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Activation of the medial prefrontal cortex by escapable stress is necessary for protection against subsequent inescapable stress-induced potentiation of morphine conditioned place preference

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Abstract

Stress can be a predisposing factor in the development of psychiatric disorders. However, not all individuals develop psychiatric disorders following a traumatic event. An attempt to understand these individual differences has led to a focus on factors that produce resistance. Interestingly, in rats, an experience with escapable tailshock (ES) before inescapable tailshock (IS) prevents the typical anxiety-like behavioral outcomes of IS. This type of resistance has been termed “behavioral immunization” and it depends on activation of the medial prefrontal cortex (mPFC) during ES. However, one outcome of IS that is not anxiety-related is potentiation of morphine conditioned place preference (CPP). The present experiments investigated whether prior ES would block IS-induced potentiation of morphine CPP. Rats received either ES, IS, or homecage control treatment on Day 1 and then either IS or homecage on Day 2. Twenty-four hours following Day 2, rats underwent morphine conditioning and CPP was subsequently assessed. In a second experiment, rats received ES 3, 14, or 56 days prior to IS to determine the duration of behavioral immunization. In a final experiment, rats were microinjected with the GABA_A agonist muscimol (50 ng/ 0.5 µl) or saline in the mPFC before Day 1 of stress. Prior ES blocked IS-induced potentiation of morphine CPP. This immunizing effect of ES lasted for at least 56 days. Additionally, intra-mPFC muscimol during ES prevented behavioral immunization. These results suggest that prior experience with ES activates the mPFC and produces long-lasting neural alterations that block subsequent IS-induced potentiation of morphine CPP.

Keywords

resilience; addiction; serotonin; learned helplessness; rat

Introduction

Exposure to stressors can augment the rewarding properties of drugs of abuse in both animals and humans (Lu *et al.*, 2003; Ouimette *et al.*, 2007; Briand & Blendy, 2010; George & Koob, 2010). We have previously reported that a single session of inescapable tailshock

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(IS) potentiates morphine conditioned place preference (CPP) as compared to homecage (HC) controls (Will et al., 1998; Der-Avakian et al., 2005). Moreover, potentiation of morphine CPP occurs even if the IS precedes CPP training by a number of days and occurs in a different environment than the CPP environment (Will et al., 1998). Interestingly, exactly equal escapable tailshock (ES) does not potentiate morphine CPP (Will et al., 1998; Rozeske et al., 2009). Thus, only the behaviorally uncontrollable stressor potentiated morphine CPP in this enduring trans-situational fashion.

There has been a growing interest in the factors that promote stress-resistance/resilience (Charney, 2004; Agaibi & Wilson, 2005; Lyons & Parker, 2007; Wingo *et al.*, 2010). This issue has been primarily pursued with regard to the effects of stressors on processes and behaviors other than drug reward (e.g. anxiety-related behaviors). A variety of manipulations have been shown to blunt the impact of subsequent stressors. For example, voluntary exercise reduces the behavioral impact of later exposure to an uncontrollable stressor (Greenwood et al., 2003). In this context it is interesting to note that experience with behavioral control over a stressor (ES) also blocks several behavioral (reduced juvenile social exploration, shuttlebox escape deficits) and neurochemical (increased serotonergic (5-HT) activity in the dorsal raphe nucleus (DRN)) effects of IS occurring as much as one week later (Amat *et al.*, 2006; Amat et al., 2008; Christianson *et al.*, 2008b). This "immunizing" effect of ES appears to be quite general, as ES blocks the behavioral and neurochemical effects of a stressor as different from shock as social defeat (Amat et al., 2010).

Whether an experience with ES would block later IS-induced potentiation of opioid reward is unknown. Thus, here we determined whether exposure to ES might alter the enhancing effects of IS on morphine CPP, as CPP is one reliable measure for assessing the acute rewarding effects of drugs (Bardo & Bevins, 2000). In addition, the duration of the protection afforded by ES is unknown in any paradigm, and so this issue was also investigated. Lastly, although the mechanisms responsible for the immunizing effects of ES are not fully understood, the ventral medial prefrontal cortex (mPFC) has been shown to play a critical role. For example, inactivation of the mPFC with the GABA_A receptor agonist muscimol during the ES experience prevents ES from producing its proactive protective effects (Amat *et al.*, 2006). So, here we explored whether mPFC activation during ES is also necessary for any ES protection against the behavioral effects of IS on morphine CPP.

