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Time-dependent protective effects of morphine against behavioral and morphological deficits in an animal model of posttraumatic stress disorder

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ABSTRACT

Post-traumatic stress disorder (PTSD) arises after an individual has experienced a major traumatic event. Recent evidence suggests that acute morphine treatment may serve as a strategy to reduce PTSD development. In the present study, we investigated the time-dependent effects of morphine on behavioral and morphological deficits induced by the single prolonged stress (SPS), an experimental model of PTSD, in adult male rats. The rats were exposed to SPS (restraint for 2 h, forced swimming for 20 min, and ether anesthesia), and kept undistributed for 11 days. Morphine was injected immediately, 6, 12 and 24 h after SPS. Anxiety profile was evaluated using the elevated plus maze11 days after SPS. Then, animals were conditioned in a fear conditioning task and extinction training was performed on days 1, 2, 3, 4 and 11 after fear conditioning which followed by morphological assessments in the medial prefrontal cortex (mPFC). SPS rats showed increased anxiety levels and impaired contextual fear extinction retention. SPS also decreased dendritic length in the infra-limbic (IL) and dendritic spines in the IL and pre-limbic (PL) regions of the mPFC. Conversely, morphine treatment 6, 12 and 24 h but not immediately after SPS significantly improved anxiety-like behaviors, fear extinction, increased dendritic length, and spines in the mPFC. Morphine-induced much stronger response when injected 24 h after the SPS, and this effect was blocked by naloxone. Our findings show that morphine within a restricted time window selectively reversed the SPS-induced deficits in anxiety profile, fear extinction, and dendritic morphology in the mPFC. Finally, these findings suggest that the time point of morphine injection following a traumatic event is an important determinant of the full therapeutic effect of morphine against PTSD.

1. Introduction

Most of the people experience, at least, one tremendous traumatic event throughout their lives [1,2]. Fortunately, most of them will forget about the traumatic event after a while [3]. Although more than half of these traumatic events such as war, rape, burn and dangerous car accident are very fearful by nature [4,5], a small but significant minority of people go through post-traumatic stress disorder (PTSD) [3,4,6]. PTSD arises after an awful traumatic experience [7] and according to the 5th edition of the diagnostic and statistical manual (DSM-5), characterized by continuous re-experience of the traumatic event, avoidance of trauma associated stimuli, negative affect emotional numbing and hyperarousal [8]. All these symptoms should be last for at least one month and cause serious problems in daily life activity [8]. Moreover, PTSD patients often experience a flashback of the events and general anxiety.

Given to wrecking trait of PTSD and because of lack of effective available treatment for patients whose disease has been diagnosed with PTSD, many researchers have been targeted at the early pharmacological interventions immediately after traumatic events. The data suggest that some medications such as propranolol [9], hydrocortisone [10] and oxytocin [11] soon after traumatic events may protect individuals against PTSD or reduce the severity of their symptoms [12,13].

Recent evidence has shown that acute morphine treatment may serve as a strategy to reduce PTSD development [14]. Some clinical studies have shown that acute morphine administration within hours of injury leads to a significant lower PTSD prevalence and mood disorders

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[15,16]. The data have been gathered after the Iraq war showed that use of morphine during early trauma care leads to lower risk of PTSD [16]. Again, morphine treatment after the single traumatic event and burn was associated with a reduced risk of the PTSD in children [17,18]. These clinical studies suggest that morphine treatment after a traumatic event may be associated with a lower probability of the PTSD; however, such a claim is largely difficult in the case of the human. On the other hand, some studies have investigated the interaction between morphine and PTSD in the experimental models of PTSD [19,20]. Long-term memory formation, which lasts from several hours to several days and even more, needs a remarkable consolidation process, which takes several hours [21]. It has been reported that opioids may negatively modulate memories during this important time [22,23]. Moreover, morphine impairs long-term acquisition in contextual fear conditioning (CFC), and radial and Morris water maze [24,25]. A recent study has shown that repeated morphine administration after the severe stressor or single injection of morphine at 48 h after the severe stressor prevents the development of stress - enhanced fear learning [20]. Altogether, these studies emphasize the protective effects of morphine on PTSD but the time window of morphine administration has not been precisely defined.

A large body of literature shows that PTSD might be associated with extinction deficits [26,27]. Fear extinction is a form of learning and occurs when the fearful conditioned stimulus (CS) no longer leads to a fear response [28,29]. Fear extinction deficits were precisely investigated with the single prolonged stress (SPS), which is one of the privileged animal models of PTSD [30,31]. SPS animals show behavioral and neurobiological hallmarks of PTSD [32] and increased arousal [31,33]. It has been reported an enhanced fast negative feedback of the hypothalamic-pituitary-adrenal (HPA) in SPS rats [30]. SPS rats show extinction retention deficits like PTSD patients [30,34]. SPS causes remarkable morphological changes in the amygdala, medial prefrontal cortex (mPFC) and hippocampus, the main brain regions critical for extinction retention, which may be involved in the pathogenesis of PTSD [35,36].

In the present study, we investigated the time-dependent effects of morphine on anxiety and fear extinction impairment and morphological deficits in the mPFC induced by SPS, as an experimental model of PTSD in adult male rats.

2. Materials and methods

2.1. Experimental animal and housing condition

Adult male Wistar rats (200–250 g) were obtained from the breeding colony of the Semnan University of Medical Sciences (SUMS), Semnan, Iran. The animals were housed in groups of five and maintained in a room with 12-h light, 12-h dark cycle (lights on at 6:00 am), standard temperature (24 ± 2 °C) and humidity (50 ± 5%). Rats were given ad libitum access to water and food. The Ethical Review Board of SUMS (IRSEMUMS.REC.1394.117) approved the experimental protocol of this study. All experimental procedures followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

Morphine sulfate and naloxone hydrochloride were purchased from Sigma (St. Louis, Mo, USA). Both drugs were freshly prepared before the experiments, dissolved in sterile 0.9 NaCl and administered intraperitoneally (i.p.).The doses of morphine (10 mg/kg/2 ml) and naloxone (1 mg/kg/2 ml) were chosen based on earlier researches [19,37].

2.3. SPS model

The SPS model of PTSD consists of three distinct stresses, including the restraint for 2 h, forced swim for 20 min and ether anesthesia [30,38]. Each rat was placed inside a clear polyethylene cylinder rat restrainer for 2 h. The restrainers were quite the size of each animal to achieve complete immobilization. Several holes in the restrainers caused the rats to breathe freely. This phase followed by 20 min forced swimming in a clear acrylic cylindrical tank (20 × 20 × 50 cm) filled two-thirds with 24 °C clean water. Animals recuperate for 15 min and then were exposed to ether vapor for 2–3 min, until loss of consciousness. Finally, animals were returned to their home cage and left undisturbed for 11 days.

2.4. Anxiety test in the Elevated plus maze

The elevated plus maze (EPM) is a valid tool to measure anxiety profile in the rats [39]. The EPM consisted of a plus-shaped and wooden four arm platform that was painted with gray enamel. Two open arms (50 × 10 cm) facing each other were bounded by a 0.5 cm edge to avoid animals falling and two enclosed arms (50 × 10 cm, surrounded by 40-cm high wooden walls) arranged perpendicular to the open arms. The walls began from a central neutral area (10 × 10 cm). All arms were 50 cm elevated above the floor.

