progressive Ratio Schedule Test

Rats were subject to the self-administered PR schedule for 4 h. Unlike FR1, PR was no longer a fixed number of active nose-pokes for receiving a single infusion of heroin but an increasingly variable number. PR = 5e (injectionnumber$\times$0.2)-5. The sequence of the PR schedule was: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62 ... In the first schedule, rats could obtain one heroin infusion after one effective active nose-poke; in the second schedule, rats required two effective active nose-pokes to obtain a single heroin infusion; then four effective active nose-pokes to obtain one infusion, six effective active nose-pokes to obtain an infusion, and so on; the more infusions given, the more active nose-pokes that were required for the next schedule. In short, as the procedure progressed, a rat received one injection of heroin throughout the series of 1, 2, 4, 6, 9, 12 … consecutive active nose pokes. PR expressed the strength value of the animal's motivation to obtain the drug [17]. If the animal did not obtain any drug injections within one hour, a break point (BP) was defined. The BP was used to evaluate the animal's motivation value for the drug reward [18].
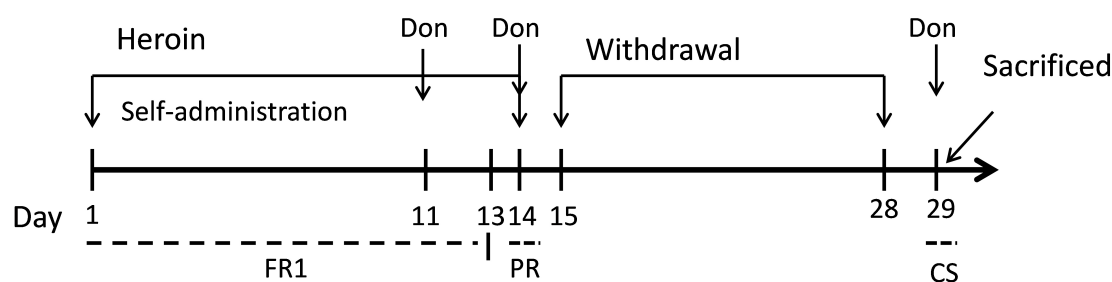
Fig. 1. Experimental procedure. All rats ($n = 28$) were subject to heroin self-administration under a FR1 schedule for 4 h/d for 13 consecutive days. On day 14, rats were subject to heroin self-administration under a PR schedule for 4 h. Donepezil was *i.p.* injected 30 min prior to self-administration on days 11 and 14. All rats were then subject to a 14 d withdrawal period. On day 29 donepezil was injected (*i.p.*) 30 min prior to a CS for heroin seeking. After the CS stimulus, rats were sacrificed.

2.6 The Effect of Donepezil on Cue-Induced Reinstatement of Heroin Seeking

After 14 d heroin self-administration followed by a two week withdrawal, the effects of donepezil on cue-induced reinstatement of heroin seeking behaviour were evaluated. Rats were injected with either donepezil (0.3, 1, or 3 mg/kg) or vehicle (n = 7 per group) 30 min before they were re-introduced into the training chamber. In the reinstatement induced by cues, the discrete CS was presented for 5 s, after which each active nose-poke response resulted in another presentation of the CS. The same schedule as for self-administration under FR1 was employed, but without any infusion. Nose-pokes during the phase of CS reinstatement were accumulated for 120 min.

2.7 Oral Sucrose Self-Administration

Twenty-four naïve rats were trained to nose-poke for sucrose pellets. The paradigm was similar to the heroin self-administration described above, except that rats received a 45 mg sucrose pellet (D12450B, Research Diets, Inc., New Brunswick, NJ, USA) delivered via a sucrose cup instead of the heroin injection. Rats were trained to nose-poke on a FR1 schedule for a sucrose pellet for 60 min each day until they finished 100 pellets or timed out. They received a sucrose pellet following completion of the ratio requirement in the active hole, which was followed by a 20-s timeout signalled by illumination of the house lights. After they acquired sucrose self-administration for 5 d when all rats finished the 100 pellet task within 20 min, the rats were randomly divided into four groups (n = 6 each). Each group was i.p. treated with vehicle or donepezil (0.3, 1, or 3 mg/kg) 30 min prior to the training session on day six.

2.8 Collection of the Brain of Rats

For Western blot analysis, once the heroin seeking task was completed, rats were anaesthetized with sodium pentobarbital and their brains were immediately removed. The NAc were dissected, flash frozen and kept at –80 °C until use. For immunohistochemistry, the rats were anaesthetized and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde solution. Brains were sectioned on a Cryostat Microtome (CM1850, Leica, Mannheim, Germany) in the coronal plane at a thickness of 30 μm. The slices were stored in PBS at 4 °C.