Materials and Methods

Subjects

Adult male Sprague-Dawley rats weighing 325–425 g (Harlan Laboratories, Indianapolis, IN, USA) were housed in pairs with food and water available *ad libitum*. The colony room was maintained at 22 °C with a 12 hour light-dark cycle (lights on at 0700). Rats were allowed to acclimate to the colony at least 1 wk before any experimentation. All experimental procedures were approved by the University of Colorado Institutional Animal Care and Use Committee.

Surgery and cannulation

Surgery was performed under isoflurane (Webster Veterinary, Sterling, MA, USA) anesthesia. Rats in Experiment 3 were implanted with a 26-gauge dual guide cannula (Plastics One, Roanoke, VA, USA) 1 mm center-to-center distance, which was secured to the skull with 4 screws and dental acrylic. The tips of the cannulae were aimed at 1 mm above the border of the infralimbic and prelimbic cortices of the mPFC (AP +2.7 mm, DV –3.3 mm, ML +0.0 mm from dura, relative to Bregma) (Paxinos & Watson, 1998). Rats were given 1–2 weeks of recovery time following surgery.

Drugs

Morphine sulfate (NIDA) was dissolved in 0.9% saline at a dose of 3.0 mg/kg and injected subcutaneously at a volume of 1 ml/kg of body weight. This dose was chosen as previous studies have demonstrated that it produced minimal morphine CPP in non-stressed controls (Will et al., 1998). Muscimol (Sigma, St. Louis, MO, USA) was also dissolved in 0.9% saline and 0.5 μ l was microinjected per hemisphere into the mPFC at a dose of 50 ng.

Microinjections

A 33-gauge microinjector tip (Plastics One) connected to a Kopf Instruments Model 5000 microinjector (Tujunga, CA, USA) via polyethylene 50 tubing was used for microinjections. Rats were gently restrained in a towel and the cannula stylet was removed. Microinjections were given 45 minutes before ES or HC treatment. Of note, muscimol does not interfere with escape learning during ES (Amat et al., 2005). The microinjector tips (Plastics One) were inserted into the cannula and extended 1 mm beyond the cannula tips. Rats then received 0.5 μ l /hemisphere of muscimol or saline over the course of 1 minute. Following infusion, the microinjector tips were left in place for 2 minutes to allow for diffusion. Microinjections were considered successful if fluid was readily dispensable from the microinjector tip following microinjection.

Stressor controllability

All stress procedures were administered in a distinct and separate room from that used for CPP behavioral testing. Rats received two experiences with stress to assess whether prior ES produces behavioral immunization. Day 1 of stress consisted of ES or IS. Rats were placed in clear Plexiglas wheel-turn boxes ($14 \times 11 \times 17$ cm) with a wheel located in the front of the box and a Plexiglas rod extending from the rear. Rat tails were taped to the extending rod and copper electrodes augmented with electrode paste were affixed to the tails. Shocks were delivered to yoked pairs of rats using a Precision Regulated Animal Shocker with Graphic State 3.0 software (Coulbourn Instruments, Whitehall, PA, USA). Day 1 stress consisted of 80 tailshocks with an average inter-trial interval of 60 seconds. Only ES subjects were able to behaviorally control the termination of the shock, initially by moving the wheel 1/4 of a full turn. Upon performing this operant response the shock terminated for both the ES and IS rats simultaneously. The response requirement increased throughout the session. Initially, the response increased a 1/4 turn following three previous escape responses that were completed in less than 5 seconds. Subsequent escape responses that were performed in less than 5 seconds produced a 50% increase in the wheel-turn requirement until a maximum of 4 wheel-turns was reached. If the operant response was not performed within 10 seconds, the wheel-turn requirement for the subsequent shock was incrementally decreased. If the escape response was not performed in less than 30 seconds the shock was terminated and the escape requirement was reset to 1/4 wheel-turn. Shock intensity was increased throughout the session to maintain consistent escape behavior of ES rats (1.0 mA for 30 minutes, 1.3 mA for 30 minutes, and 1.6 mA for the remainder of the session).