At the beginning of each test, animals were gently placed in the center square always facing one of the open arms and were allowed to discover the maze for 5 min. When the hind limbs of the rats crossed the arms considered as an entrance. A total number of entries and total time spent into open arms were used as a measure of anxiety [39]. Anxiogenic influences selectively decrease the open arm entry and/or open arm time and, in contrast, anxiolytic influences selectively increase the open arm entry and/or open arm time. The number of total arm entries was used as a measure of spontaneous locomotor activity.

2.5. Contextual fear conditioning and extinction

In order to study contextual fear conditioning (CFC) in the rats, an automated rodent fear conditioning system (TSE, Bad Homburg, Germany) was used. CFC was performed in a conditioning box as before described [40]. The conditioning box was made of clear Plexiglas, which its floor was constructed of 25 stainless steel rods (6 mm in diameter, 12 mm apart) to deliver foot shock. On day zero (fear conditioning session), the rats were placed on the conditioning chamber for 3 min and then received 3 foot-shocks (1 mA, 2 s duration) with 40 s intervals. The animals remained an additional minute in the chamber before being returned to their home cages. A continuous background noise and light illumination were provided during this session. The chamber was cleaned with 5% ethanol after each session.

Extinction training was performed on days 1, 2, 3, 4 and 11 after fear conditioning. During extinction training, the animals were placed in the same chamber with the same conditions for 5 min, but they did not get any shock. A trained technician looked at the rats every five seconds and marked on a sheet if there was freezing. Freezing means the complete immobility of the rats in a stereotyped crouching position except for movements necessary for respiration [41]. The extinction index is the percentage of freezing reduction from the first to the last extinction day and was calculated with the following formula: the freezing level during the first extinction day - the last extinction day/ the freezing levels in the first extinction day multiplied by 100.

2.6. Morphological analysis

Golgi-Cox is the most reliable method in determining dendritic arborization [42]. After the completion of the tests, 6 animals of each group were randomly selected, deeply anesthetized with carbon dioxide and rapidly decapitated. The brains were gently removed and placed in

20 mL Golgi–Cox solution (potassium dichromate, mercuric chloride and potassium chromate), where they were stored in the dark for 14 days, and then transferred to a 30% sucrose solution for at least three days [43]. Coronal 200 μm sections were obtained using a vibratome collected onto gelatin-coated slides and dehydrated through a graded series of ethanol, cleared in xylene and coverslipped. An experimenter visualized individual neurons at 100× using camera lucida. Pyramidal neurons of the mPFC were selected on the basis of the following criteria: (1) the cell type must be identical; (2) neurons must be dark and consistently sliver impregnated throughout the extent of all dendrites; (3) dendrites must be untruncated; and (4) stained neurons must be relatively free from the neighboring impregnated neurons. Pyramidal neurons were defined by the presence of a basilar dendritic tree, a distinct, single apical dendrite, and dendritic branch points [44,45]. For each animal, an average apical dendritic length and branch points within a 100-μm thick section of each dendritic tree of 6–8 selected pyramidal neurons was calculated. From each experimental group, six animals randomly were selected for morphology analysis.

2.7. Experimental groups

In order to determine the time window of acute morphine treatment, morphine was injected immediately (0), 6, 12, and 24 h after SPS. Sham groups were injected with saline in the same way. For each time point, animals were divided into 4 groups (n = 10 in each group): Sham + Saline, Sham + Morphine, SPS + Saline and SPS + Morphine. The SPS animals were left 10 days after SPS to develop symptoms of PTSD. Sham groups were left undisturbed in their home cages. Anxiety-like behaviors were assessed on day 11 after SPS with the EPM. Two weeks after SPS, fear conditioning and extinction training were performed as described earlier (Fig. 1).

To confirm the specificity of the protective effects of morphine on SPS animals, two additional groups were as used to test whether naloxone could prevent the therapeutic effects of morphine administrated 24 h after SPS. Naloxone was injected 30 min before saline (n = 10) or morphine (n = 10) injections and SPS animals were tested according to procedures described the above.

2.8. Statistical analysis

All data were expressed as a means ± SEM. The results were analyzed by one-way, or two-way (ANOVA) followed by Bonferroni's post hoc test, when appropriate. A $p < 0.05$ was considered significant.

3. Results

3.1. Anxiety profile

EPM data are shown in Fig. 2. A two-way ANOVA on open arms time (OAT) demonstrated a significant effect of SPS ($F_{1,144} = 37.422$, $P < 0.001$) but not the treatment ($F_{7,144} = 1.635$, $P = 0.130$), and an interaction between SPS and treatment ($F_{7,144} = 7.731$, $P < 0.001$). Between groups comparisons revealed that OAT was significantly decreased in the SPS + Saline 0 h than the Sham + Saline 0 h ($P < 0.05$), SPS + Saline 6 h than the Sham + Saline 6 h ($P < 0.05$), SPS + Saline 12 h than the Sham + Saline 12 h ($P < 0.01$) and SPS + Saline 24 h than the Sham + Saline 24 h ($P < 0.01$) groups. Moreover, OAT was significantly increased in the SPS + Morphine 12 h than the SPS + Saline 12 h, and SPS + Morphine 24 h than the SPS + Saline 24 h (both, $P < 0.05$) groups.

A two-way ANOVA on open arms entry (OAE) showed significant effects of SPS ($F_{1,144} = 12.483$, $P < 0.01$), treatment ($F_{7,144} = 2.682$, $P < 0.05$) and an interaction between SPS and treatment ($F_{7,144} = 8.796$, $P < 0.001$). Between groups comparisons revealed that OAE was significantly decreased in the SPS + Saline 6 h than the Sham + Saline 6 h ($P < 0.01$) and SPS + Saline 24 h than the Sham + Saline 24 h ($P < 0.01$). OAE was significantly increased in the SPS + Morphine 6 h than the SPS + Saline 6 h ($P < 0.01$), SPS + Morphine 12 h than the SPS + Saline 12 h ($P < 0.01$), and SPS + Morphine 24 h than the SPS + Saline 24 h ($P < 0.001$).

A two-way ANOVA on total arms entry (TAE) demonstrated no significant effects of SPS ($F_{1,144} = 3.091$, $P = 0.081$) or treatment ($F_{7,144} = 1.654$, $P = 0.125$).

These results demonstrated that SPS increased anxiety-like behaviors in rats, which blocked by morphine administration, particularly 24 h after the SPS.

3.2. Contextual fear conditioning and extinction

Extinction data are shown in Fig. 3A-D. A one-way ANOVA analysis on freezing scores during the initial 3 min period (data not shown) in the conditioning chamber before the presentation of the first foot shock showed no significant differences among the groups ($F_{17,164} = 0.194$, $P > 0.05$), which shows no differences in novelty-induced exploratory behavior between sham and SPS rats. Also, the analysis of the freezing scores during one min (data not shown) after the last foot shock, a measure of the acquisition of contextual fear conditioning, demonstrated no significant differences among the groups ($F_{17,164} = 0.473$, $P > 0.05$), which shows no differences in contextual fear conditioning acquisition between the SPS and Sham rats.