2.9 Western Blot Analysis

Tissues from the NAc were homogenized in RIPA buffer and Western blotting was performed as previously described [19]. Primary antibodies were against choline acetyltransferase (ChAT, 1:3000, ab181023, Abcam, Cambridge, UK) and β-actin (1:1000, bsm-33036M, Bioss, Beijing, China). The secondary antibodies were anti-rabbit and anti-mouse (1:5000, 926-32211 and 926-32212, Li-cor, Lincoln, NE, USA) antibodies. Immunoreactive protein bands were detected by using the Odyssey CLx infrared imaging system. The darkness of the blots was evaluated by analysis of the intensity of each band using ImageJ software (Version 1.8.0, NIH Image, Bethesda, MD, USA). Data were expressed as ratios of optical density (OD) compared with controls (β-actin) for statistical analysis.

2.10 Immunohistochemistry

Coronal slices were prepared and blocked for 1 h at room temperature in 1% albumin and 0.5% Triton-X in PBS. Slices were then incubated overnight at 4 °C in 1% albumin in PBS with the following antibodies: anti-ChAT (1:200, ab181023, Abcam, Cambridge, UK); anti-dopamine receptor 1 (D1R, 1:100, Ab20066, Abcam, Cambridge, UK) and anti-dopamine receptor 2 (D2R, 1:100, sc-5303, Santa Cruz, Dallas, TX, USA). Sections were then dark incubated 1 h at room temperature in blocking buffer with AlexaFluor 488 donkey anti-goat IgG (1:400, ab150077, Abcam, Cambridge, UK), AlexaFluor 555 donkey anti-mouse IgG (1:400, ab150106, Abcam, Cambridge, UK) and AlexaFluor 647 donkey anti-rabbit IgG (1:400, ab150075, Abcam, Cambridge, UK). Sections were washed with PBS ($\times$3, 5 min each) after each incubation step. Samples were visualized using the Leica SP8 laser scanning confocal microscope and Leica LAS X image acquisi-
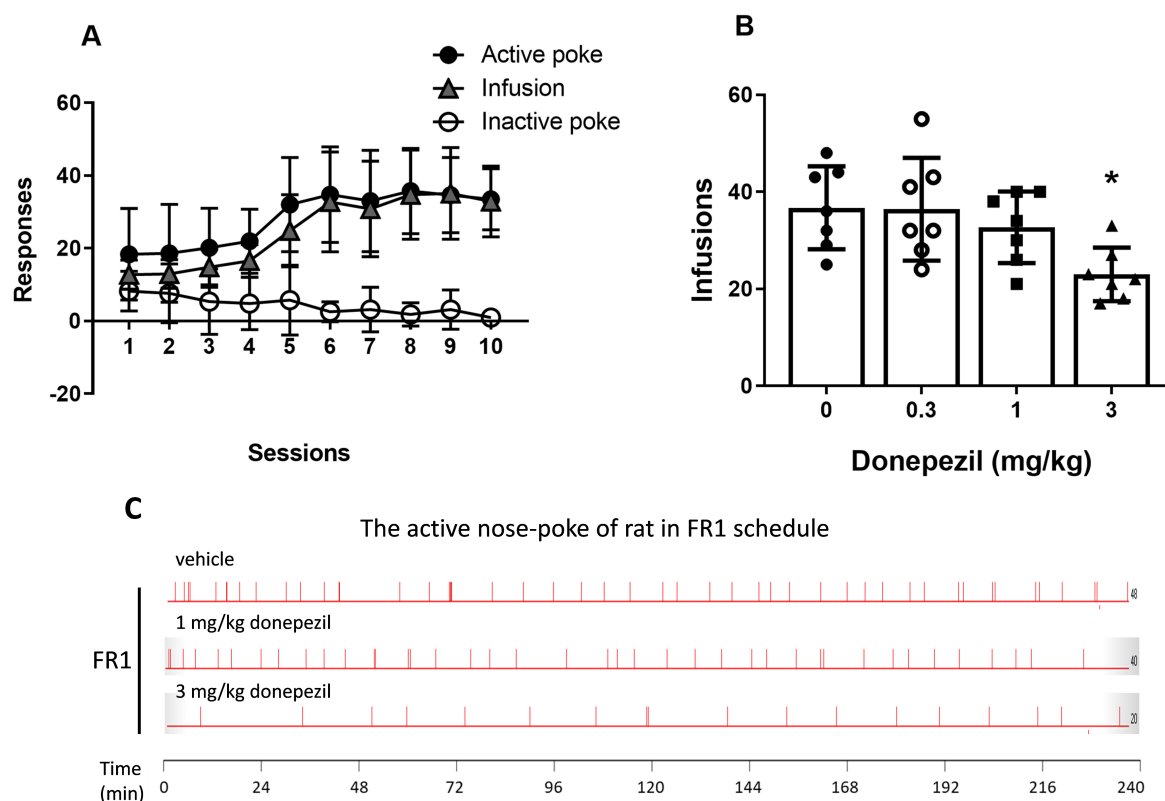
Fig. 2. Effects of donepezil on heroin reinforcement in self-administration. (A) Rats were successfully trained within 10 days to self-administer heroin 4 h/d (infusion dose 50 μg/kg) under the FR1 reinforcing schedule and learned to associate active nose-poke with heroin reward. (B) Rats were *i.p.* administered with vehicle, 0.3, 1 or 3 mg/kg donepezil 30 min prior to 4 h training session on day 11. Donepezil (0.3–3 mg/kg) tended to decrease heroin infusions while 3 mg/kg donepezil significantly decreased the number of infusions compared with vehicle control. (C) The raster plot of the active nose-poke under the FR1 reinforcing schedule. Data shown are means $\pm$ SD; $n = 7$ per group. * Significant difference from vehicle ($p < 0.05$).