Day 2 stress consisted of a session of IS. Rats were placed in a clear Plexiglas restraint tube that measured 17 cm in length and 7 cm in diameter. The restraint tube contained a rod extending from the rear where rat tails were taped and electrodes were affixed and augmented with electrode paste. Day 2 stress consisted of 100, 5 second inescapable shocks at an intensity of 1.0 mA, separated by an average inter-trial interval of 60 seconds. Prior studies (Will et al., 1998) indicated that restraint in the absence of shock did not potentiate morphine CPP, therefore non-stressed HC controls remained in the colony during stress. Stress group designations were expressed as Day 1/Day 2 stress, e.g. a rat that received ES on Day 1 and IS on Day 2 was represented as ES/IS. Lastly, since Experiment 1

demonstrated no significant difference between HC/IS and IS/IS groups, the IS/IS group was eliminated.

Conditioned place preference

Apparatus—Rectangular black Plexiglas boxes ($72 \times 30 \times 30$ cm) consisting of 2 distinct conditioning environments and a central neutral chamber were used for CPP. The conditioning environments ($30 \times 30 \times 30$ cm) contained different tactile and visual cues. One conditioning environment contained vertically oriented alternating black and white stripes (2 cm wide) and the floor consisted of a 3 mm wire mesh. The other conditioning environment contained horizontally oriented black and white stripes (2 cm wide) and the floor consisted of a 2 cm wire grid. The neutral area ($12 \times 30 \times 30$ cm) was painted gray. During the conditioning phase Plexiglas partitions ($30 \times 30 \times 30$ cm) containing horizontal or vertical black and white stripes were inserted to confine the rat to a single conditioning environment. Rat activity in the CPP apparatus was monitored with a Philips TC352A video camera (Lancaster, PA, USA) mounted 1.5 meters above the CPP apparatus. The rat's location was relayed to a PC installed with Chromotrack Version 4.02 tracking software (Prototype Systems Ltd., Boulder, CO, USA). A SA-3 Tracker (San Diego Instruments, San Diego, CA, USA) simultaneously measured the amount of time spent in each of the CPP environments.

Procedure—Prior to experimentation rats were handled and loosely fitted with a collar (BAS, West Lafayette, IN, USA) affixed with reflective tape to aid video tracking. The first day of the CPP procedure was a 20 minute pre-exposure to the entire CPP apparatus. Rats that spent less than 4 minutes in either conditioning environment were eliminated from the study. The following day consisted of either ES, IS, or HC treatment. The amount of time between Day 1 stress and Day 2 stress varied depending on the experiment, but at minimum was 24 hours unless otherwise noted. Day 2 stress consisted of either IS or HC. Twenty-four hours after Day 2 stress rats were weighed and randomly assigned to a conditioning environment using a counterbalanced procedure. Conditioning sessions lasted 45 minutes each and were separated by 4 hours. During the first day of conditioning half of the rats received morphine in the AM, while the other half received equivolume saline. Over the course of 1 minute, all rats were injected with drug or saline and then immediately placed in their respective conditioning chamber. During PM conditioning the injections were alternated such that a rat that had received saline in the AM now received morphine in the PM. The following day of conditioning was similar to the previous day except the order in which the rat was presented morphine and saline on the previous conditioning day was reversed. The final day of the CPP procedure was a 20 minute test of preference. Rats were placed in the neutral area and allowed to explore the entire apparatus in a drug-free state. The amount of time spent in each compartment was recorded. The dependent measure for CPP was the difference of time spent in the drug-paired environment before morphine conditioning (pre-exposure) and after conditioning (test of preference). Thus, a positive score indicates a preference for the drug-paired environment.