When morphine was administered immediately after SPS (Fig. 3A), mixed ANOVA (groups × days (4 × 5)) revealed main significant effects of groups ($F_{3,180} = 17.850$, $P < 0.001$) and days ($F_{4,180} = 55.024$, $P < 0.001$) but not an interaction between groups and days ($F_{12,180} = 1.410$, $P > 0.05$). Post-hoc analysis indicated that percentage of freezing time of the SPS + Saline 0 h was significantly higher than the Sham + Saline 0 h in the extinction 4 ($P < 0.01$) and extinction 5 ($P < 0.05$), but no differences were found between the SPS + Morphine 0 h and the SPS + Saline 0 h groups. One - way ANOVA revealed that the extinction occurred across the multiple extinction tests in the Sham + Saline 0 h group ($F_{4,54} = 20.320$, $P < 0.001$). Post-hoc analysis indicated significant differences in the freezing scores of the extinction 1 with the extinction 2 ($P < 0.05$), and extinction 3–5 (all, $P < 0.001$). The differences in the freezing scores between the extinction 2 and extinction 4 ($P < 0.05$), and extinction 2 and extinction 5 ($P < 0.001$) and the extinction 3 and extinction 5 ($P < 0.05$) were significant (Fig. 3A).

When morphine was administered 6 h after SPS (Fig. 3B), mixed ANOVA (groups × days (4 × 5)) revealed main significant effects of groups ($F_{3,185} = 55.556$, $P < 0.001$), days ($F_{4,185} = 61.223$, $P < 0.001$) and an interaction between groups and days ($F_{12,185} = 2.381$, $P < 0.01$). Post-hoc analysis indicated that percentage of freezing time of the SPS + Saline 6 h was significantly higher than the Sham + Saline 6 h in the extinction 4 ($P < 0.01$) and extinction 5

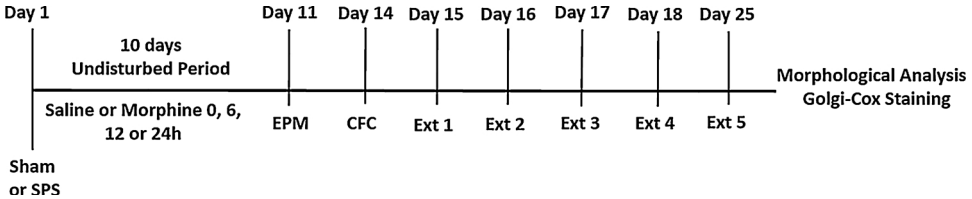

Fig. 1. Timeline of the experiment (see Martials and Methods for more detail).

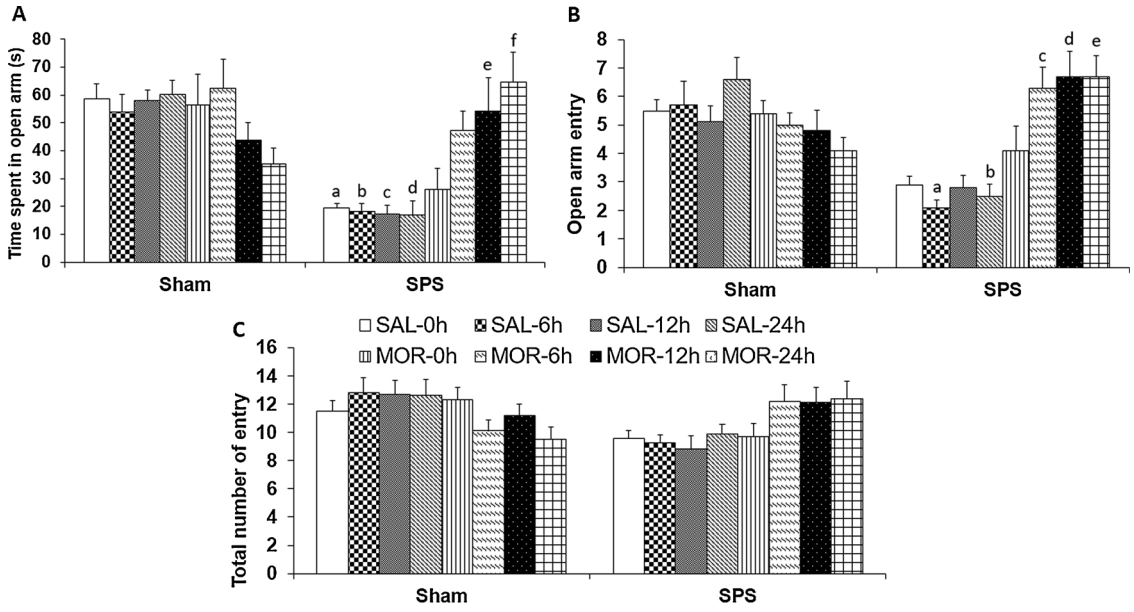

Fig. 2. Anxiety-like behaviors were assessed in the EPM. (A) Time spent in open arm, (B) open arm entry and (C) total arm entry as a measure of spontaneous locomotor activity. In (A); a: $P < 0.05$ than the Sham + SAL 0 h group; b: $P < 0.05$ than the Sham + SAL 6 h group; c: $P < 0.01$ than the Sham + SAL 12 h group; d: $P < 0.01$ than the Sham + SAL 24 h group; e: $P < 0.05$ than the SPS + SAL 12 h group and f: $P < 0.001$ than the SPS + SAL 24 h group. In (B); a: $P < 0.01$ than the Sham + SAL 6 h group, b: $P < 0.01$ than the Sham + SAL 24 h group; c: $P < 0.01$ than the SPS + SAL 6 h group, d: $P < 0.01$ than the SPS + SAL 12 h group and e: $P < 0.001$ than the SPS + SAL 24 h group. Data represent the mean ± SEM.

($P < 0.001$). The freezing response in the SPS + Morphine 6 h group was lower than the SPS + Saline 6 h group in the extinction 5 ($P < 0.01$). Moreover, to examine the occurrence of extinction across extinction tests in the Sham + Saline 6 h group, a one-way ANOVA on the extinction data revealed significant effects of extinctions days ($F_{4,39} = 50.356$, $P < 0.001$). Post-hoc analysis indicated a significant difference in the percentage of freezing time between the extinction 1 and the extinctions 2–5 (all, $P < 0.001$) tests. Significant differences in the freezing scores were also found between the extinction 2 and extinctions 4–5 (both, $P < 0.001$), and the extinction 3 and extinction 5 ($P < 0.001$) sessions (Fig. 3B).

When morphine was administered 12 h after SPS (Fig. 3C), mixed ANOVA (groups × days (4 × 5)) revealed main significant effects of groups ($F_{3,190} = 46.159$, $P < 0.001$), days ($F_{4,190} = 97.518$, $P < 0.001$) and an interaction between groups and days ($F_{12,190} = 3.193$, $P < 0.01$). Post-hoc analysis indicated that percentage of freezing time of the SPS + Saline 12 h was significantly higher than the Sham + Saline 12 h group in the extinction 3 ($P < 0.05$), extinction 4 ($P < 0.01$) and extinction 5 ($P < 0.001$). The freezing response in the SPS + Morphine 12 h group was lower than the SPS + Saline 12 h