tion software. Quantitative analysis (cell counts) was performed using LAS X software (Version 2.0.1, Mannheim, Germany).

2.11 Data Statistics

Data are expressed as mean $\pm$ SD. All statistical tests were performed using SPSS (Version 18.0, IBM, Armonk, NY, USA). The mean number of "active" and "inactive" nose-pokes and infusions during self-administration and in the drug-seeking test were analysed via one-way Analysis of Variance (ANOVA) with Bonferroni post hoc testing when appropriate. The *t*-test was used to test for any differences between the two groups. A *p*-value < 0.05 was considered to indicate statistically significant differences.

3. Results

### 3.1 Donepezil at a High Dose Reduces Heroin-Taking in Rats

The experimental procedure is given in Fig. 1. Rats were successfully trained to self-administer heroin under an FR1 reinforcing schedule and learned to associate active nose-poke with heroin reward (Fig. 2A). As shown in

Fig. 2B,C, one-way ANOVA revealed a significant effect of donepezil on infusions ($F_{(3,24)} = 4.26$, $p < 0.05$) and *post hoc* comparison revealed a significant decrease in infusions by pretreatment with donepezil at 3.0 mg/kg ($p < 0.05$) but not 0.3 or 1 mg/kg.

3.2 Donepezil Reduces Heroin Motivation Value in Rats

To determine whether donepezil attenuated the reward motivation of heroin self-administration, rats were examined under the PR schedule following donepezil treatment. One-way ANOVA revealed a significant effect of donepezil on the accumulated responses of active nose-pokes ($F_{(3,24)} = 5.90$, $p < 0.01$), but not for that of inactive nose-pokes ($F_{(3,24)} = 0.1$, $p = 0.96$; Fig. 3A). *Post-hoc* comparison