Cannula verification

Following morphine CPP, rats were overdosed with an injection of sodium pentobarbital (Vortech Pharmaceuticals, Dearborn, MI, USA). Animals were decapitated and brains were rapidly removed and chilled in ice-cold isopentane and stored at -80°C . A cryostat was used to section brains at $40\text{ }\mu\text{m}$; slices were mounted on gelatin-coated slides for subsequent Cresyl Violet staining. Cannula placements were considered successful if the tips of the cannulae terminated in the infralimbic or prelimbic cortices of the mPFC. Data from animals with misplaced cannulae were eliminated from statistical analyses.

Statistical analysis

All data were analyzed by ANOVA to determine statistically significant differences between experimental groups. All statistically significant main effects and interactions were followed by a Newman-Keuls multiple comparison *post hoc* test ($\alpha = 0.05$).

Results

Experiment 1: Effect of behavioral immunization on morphine conditioned place preference

Preference for the morphine-conditioned environment following behavioral immunization is shown in Figure 1 ($n = 8-9/\text{group}$). Stress treatments were separated by 24 hours. As previously observed (Will *et al.*, 1998), IS potentiated morphine CPP (HC/IS versus HC/HC). IS 24 hours prior to additional IS (IS/IS) did not alter the magnitude of this effect. However, ES prior to IS (ES/IS) blocked IS-induced potentiation of morphine CPP. A one-way ANOVA revealed a significant main effect of group ($F_{(3, 31)} = 5.268, p < 0.01$). A subsequent Newman-Keuls *post hoc* comparison revealed a significant difference between ES/IS and the HC/IS & IS/IS groups. However, there were no significant differences between ES/IS & HC/HC or between HC/IS & IS/IS groups. Lastly, there was a significant difference between HC/HC and HC/IS & IS/IS groups.

Experiment 2: Timecourse of behavioral immunization on morphine conditioned place preference

The length of time between initial ES and subsequent IS was manipulated to assess the duration of behavioral immunization produced by ES. Either 3, 14, or 56 days were allowed to intervene between ES and later IS. To control for the differing amounts of time spent in the colony room during the intervening time between ES and IS, all time points contain a different HC/HC group. For this reason, Experiment 2 is presented as 3 different data sets. As shown in Figure 2A ($n = 10-11/\text{group}$), behavioral immunization to IS-induced potentiation of morphine CPP lasts at least 3 days. A one-way ANOVA confirmed this finding ($F_{(2, 29)} = 3.986, p < 0.05$). Figure 2B ($n = 10-12/\text{group}$) shows that behavioral immunization lasts at least 14 days ($F_{(2, 30)} = 5.379, p < 0.02$). Lastly, Figure 2C ($n = 12/\text{group}$) shows that a 56 day separation between ES and IS still produces behavioral immunization ($F_{(2, 33)} = 7.244, p < 0.01$). Subsequent Newman-Keuls *post hoc* comparisons all revealed that ES/IS & HC/HC groups were significantly different from HC/IS, but ES/IS & HC/HC groups were not significantly different from each other. Lastly, the difference between HC/HC and ES/IS groups in Figure 2C trended toward, but did not reach, significance.

Experiment 3: The role of the medial prefrontal cortex during behavioral immunization on morphine conditioned place preference

Figure 3A shows cannula placements within the mPFC for the animals tested in Experiment 3. Five subjects were removed due to misplaced cannulae. As shown in Figure 3B, activation of the mPFC during initial ES is necessary for behavioral immunization to IS-induced potentiation of morphine CPP ($n = 9-11/\text{group}$). A two-way ANOVA revealed significant effects of *stress* ($F_{(2, 53)} = 11.300, p < 0.0001$), and *microinjection* ($F_{(1, 53)} = 4.336, p < 0.05$), and an interaction of *stress* $\times$ *microinjection* ($F_{(2, 53)} = 3.379, p < 0.05$). Subsequent Newman-Keuls *post hoc* comparisons revealed significant differences between (i) saline-ES/IS and muscimol-ES/IS groups (ii) saline-HC/IS and the saline-ES/IS & saline-HC/HC groups (iii) and muscimol-HC/HC and the muscimol-ES/IS & muscimol-HC/IS groups.