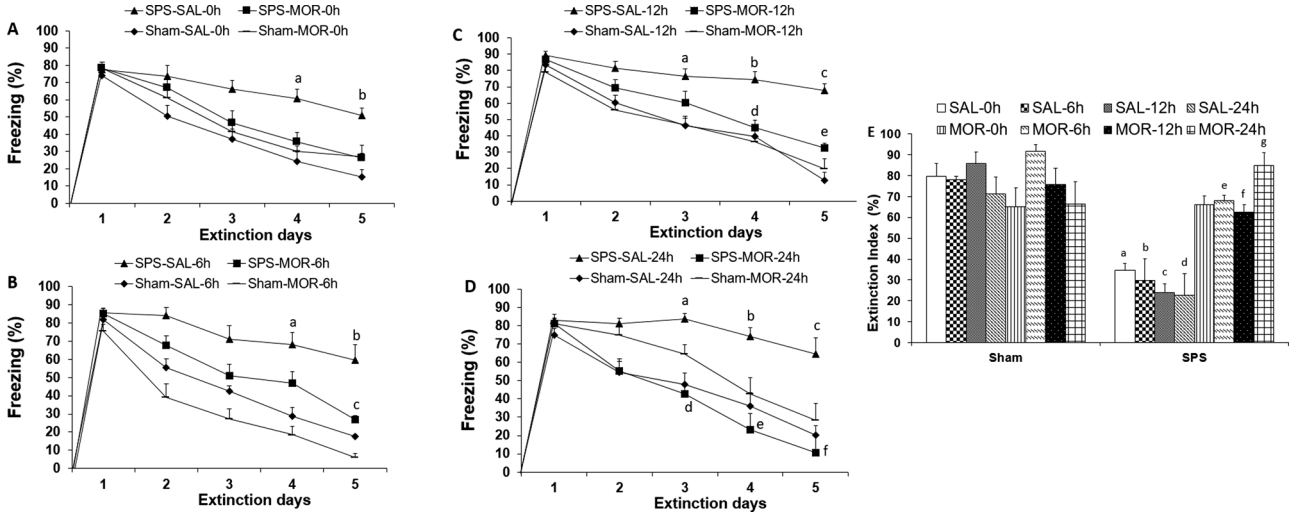

Fig. 3. Effects of morphine injection in different time points following the SPS on subsequent contextual fear extinction in sham and SPS animals. In A: a:$P < 0.01$ and b:$P < 0.05$ than the Sham + Saline 0 h group. In B: a:$P < 0.01$ and b:$P < 0.05$ compared with the Sham + Saline 6 h group. c:$P < 0.01$ compared with the SPS + Saline 6 h animals. In C: a:$P < 0.05$, b:$P < 0.01$ and 5 c:$P < 0.001$ compared with the Sham + Saline 12 h group; d:$P < 0.05$ and e:$P < 0.01$ compared with the SPS + Saline 12 h animals. In D: a:$P < 0.001$, b:$P < 0.001$ and c:$P < 0.001$ compared with the Sham + Saline 24 h group; d:$P < 0.001$, e: $P < 0.001$ and f: $P < 0.001$ compared with the SPS + Saline 24 h animals. In E: Extinction index; a:$P < 0.01$ than the Sham + Saline 0 h group, b:$P < 0.01$ than the Sham + Saline 6 h group, c:$P < 0.001$ than the Sham + Saline 12 h group and d:$P < 0.001$ than the Sham + Saline 24 h group, e:$P < 0.05$ than the SPS + Saline 6 h, f: $P < 0.01$ than the SPS + Saline 12 h and g:$P < 0.001$ than the SPS + Saline 24 h. Data represent the mean ± SEM. SAL: Saline, MOR: Morphine, SPS: Single prolonged stress.

groups in the extinction 4 ($P < 0.05$) and extinction 5 ($P < 0.01$). A one-way ANOVA revealed that extinction occurred across multiple extinction tests in the Sham + Saline 12 h group ($F_{4,54} = 26.488$, $P < 0.001$). Post-hoc analysis indicated significant differences in the percentage of freezing between the extinction 1 and the extinction 2 ($P < 0.05$), and extinction 3–5 (all, $P < 0.001$) tests. Significant differences also were found between the extinction 2 and extinction 5 ($P < 0.001$), the extinction 3 and extinction 5 ($P < 0.001$), and the extinction 4 and extinction 5 ($P < 0.01$) tests (Fig.3C).

When morphine was administered 24 h after SPS (Fig. 3D), mixed ANOVA (groups × days (4 × 5)) revealed main significant effects of groups ($F_{3,190} = 30.610$, $P < 0.001$), days ($F_{4,190} = 38.722$, $P < 0.001$) and an interaction between groups and days ($F_{12,190} = 2.264$, $P < 0.01$). Post-hoc analysis indicated that percentage of freezing time of the SPS + Saline 24 h was significantly higher than the Sham + Saline 24 h in the extinction 3 ($P < 0.01$), extinction 4 ($P < 0.05$) and extinction 5 ($P < 0.01$). The freezing response of the SPS + Morphine 24 h group was lower than the SPS + Saline 24 h groups in the extinction 3 ($P < 0.001$), extinction 4 ($P < 0.001$) and extinction 5 ($P < 0.001$). An ANOVA revealed that extinction occurred in the Sham + Saline 24 h group across the multiple extinctions tests ($F_{4,54} = 10.872$, $P < 0.001$). Post-hoc analysis showed significant differences in the percentage of freezing time between the extinction 1 with the extinction 3 ($P < 0.05$), and extinctions 4–5 (both, $P < 0.01$), and the extinction 2 and the extinction 5 ($P < 0.01$) and the extinction 3 and extinction 5 ($P < 0.05$) (Fig. 3D).

Extinction index data are shown in Fig. 3E. A two-way ANOVA analysis demonstrated main significant effects of SPS ($F_{1,149} = 63.997$, $P < 0.001$) and treatment ($F_{7,149} = 5.665$, $P < 0.001$) and an interaction between SPS and treatment ($F_{7,149} = 8.415$, $P < 0.001$). Post hoc analysis demonstrated that the percentage of extinction was significantly higher in the Sham + Saline 0 h than SPS + Saline 0 h ($P < 0.01$), Sham + Saline 6 h than SPS + Saline 6 h ($P < 0.01$), Sham + Saline 12 h than SPS + Saline 12 h ($P < 0.001$) and Sham + Saline 24 h than SPS + Saline 24 h groups ($P < 0.001$). The percentage of extinction was significantly higher in the SPS + Morphine 6 h than SPS + Saline 6 h ($P < 0.05$), SPS + Morphine 12 h than SPS + Saline 12 h ($P < 0.01$) and SPS + Morphine 24 h than SPS + Saline 24 h ($P < 0.001$) groups.

These findings together that SPS impaired contextual fear extinction and morphine administration 24 h after SPS had a protective effect against SPS-induced impairment in fear extinction.

Fig. 4A showed the effects of naloxone pretreatment on morphine influences on extinction response in SPS rats. Data of SPS + Saline 24 h and SPS + Morphine 24 h groups from previous experiment were included in this analysis. A two-way repeated measure ANOVA analysis revealed main significant effects of extinction ($F_{4,152} = 59.862$, $P < 0.001$) and treatment ($F_{3,38} = 23.216$, $P < 0.001$), an interaction between extinctions and treatment ($F_{12,152} = 7.686$, $P < 0.001$). Post hoc analysis indicated that the percentage of freezing time of the SPS + Morphine 24 h group was significantly lower than the SPS + Saline 24 h in the extinction 2 ($P < 0.01$), extinction 3 ($P < 0.001$), extinction 4 ($P < 0.001$) and extinction 5 ($P < 0.01$). The percentage of freezing time of the SPS + Naloxone + Morphine 24 h group was significantly higher than the SPS + Morphine 24 h in the extinction 2 ($P < 0.01$), extinction 3 ($P < 0.001$), extinction 4 ($P < 0.001$) and extinction 5 ($P < 0.01$).