indicated that donepezil at 1–3 mg/kg significantly decreased active responses ($p < 0.05$) in a dose-dependent manner, but it did not affect the inactive responses relative to the corresponding vehicle control. Donepezil at the same doses decreased the total number of heroin injections and observed BPs in dose-dependent manner ($F_{(3,24)} = 5.05$, $p < 0.01$; Fig. 3B,C). Additionally, donepezil at 1–3 mg/kg significantly decreased the BP under the PR schedule ($p < 0.05$).
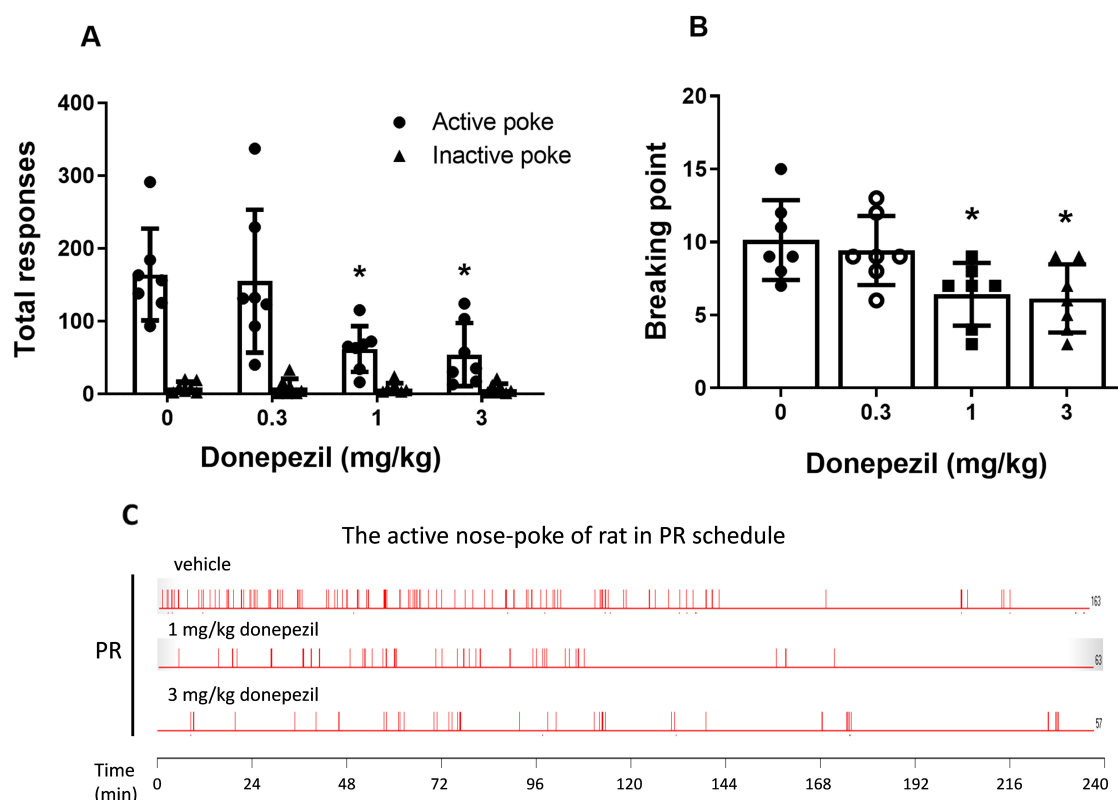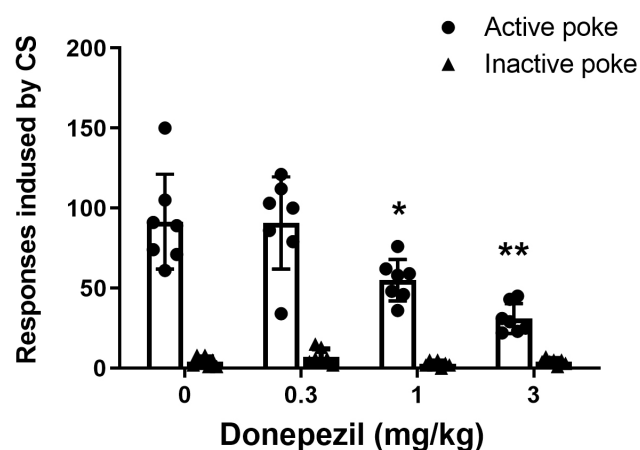
Fig. 3. Donepezil reduces heroin motivation value in rats. Rats were *i.p.* administered with vehicle, 0.3, 1 or 3 mg/kg donepezil 30 min prior to a 4 h training session on day 14. Donepezil at 1 and 3 mg/kg decreased (A) the mean total responses of active nose-pokes, and (B) the infusion breakpoints for intravenous heroin during PR schedule of reinforcement. (C) The raster plot of the active nose-poke under the PR reinforcing schedule. Data shown are mean $\pm$ SD; $n = 7$ per group. * Significant difference from vehicle ($p < 0.05$).

### 3.3 Donepezil Reduces Heroin Seeking Induced by Cues in Rats

The effects of donepezil on cue-induced reinstatement of heroin seeking were examined after extinction for two weeks. As shown in Fig. 4, one-way ANOVA revealed that the cue-induced active nose-pokes were reduced by donepezil in a dose-dependent manner ($F_{(3,24)} = 12.3$, $p < 0.01$), while inactive nose-pokes were not significantly affected ($F_{(3,24)} = 1.67$, $p = 0.20$; Fig. 4). *Post hoc* comparison revealed that donepezil at 1–3 mg/kg significantly decreased cue-induced active responses ($p < 0.05$ or 0.01).