Extinction index data are shown in Fig. 4B. A one-way ANOVA analysis showed significant differences among groups ($F_{3,36} = 16.192$, $P < 0.001$). Post hoc analysis indicated that the percentage of extinction was significantly higher in the SPS + Morphine 24 h than the SPS + Saline 24 h ($P < 0.001$), but it was significantly lower in the SPS + Naloxone + Morphine 24 h ($P < 0.01$) than the SPS + Morphine 24 h ($P < 0.001$).

These findings indicate that the protective effects of morphine against SPS - induced fear extinction deficits are mediated through via opioid receptors.

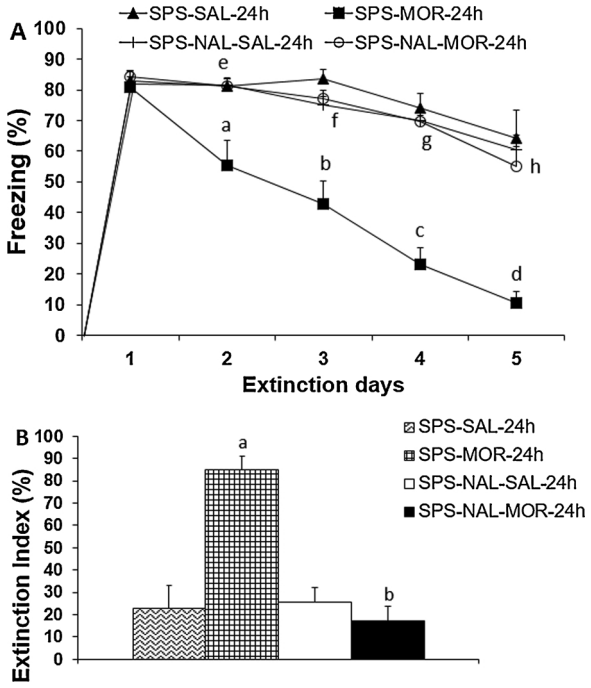

Fig. 4. Naloxone pretreatment blocks morphine effects on fear extinction in SPS rats. (A); a: $P < 0.01$, b: $P < 0.001$, c: $P < 0.001$ and d: $P < 0.001$ compared with the SPS + Saline animals. e: $P < 0.01$, f: $P < 0.001$, g: $P < 0.001$ and h: $P < 0.001$ compared with the SPS + Morphine 24 h group. (B); Extinction index; a: $P < 0.001$ compared with the SPS + Saline 24 h group, b: $P < 0.001$ compared with the SPS + Morphine 24 h group. Data represent the mean ± SEM. SAL: Saline, MOR: Morphine, NAL: Naloxone; SPS: Single prolonged stress.

3.3. Morphological analysis

Data of dendritic length of neurons in the IL are shown in Fig. 5A. A two-way ANOVA analysis on dendritic length of neurons showed main significant effects of SPS ($F_{1,80} = 365.3307$, $P < 0.001$) and treatment ($F_{7,80} = 760.6946$, $P < 0.001$). Furthermore, a significant interaction between SPS and treatment ($F_{7,80} = 133.888$, $P < 0.001$) was shown. Post hoc analysis showed that dendritic length of the IL pyramidal neurons was significantly decreased in the SPS + Saline 0 h than the Sham + SAL 0 h ($P < 0.001$), SPS + Saline 6 h than the Sham + SAL 6 h ($P < 0.001$), SPS + Saline 12 h than the Sham + SAL 12 h ($P < 0.001$) and SPS + Saline 24 h than the Sham + SAL 24 h ($P < 0.001$). Dendritic length of the IL neurons increased in the SPS + Morphine 0 h than the SPS + SAL 0 h ($P < 0.001$), SPS + Morphine 6 h than the SPS + SAL 6 h ($P < 0.001$), SPS + Morphine 12 h than the SPS + SAL 12 h ($P < 0.001$) and SPS + Morphine 24 h than the SPS + SAL 24 h ($P < 0.001$). The same effect was shown between the Sham + Morphine 0 h than the Sham + SAL 0 h ($P < 0.001$), Sham + Morphine 6 h than the Sham + SAL 6 h ($P < 0.001$), Sham + Morphine 12 h than the Sham + SAL 12 h ($P < 0.001$) and Sham + Morphine 24 h than the Sham + SAL 24 h ($P < 0.001$).

Data from dendritic spines of the IL neurons are shown in Fig. 5B. A two-way ANOVA analysis on dendritic spines showed main significant effects of SPS ($F_{1,80} = 110.360$, $P < 0.001$), treatment ($F_{7,80} = 84.479$, $P < 0.001$) and a significant interaction between SPS and treatment ($F_{7,80} = 2.561$, $P < 0.05$). Post hoc analysis showed that dendritic spines of the IL neurons were significantly decreased the SPS + Saline 0 h than the Sham + SAL 0 h ($P < 0.001$), SPS + Saline 6 h than the Sham + SAL 6 h ($P < 0.01$), SPS + Saline 12 h than the

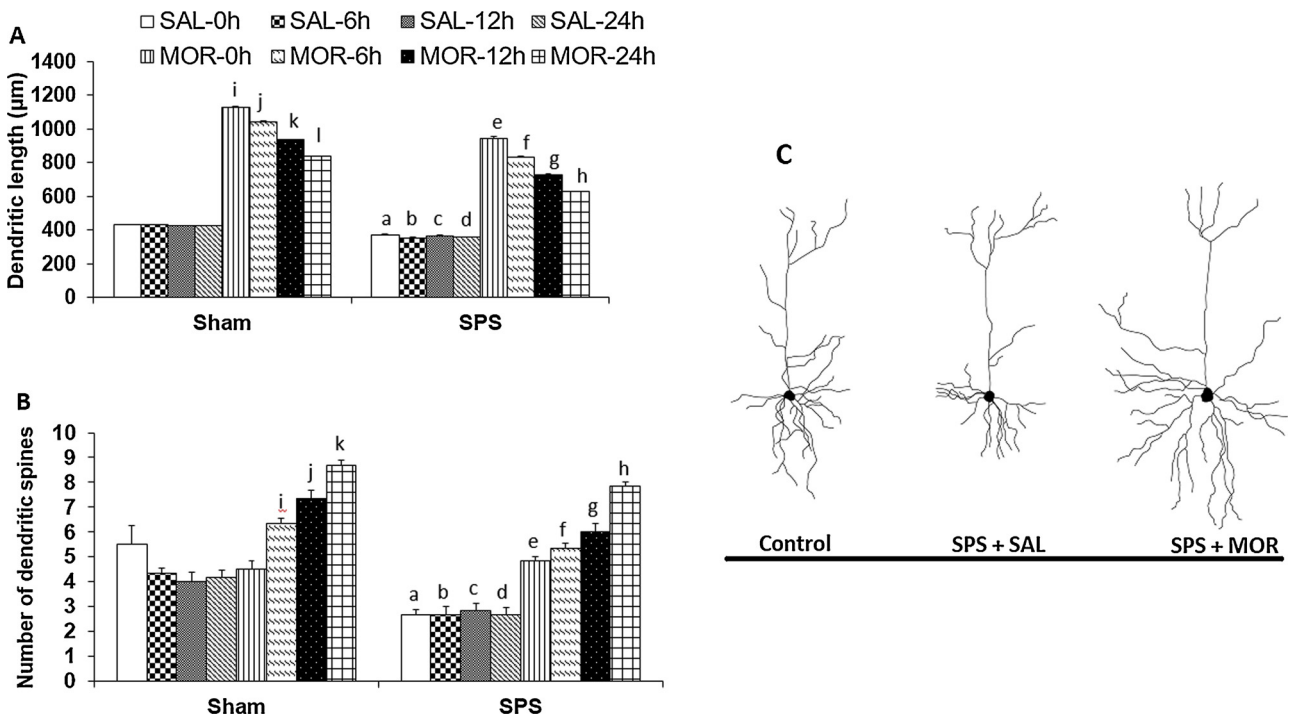

Fig. 5. Dendritic length and spines of neurons in the infralimbic (IL) of the medial prefrontal cortex in sham and SPS rats in the presence and absence of morphine. (A); Dendritic length of neurons in the IL; a: $P < 0.001$ than the Sham + SAL 0 h group, b: $P < 0.001$ than the Sham + SAL 6 h group c: $P < 0.001$ than the Sham + SAL 12 h group and d: $P < 0.001$ than the Sham + SAL 24 h group, e: $P < 0.001$ than the SPS + SAL 0 h group, f: $P < 0.001$ than the SPS + SAL 6 h group, g: $P < 0.001$ than the SPS + SAL 12 h, h: $P < 0.001$ than the SPS + SAL 24 h group, i: $P < 0.001$ than the Sham + SAL 0 h group, *j*: $P < 0.001$ than the Sham + SAL 6 h group, k: $P < 0.001$ than the Sham + SAL 12 h group and l: $P < 0.001$ than the Sham + SAL 24 h group. (B); Dendritic spines of neurons in the IL a: $P < 0.001$ than the Sham + SAL 0 h group, b: $P < 0.01$ than the Sham + SAL 6 h group, c: $P < 0.05$ than the Sham + SAL 12 h group, d: $P < 0.05$ than the Sham + SAL 24 h group, e: $P < 0.001$ than the SPS + SAL 0 h group, f: $P < 0.001$ than the SPS + SAL 6 h group, g: $P < 0.001$ than the SPS + SAL 12 h group, h: $P < 0.001$ than the SPS + SAL 24 h group, i: $P < 0.001$ than the Sham + Saline 6 h, *j*: $P < 0.001$ than the Sham + Saline 12 h and k: $P < 0.001$ than the Sham + Saline 24 h. C; Camera lucida drawings of representative Golgi-impregnated IL pyramidal neurons from control, SPS + saline, and SPS + morphine rats. SAL: Saline, MOR: Morphine, SPS: Single prolonged stress.

Sham + SAL 12 h ($P < 0.05$), SPS + Saline 24 h than the Sham + SAL 24 h ($P < 0.05$). Moreover, dendritic length of the IL neurons increased in the SPS + Morphine 0 h than the SPS + SAL 0 h ($P < 0.001$), SPS + Morphine 6 h than the SPS + SAL 6 h ($P < 0.001$), SPS + Morphine 12 h than the SPS + SAL 12 h ($P < 0.001$) and SPS + Morphine 24 h than the SPS + SAL 24 h ($P < 0.001$). The same effect was shown between the Sham + Morphine 6 h than the Sham + SAL 6 h ($P < 0.001$), Sham + Morphine 12 h than the Sham + SAL 12 h ($P < 0.001$) and Sham + Morphine 24 h than the Sham + SAL 24 h ($P < 0.001$). Representative camera lucida drawings of Golgi-impregnated PL pyramidal neurons from control, SPS + saline and SPS + morphine rats are depicted in Fig. 5C.

Data from dendritic length of neurons in the PL are shown in Fig. 6A. A two-way ANOVA showed main significant effects of SPS ($F_{1,80} = 477.267$, $P < 0.001$) and treatment ($F_{7,80} = 134.5304$, $P < 0.001$). Moreover, a significant interaction between SPS and treatment ($F_{7,80} = 65.210$, $P < 0.001$) was shown. Between groups comparisons revealed the dendritic length of the PL neurons was increased in the SPS + Morphine 0 h than the SPS + SAL 0 h ($P < 0.001$), SPS + Morphine 6 h than the SPS + SAL 6 h ($P < 0.001$), SPS + Morphine 12 h than the SPS + SAL 12 h ($P < 0.001$) and SPS + Morphine 24 h than the SPS + SAL 24 h ($P < 0.001$). The same effect was found between the Sham + Morphine 0 h than the Sham + SAL 0 h ($P < 0.001$), Sham + Morphine 6 h than the Sham + SAL 6 h ($P < 0.001$), Sham + Morphine 12 h than the Sham + SAL 12 h ($P < 0.001$) and Sham + Morphine 24 h than the Sham + SAL 24 h ($P < 0.001$).

Data from dendritic spines of neurons in the PL are shown in Fig. 6B. A two-way ANOVA showed main significant effects of SPS ($F_{1,80} = 134.140$, $P < 0.001$) and treatment ($F_{7,80} = 77.618$, $P < 0.001$). Also, a significant interaction between SPS and treatment ($F_{7,80} = 2.996$, $P < 0.01$) was seen. Post hoc analysis showed that dendritic spines of neurons were significantly decreased in the SPS + Saline 0 h than the Sham + SAL 0 h ($P < 0.001$), SPS + Saline 6 h than the Sham + SAL 6 h ($P < 0.001$), SPS + Saline 12 h than the Sham + SAL 12 h ($P < 0.001$), and SPS + Saline 24 h than the Sham + SAL 24 h ($P < 0.05$). Moreover, dendritic spines of neurons in the PL was increased in the SPS + Morphine 0 h than the SPS + SAL 0 h ($P < 0.01$), SPS + Morphine 6 h than the SPS + SAL 6 h ($P < 0.001$), SPS + Morphine 12 h than the SPS + SAL 12 h ($P < 0.001$) and SPS + Morphine 24 h than the SPS + SAL 24 h ($P < 0.001$). The same effect was shown between the Sham + Morphine 6 h than the Sham + SAL 6 h ($P < 0.01$), Sham + Morphine 12 h than the Sham + SAL 12 h ($P < 0.001$) and Sham + Morphine 24 h than the Sham + SAL 24 h ($P < 0.001$). Representative camera lucida drawings of Golgi-impregnated PL pyramidal neurons from control, SPS + saline and SPS + morphine rats are depicted in Fig. 2 6C.

These data showed that SPS decreased both dendritic length and spines in the IL and dendritic length in the PL regions of the mPFC. Morphine injection, particularly 24 h after SPS, significantly blocked the effect of SPS on dendritic morphology of the pyramidal neurons in the mPFC.