3.4 Effect of Donepezil on Sucrose Reinforcement

To determine whether donepezil specifically affected heroin taking, the effect of donepezil on sucrose self-administration was examined in a separate set of rats using similar procedures. One-way ANOVA revealed a significant effect of donepezil on the time to finish the sucrose reinforcement task $F_{(3,20)} = 7.28$, $p < 0.01$). *Post hoc* comparisons indicated that donepezil significantly prolonged the time of the sucrose task only at a high dose (3 mg/kg; $p < 0.01$) (Fig. 5).

Fig. 4. Donepezil reduces heroin seeking induced by cues in rats. The test was performed two weeks after withdrawal following a 14 d self-administration training. Rats were *i.p.* administered with vehicle, 0.3, 1 or 3 mg/kg donepezil 30 min before being returned to the operant chambers and exposed to the cues previously paired with heroin infusions. Data shown are mean $\pm$ SD; $n = 7$ per group. * and ** significant difference from vehicle ($p < 0.05$ and $p < 0.01$, respectively).
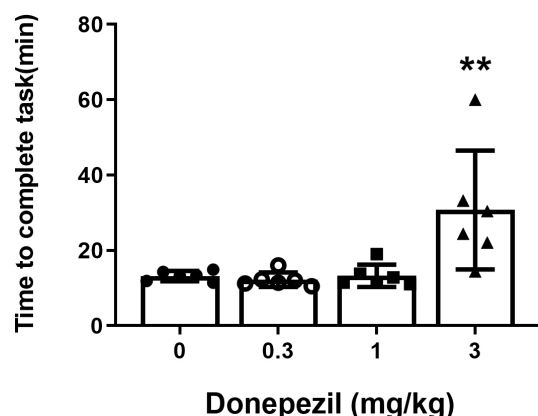
Fig. 5. Effect of donepezil on sucrose self-administration. Rats were *i.p.* administered with vehicle, 0.3, 1, or 3 mg/kg donepezil 30 min prior to the 60 min FR1 training session until they finished 100 pellets or timed out. Donepezil at 0.3 or 1 mg/kg failed to affect sucrose self-administration. Donepezil at 3 mg/kg prolonged the time to complete the sucrose pellet self-administration task under the FR1 schedule. Data shown are mean ± SD; *n* = 6 per group. ** Significant difference from vehicle ($p < 0.01$).

3.5 Donepezil Prevents Heroin-Induced Biochemical Changes in Rats

The effect of donepezil on dopaminergic and cholinergic systems was further measured. NAc is the core brain area directing heroin seeking and taking [20]. Immunohistochemistry showed the expression of D1R in the NAc significantly decreased in heroin trained rats compared with that in control rats, while donepezil significantly increased the expression of D1R in the NAc of heroin trained rats (one-way ANOVA, *n* = 3, Bonferroni *post hoc* test, $p <$ 0.01, Fig. 6A,B). Moreover, donepezil significantly increased the expression of D2R in the NAc of heroin trained rats (one-way ANOVA, *n* = 3, Bonferroni *Post hoc* test, $p < 0.05$, Fig. 6A,B). However, the decreased expression of D2R in the NAc of heroin trained rats was not significantly changed compared to that of control rats, neither was the expression ChAT in the NAc significantly changed among control, heroin and donepezil + heroin groups ($F_{(2,6)}$ = 0.17, $p = 0.85$, Fig. 6C,D). Western blot analysis also showed that donepezil did not significantly change ChAT levels in the NAc of heroin trained rats ($t_{(4)} = 2.2$, $p = 0.09$, Fig. 6E,F). Interestingly, ChAT levels in the VTA significantly decreased in heroin trained rats when compared to that in control rats, while donepezil significantly increased the expression of ChAT level in the VTA of heroin trained rats (one-way ANOVA, *n* = 3, Bonferroni *post hoc* test, $p <$ 0.05, Fig. 6G,H).

4. Discussion

The main findings of this study were that the systemic administration of donepezil at 3 mg/kg not only reduced heroin infusions but also decreased sucrose consumption. Moreover, donepezil dose-dependently reduced the motivation to seek heroin under a PR schedule and blocked the reinstatement behaviour in heroin-seeking induced by heroin cues after prolonged withdrawal, with the minimum effective dose at about 1 mg/kg. These results suggested that there is a specific role played by acetylcholine in heroin motivation and reinstatement but not in heroin reinforcement. Moreover, results indicated that donepezil reversed the decrease of D1R and D2R expression in the NAc of heroin withdrawal rats. Additionally, donepezil significantly increased ChAT expression in the VTA but not in the NAc.

It is indicated that enhanced cholinergic signalling is critical in opioid reinforcement [21,22]. Moreover, the gene variants of AChE have been associated with heroin addiction vulnerability in humans [23]. Donepezil increases cholinergic transmission in the brain by inhibiting AChE, thereby elevating endogenous acetylcholine levels. Extracellular acetylcholine levels in the brain were significantly increased at 20 min following acute systemic administration of donepezil and remain elevated for at least three hours [24]. Previous research by the authors has shown the cholinergic system was disrupted during the development of heroin addiction [13]. Additionally, 3 mg/kg donepezil prior to 10 mg/kg cocaine abolished conditioned place preference for cocaine and pre-treatment with 1 mg/kg donepezil significantly inhibited locomotor sensitivity to cocaine in mice [25]. Moreover, donepezil (1–3 mg/kg, *i.p.*) attenuated nicotine self-administration maintained on a fixed-ratio five schedule of reinforcement in rats [26]. The present results support these previous reports, further suggesting that acetylcholine plays an important role in drug reinforcement, and that donepezil might be helpful for the treatment of drug misuse disorders.

The ability of 3.0 mg/kg donepezil to decrease sucrose intake is consistent with a previous report that a high dose of AChE inhibitors attenuates food intake by rats [18], although the ED_{50} value of AChE inhibitors for attenuating self-administration of drugs may be lower than that for attenuating food reinforced behaviour [27]. The results reported here showed both inhibition of donepezil on sucrose and heroin intake at 3.0 mg/kg, indicating no specific action of acetylcholine elevation by donepezil for sucrose or heroin reinforcement.

Systemic administration of donepezil at 1 mg/kg reduced both the motivation to respond for heroin and reinstatement behaviour in heroin-seeking induced by heroin cues. Interestingly, donepezil at 1 mg/kg did not significantly affect sucrose taking, suggesting that the effect of 1 mg/kg donepezil on motivation or reinstatement induced by cues might not be due to general motor disruption or sedation. DA and ACh in the NAc balance approach and avoidance behaviour, ACh may counteract excessive DA-mediated approach behaviour as revealed during withdrawal from drugs of misuse when the animal enters an
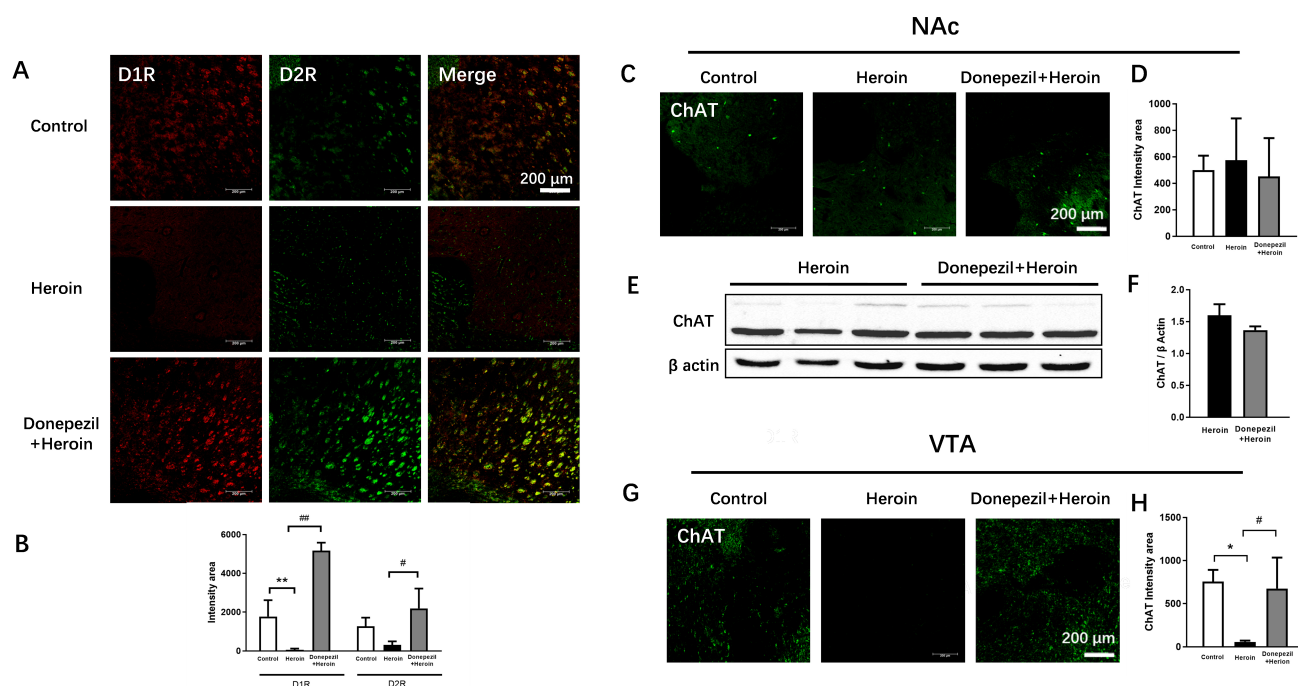
Fig. 6. Donepezil prevents heroin-induced biochemical changes in rats. (A,B) The expression of D1R or D2R in the NAc significantly decreased in heroin trained rats when compared with control rats, while donepezil significantly increased D1R or D2R in the NAc of heroin trained rats. (C,D) The expression of ChAT in the NAc was not significantly changed among control, heroin and donepezil + heroin groups. (E,F) Western blot analysis showed that donepezil did not significantly alter ChAT levels in the NAc of heroin trained rats. (G,H) ChAT levels in the VTA significantly decreased in heroin trained rats when compare with control rats, while donepezil significantly increased ChAT levels in the VTA of heroin trained rats. Bars give mean $\pm$ SD; $n = 3$; * and ** significant difference from control ($p < 0.05$ and $p < 0.01$, respectively); # and ## indicate a significant difference from the heroin trained group ($p < 0.05$ and $p < 0.01$, respectively). D1R, dopamine receptor 1; D2R, dopamine receptor 2; ChAT, choline acetyltransferase.