4. Discussion

The present study investigated the therapeutic effects of morphine injected at different time points (immediately, 6, 12 and 24 h) after SPS

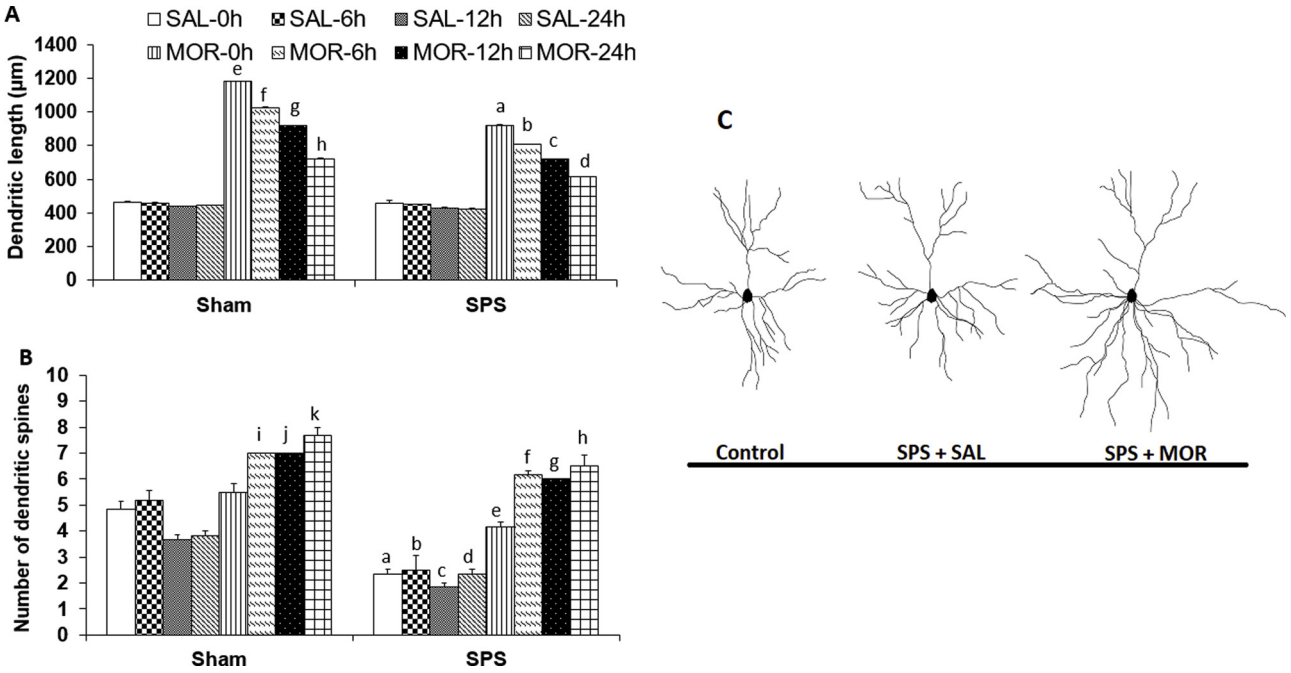

Fig. 6. Dendritic length and spines of neurons in the prelimbic (PL) of the medial prefrontal cortex in sham and SPS rats in the presence and absence of morphine.. (A); Dendritic length of neurons in the PL; a: $P < 0.001$ than the SPS + SAL0 h group, b: $P < 0.001$ than the SPS + SAL 6 h, c: $P < 0.001$ than the SPS + SAL 12 h and d: $P < 0.001$ than the SPS + SAL 24 h e: $P < 0.001$ than the Sham + SAL 0 h group, f: $P < 0.001$ than the Sham + SAL 6 h, g: $P < 0.001$ than the Sham + SAL 12 h and h: $P < 0.001$ than the Sham + SAL 24 h.(B); Dendritic spines of neurons in the PL; a: $P < 0.001$ than the Sham + SAL 0 h, b: $P < 0.001$ than the Sham + SAL 6 h, c: $P < 0.001$ than the Sham + SAL 12 h, d: $P < 0.001$ than the Sham + SAL 24 h, e: $P < 0.001$ than the SPS + SAL 0 h, f: $P < 0.001$ than the SPS + SAL 6 h, g: $P < 0.001$ than the SPS + SAL 12 h, h: $P < 0.001$ than the SPS + SAL 24 h group. i: $P < 0.01$ than the Sham + Saline 6 h, *j*: $P < 0.001$ than the Sham + Saline 12 h and k: $P < 0.001$ than the Sham + Saline 24 h. C; Camera lucida drawings of representative Golgi-impregnated PL pyramidal neurons from control, SPS + saline and SPS + morphine rats. SAL: Saline; MOR: Morphine, SPS: Single prolonged stress.

as an experimental model of PTSD in adult male rats. We found that the saline-treated SPS rats showed enhanced anxiety-like behaviors, impaired fear extinction, and dendritic retraction in the mPFC. Morphine injection particularly 24 h after the SPS selectively reversed the SPS-induced deficits in anxiety profile, fear extinction, and dendritic morphology in the mPFC. Taken together, it is reasonable to conclude that morphine had a protective and therapeutic effect on SPS rats.

4.1. SPS impairs contextual fear extinction and increases anxiety-like behaviors: Beneficial effects of morphine

In terms of the fear extinction, predictably, all groups had a similar and high percentage of freezing time in the early extinction tests, because during training, conditioned stimulus (CS) was paired to noxious unconditioned stimulus (US) in all animals. As a result, the percentage of freezing time was high on the first and/or second days after training. But after a while, the CS−US connection was gradually unpaired in the sham groups which represents unimpaired fear extinction in control animals. Consequently, the percentage of freezing time of the control groups dropped with a steep slope during following the remaining extinction tests of the experiment. On the contrary, the percentage of freezing time did not decrease in the saline-treated SPS groups even until the last day of the experiment. In addition, the extinction index of the saline-treated SPS animals was significantly lower than the sham groups. These findings show the impairment of fear extinction in SPS rats, and are in line with previous studies reporting that SPS impairs contextual fear conditioning [46]. In agreement with the previous findings, anxiety-like behaviors significantly increased in the saline-treated SPS rats than the sham groups [47]. Morphine-treated SPS animals showed an increased percentage of open arm time and entry compared with the saline-treated SPS groups. Morphine-treated SPS rats showed an extinction curve with a slope like the sham animals. Interestingly, the protective effect of morphine was time-dependent, and the most impressive effect of morphine was seen when injected 24 h after SPS. Our findings confirm the results of a recent study showing that a single dose of morphine reduces stress-enhanced fear learning (an animal model of the PTSD) when administrated 48 h after the severe stressor [20]. The observed time-dependent effect of morphine against SPS induced impairments in fear extinction is interesting and strange. Some clinical studies were mentioned earlier suggested that one interpretation of the protective effect of morphine on PTSD is probably due to analgesic effect of morphine and decreased in the pain experience [12,48]. If that was the case, why the present study did not show the most impressive effect of morphine immediately after the SPS that the analgesic effect of morphine would most be potent? The protective effect of morphine almost was not seen in the SPS + morphine 0 h group and was much weaker in the SPS + morphine 6 h group than the SPS + morphine 12 h and SPS + morphine 24 h group. In addition, morphine effect was weaker in the SPS + morphine 12 h than the SPS + morphine 24 h group. Finally, the protective effect of morphine was disappeared when naloxone was injected 30 min prior to morphine. Thus, it is reasonable to conclude that the protective effect of morphine might be mediated via its opioid receptors [19,49].