ACh-mediated state of anxiety and behavioural depression [11]. This may explain why ACh can effectively reduce the enhanced reward motivation value caused by the excessive release of DA in heroin taking.

Relapse rates for opioids are higher than any other illicit drugs, with up to 59% of individuals relapsing in the first week of abstinence and 80% relapsing in the first month [28]. In this study, donepezil significantly inhibited heroin seeking, suggesting that elevation of ACh may be involved in the inhibition of heroin seeking. This is consistent with a previous study that galantamine, an inhibitor of cholinesterase, dose-dependently blocked the reinstatement of heroin seeking induced by either cues or heroin priming after prolonged withdrawal [29].

The NAc, one of the terminal regions of the mesocorticolimbic dopamine projecting system, is necessary for the development of opioid seeking and has been shown to modulate opioid reward [30] and opioid-taking [31]. Approximately 95% of the neurons in the NAc are medium spiny neurons (MSNs) that generally express excitatory D1-like receptors or inhibitory D2-like receptors [32]. There are substantial reductions of D2R availability in the striatum in individuals with addiction that persists for months after protracted detoxification. This has also been observed in

preclinical studies in rodents and non-human primates with repeated drug exposure [33]. Results given here were consistent with these studies, indicating that prolonged heroin withdrawal decreased the expression of D1R and D2R in the NAc and that donepezil could reverse this decrease in heroin withdrawal rats.

DA release induced the activation of D1-MSNs and the inhibition of D2-MSNs, synergistically increased DA release in the NAc, and promoted greater reward [34]. There are two largely separated populations of D1-MSNs in the NAc. The activation of D1 NAc-ventral mesencephalon (VM) neurons increases dopamine release into the NAc and promotes reward, whereas activation of D1 NAc-ventral pallidum (VP) neurons reduces dopamine levels and elicits aversion [35]. D2-MSNs primarily target VP neurons that innervate the VM. Activation of D2R decreases excitability of D2-MSNs, inducing dopamine release and produces rewarding effects [36]. Activating D1-MSNs promotes drug seeking, while D2-MSN stimulation reduces drug seeking in drug-associated contexts after extinction training [37]. In parallel with the involvement of distinct subtypes, DA release in the NAc activates both D1- and D2-dopamine receptors, increases cyclic adenosine monophosphate (cAMP) /protein kinase A (PKA) ac-

tivity in D1-MSNs, and inhibits cAMP/PKA activity in D2-MSNs [38]. Previously, it has been shown that cAMP response element-binding protein (CREB) phosphorylation in the NAc decreases after withdrawal and increases CREB phosphorylation in the NAc by rolipram [39]. Vagus nerve stimulation [40] or deep brain stimulation of the NAc [41] can reverse the heroin seeking induced by cues. Importantly, D1-like receptors in the NAc were demonstrated to play an important role in morphine seeking after prolonged abstinence [42]. Reinstatement to heroin-associated cues induces synaptic adaptations at D1-MSNs within the NAc [36,37]. However, it was not clarified here, how donepezil regulated different populations of D1-MSNs and D2-MSN in the NAc. Thus, the regulative mechanism underlying inhibition of donepezil on heroin seeking warrants further study.