The mechanisms underlying the protective effects of morphine are not known. We found that the protective effect of morphine against SPS appeared when morphine was injected from 6 h after the SPS. Interestingly, the protective effect of morphine was stronger and appeared sooner when morphine was injected 24 h after the SPS. Importantly, morphine half-life is about 2–4 h. Moreover, it takes, at least, 7–14 days to develop the SPS symptoms [30]. So the question is how does morphine do this protective effect on the SPS animals? It is more likely that morphine targeted the early biochemical, cellular and neural mechanisms critical for PTSD development in the later. One important process is memory consolidation during which the gene expression and protein synthesis work in parallel pathways to consolidate the traumatic information experienced during the SPS. Morphine may

act via its receptors to inhibit the consolidation process of the traumatic event [50]. Interestingly, an immediate injection of morphine was not effective, confirming our assumption that morphine targets the consolidation process which starts at least 3–6 h after the traumatic event and lasts over the next 24 h. 24 h post training is a critical time point in terms of consolidation process and extinction of the fear memory [51]. But, the question remains, how does morphine affect the brain areas responsible for fear memory? Plastic events in the amygdala have been suggested as a key factor in the extinction process [52]. It seems that CS information projects from basolateral amygdala (BLA) to intercalated (ITC) amygdala neurons [53,54], which send inhibitory GABAergic projections to the central amygdala (CeA) [55,56]. ITC has been proposed as the main part in controlling the flow of information between the BLA and CeA. Regarding conditioned fear responses, CeA is the main part of the amygdala [57]. The IL sends projections to ITC that inhibits CeA [58,59] and accelerates extinction [60]. Recent studies showed an increased responsiveness of the IL neurons after extinction training [60,61]. Altogether, it would be possible that soon after receiving a conditioned stimulus, CS-related inputs from the BLA and IL integrate in ITC neurons. As a result, the GABAergic projection of ITC dampens the output of the CeA and leads to a reduction of fear responses. Previous western blot studies showed that morphine increases expression of the mu receptors in the amygdala. The high amount of the mu receptors are localized in the ITC [52,62]. Likhtik et al., reported that ITC lesions caused remarkable deficits in the expression of extinction [52], so it is reasonable to conclude that morphine stimulates the mu receptors in the ITC, which increases ITC inhibitory projection to CeA, culminating a less fear response and freezing. Further studies are required to test this assumption.

4.2. SPS decreases dendritic length and spines in the mPFC: Beneficial effects of morphine

We found that SPS and control animals differ in the dendritic architecture of mPFC neurons, which form the main part of the neural circuitry of extinction [63,64]. SPS decreased dendritic length and spines in the IL area and dendritic length in the PL region, showing that SPS-induced changes in neuronal morphology of the mPFC are mainly limited to the IL, but not the PL. This finding is very interesting and is in line with a previous work [65]. In terms of stress, the IL is the most sensitive part of the mPFC. Instead, the PL might be more sensitive to prolonged stress and probably involved with conditioned fear expression [66]. Surprisingly, Knox et al. reported the high PL neural activity in all animals including sham rats during extinction testing, since, except the SPS animals the levels of conditioned fear were low in the rest of the rats [67]. Some studies have shown the low PL neural activity during extinction testing [68], and some others have reported the high PL activity [69]. The IL sends an inhibitory projection to CeA and plays a critical role in extinction. In fact, inhibition of the BLA neural activity by IL is a critical part of extinction [70,71]. Thus, it would be possible that dendritic retraction of the IL could attenuate IL inhibitory function on BLA and ITC and contribute to impaired extinction in the SPS rats that needs further investigation.

Morphologically, our findings show morphine significantly increased dendritic spines in the mPFC in both sham and SPS rats. The effect of morphine was impressive when injected 24 h after the SPS. Morphine also increased dendritic length in the IL and PL in both sham and SPS rats. This effect of morphine was more remarkable when injected immediately after the SPS. These different pattern and time-point effects of morphine on the dendritic length and spines are very interesting and need to be further explored. Importantly, morphine has pronounced effects in both sham and stress conditions. Thus, it is not clear whether the effect of morphine alone is protective against effects of the SPS or simply has effects that mask the appearance of the stress effect. In other words, does morphine prevent the effect of stress on dendrites or provide an effect by itself that masks the appearance of the stress effect. The explanation of these unanswered questions and the reason of the time-dependent effects of morphine in both sham and SPS rats need further carefully designed studies.

The mPFC plays a critical role in the regulation of emotional behaviors and fear extinction [70,71]. The present study showed that the SPS led to dendritic retractions and loss in the mPFC; these changes appear to be the morphological correlate of SPS - induced impairment of the mPFC-dependent behavior(s). The present study showed that morphine administration after the SPS recovered stress induced behavioral deficits and structural abnormalities in the mPFC.

4.3. Possible intracellular pathways of beneficial effects of morphine on SPS induced behavioral and morphological impairments

Recent studies have reported some new pharmacological approaches to PTSD treatment including NMDA receptor agonists such as D-cycloserine and brain-derived neurotrophic factor (BDNF) agonists [72,73]. Administration of NMDA receptor antagonists impairs extinction retention showing that NMDA receptors are involved in fear memory extinction [74,75]. Morphine positively modulates NMDA receptors. Indeed, the interaction between mu and NMDA receptors is bidirectional [76]. Chen et al. and Heinricher et al. have reported sustained glutamate-activated NMDA receptors currents by activation of mu receptors [77,78]. Morphine may act via NMDA receptors and change long-term potentiation (LTP). LTP may underlie synaptic plasticity in the hippocampus and amygdala during fear extinction [79,80]. Moreover, BDNF has been reported to be involved in LTP probably through its interaction with TrkB [81,82]. Morphine elevates BDNF mRNA in the brain subregions such as locus coeruleus, ventral tegmental area, mPFC and amygdala [83–85]. It is reasonable to conclude that morphine may affect LTP via NMDA and BDNF and the related intracellular signaling pathways of LTP, leading to increased fear extinction.

Morphine may act via inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) that have been associated with depression, anxiety and PTSD pathogenesis [86,87]. Transgenic animals with deficient pro-inflammatory cytokines showed altered anxiety-like behavior in the EPM and open field tests [88,89]. Spivak et al. have reported an elevated level of IL-1β in PTSD patients [86]. Jones et al. have shown that severe stressor induces IL-1β expression in the dorsal hippocampus [90]. They have alleged that systemic morphine treatment reduces IL-1β expression in the dorsal hippocampus, one of the brain regions critical for extinction retention [67]. It is accepted that opioid receptors regulate the development of fear extinction [50]. Administration of opioid antagonists increase conditioned fear [91], and, the present study showed that beneficial effect of morphine on fear extinction of the SPS animals was disappeared when naloxone was injected before morphine. Even microinjection of morphine into the amygdala diminished the expression of fear responses. Opioid signaling in the ventrolateral periaqueductal gray matter has been proposed to regulating conditioned fear extinction [92,93]. Conversely, Parsons et al, have emphasized the role of NMDA but not opioid receptors in the amygdala for fear conditioning extinction [93]. Miller et al., have reported that the binding of morphine to its mu receptors time-dependently changes AMPA receptor subunit composition and Ca^{2+} dynamics, which lead to morphine-induced alteration in dendritic spines amount [94]. However, there is a need for more studies to determine the likely mechanisms by which morphine prevents the development of PTSD.

Briefly, we found that SPS gave rise to more anxiety-like behaviors and impaired fear extinction in male rats. SPS decreased dendritic length and spines of neurons in the mPFC. Conversely, morphine administration within a restricted time window selectively improved anxiety-like behaviors, fear extinction, and increased dendritic length, and spines in the mPFC. Our findings suggest that the time point of morphine injection following a traumatic event is an important

determinant of the full therapeutic effect of morphine against PTSD.

Conflict of interest

The authors report no biomedical financial interests or potential conflicts of interest about this work.

Author's contributions

P.R.A. and A.R.P. designed the overall study and wrote the paper. P.R.A., A. A.V, A.G and M.D collected data and carried out the lab work. P.R.A. and A.R.P. carried out the statistical analysis and mostly drafted the manuscript. A.R.P. coordinated and supervised the study. All authors approved the manuscript.

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