The cholinergic system has been shown to be involved in drug misuse [22,43] and has also been proposed as a novel target for treating addiction [44,45]. Cholinergic interneurons comprise less than 1% of neurons in the NAc [46], while cholinergic projection to the NAc is mainly from laterodorsal tegmentum (LDTg), which provides cholinergic modulation together with the NAc cholinergic interneurons [47]. The results reported here showed that donepezil did not significantly change the expression of ChAT in the NAc, suggesting that donepezil might not directly act on these neurons. Results further showed that the expression of ChAT was lower in the VTA of heroin withdrawal rats and the ChAT level in VTA was increased in donepezil-treated animals. It has previously been demonstrated that dopaminergic neurons originating in the VTA project primarily to the NAc and prefrontal cortex, forming the mesolimbic and mesocortical systems, respectively [48]. Acetylcholine might locally regulate dopaminergic function via the muscarinic or nicotine receptors in the VTA. The muscarinic receptors M2 and M4 are autoreceptors that locate presynaptically, and their activation decreases the release of acetylcholine at the synapse. In NAc-specific M4 knockout mice, psychostimulant-induced DA basal values were elevated and the psychostimulant-induced DA response was enhanced [49]. The M5 muscarinic receptor is the only muscarinic receptor subtype expressed in mesencephalic dopamine neurons and provides an important excitatory input for mesolimbic and nigrostriatal dopamine projections. Depletion of M5 receptors on VTA dopamine neurons reduces heroin-induced sensitization [50]. Thus, the elevation of acetylcholine by donepezil may activate muscarinic receptors to regulate dopaminergic neurons in the VTA. An alternative explanation is that elevation of acetylcholine by donepezil may activate the nicotine receptor. Nicotine activates neurons that project to the NAc, whereas nicotine inhibits neurons that project to the amygdala nuclei [51]. The pathway from the amygdala has been identified as a common element involved in the incubation of drug seeking for different substances [52]. Thus, it was suggested that donepezil might regulate D1R and D2R in the NAc at least partially via affecting cholinergic neurons in the VTA.

Currently, there are only a few drugs clinically used for heroin addiction, including methadone (a full opioid agonist), buprenorphine (a partial agonist), and naltrexone (an opioid antagonist) [53]. Preclinical studies by the authors have shown that donepezil inhibited motivation for heroin intake and drug seeking induced by cues, suggesting that that donepezil might potentially be useful for the treatment of heroin use disorder. Moreover, as a first-line clinical medicine for AD patients, the safety and reliability of donepezil are guaranteed in clinical trials.

Recent research suggests cognitive deficits may underline heroin relapse and addiction, and that cognitive enhancers appear to be a valuable therapeutic target for heroin addiction treatment by preclinical research. The different kinds of cognitive enhancers used as a treatment for heroin relapse and addiction may act on cholinergic, glutamatergic, dopaminergic or adrenergic pathways [54]. However, the benefits or harms of the use of pharmacological cognitive enhancers in healthy individuals should be studied further [55]. As a cognitive enhancer, this study has shown that donepezil has the potential to treat heroin addiction, but the regulative mechanism underlying inhibition of donepezil on heroin seeking and taking warrants further study. Meanwhile, these data obtained from a small cohort and need to be replicated in a greater number of animals under different conditions, such as in opioid naïve animals and in opioid experienced animals with chronic heroin use both current use and those exposed to relapse/reinstatement.

In summary, the present study demonstrated that donepezil inhibited heroin intake and heroin-seeking behaviour possibly via acting on dopamine receptors. This indicates that development of cognitive enhancers may provide potential approaches for the treatment of heroin misuse.

5. Conclusions

(1) High dose of donepezil (3 mg/kg) can inhibit heroin intake.

(2) Donepezil can dose-dependently reduce the motivation to seek heroin.

(3) Donepezil can dose-dependently block the reinstatement behaviour in heroin-seeking induced by heroin cues after prolonged withdrawal.

(4) Donepezil can regulate dopamine receptors in the NAc via an increase of acetylcholine.

(5) The way donepezil regulates D1R and D2R in the NAc may at least partially via affecting cholinergic neurons in the VTA.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

IMR Press

Author Contributions

Ethics Approval and Consent to Participate

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