Discussion

These experiments, along with others (Williams & Maier, 1977; Amat *et al.*, 2006; Amat *et al.*, 2008; Christianson *et al.*, 2008b), clearly indicate that an initial experience with controllable stress can produce resistance to the behavioral effects normally produced by uncontrollable stress. However, here the behavior assessed was opioid reward and not an anxiety-related measure. Moreover, the present results corroborate previous findings in that behavioral immunization of IS-induced potentiation of morphine reward depends on activation of the mPFC during the initial ES exposure (Amat *et al.*, 2006; Amat *et al.*, 2008). Further, the present experiments extend our understanding of the breadth and longevity of the behavioral immunization paradigm.

As noted above, previous investigations of behavioral immunization have focused on the role of the mPFC during the initial stressor. The current experiments demonstrate that activation of the mPFC during initial ES is necessary for ES to block IS-induced potentiation of morphine CPP. The GABA_A receptor agonist muscimol was used to inhibit output activity from the mPFC as muscimol is known to produce neuronal hyperpolarization (Andrews & Johnston, 1979). Indeed, muscimol has previously been used in the stressor controllability paradigm to support the idea that the mPFC becomes activated during ES, but not IS, and that inactivation of the mPFC during ES produces behaviors typical of IS exposure (Amat *et al.*, 2005; Rozeske *et al.*, 2009).

Activation of the mPFC during the experience of behavioral control likely serves two purposes. Acutely, activation of the mPFC during ES leads to inhibition of stress responsive limbic and brainstem regions (Baratta *et al.*, 2009), thereby blunting the impact of the stressor being experienced. For example, IS relative to ES produces intense activation of DRN 5-HT neurons as assessed by extracellular levels of 5-HT in the DRN as well as c-Fos protein in 5-HT labeled cells (Amat *et al.*, 2006). However, inactivation of the mPFC during ES leads ES to produce the same high levels of 5-HT activation that is normally only produced by IS (Amat *et al.*, 2006). This stress-induced hyperactivation of 5-HT cells in the DRN by IS is thought to lead to sensitization of 5-HT cells in the DRN (Amat *et al.*, 1998; Bland *et al.*, 2003a). It is this sensitization of 5-HT cells in the DRN that leads to potentiation of morphine CPP (Will *et al.*, 2004) as well as potentiation of extracellular dopamine in the nucleus accumbens following subcutaneous morphine (Bland *et al.*, 2004a; Bland *et al.*, 2004b). For these reasons, pharmacological inhibition of the mPFC during ES leads ES to sensitize 5-HT cells in the DRN (Rozeske *et al.*, 2010), and consequently, the rewarding properties of morphine are now potentiated due to elevated extracellular dopamine in the nucleus accumbens in IS, but not ES, rats.

In addition, activation of the mPFC during ES has another more enduring effect, namely the induction of long-term plasticity within the mPFC. This plasticity is thought to alter the mPFC in such a way that now even uncontrollable stressors produce activation. Again, this activation leads to inhibition of stress-responsive limbic and brainstem structures, the mediator of immunization. Two types of evidence have been provided for this plasticity phenomenon. First, inhibition of *de novo* protein synthesis in the mPFC during initial ES blocks behavioral immunization (Amat *et al.*, 2006). Second, inactivation of the mPFC during subsequent IS prevents an initial ES exposure from producing immunization (Amat *et al.*, 2006). Thus, the mPFC is required both at the time of initial ES and later IS for immunization to occur.

Nevertheless, measures of neuronal activation that correlate with drug reward were not made in the present studies. Phosphorylated extracellular signal-regulated kinase would be an appropriate marker as drugs of abuse elevate its expression and it is required for morphine

CPP (Valjent *et al.*, 2004; Valjent *et al.*, 2006). Assessing phosphorylation of extracellular signal-regulated kinase could provide information concerning the extent of cellular activation in reward-related brain regions during time points where the strength of behavioral immunization begins to diminish, such as the 56 day time point.

In addition to the interaction between the mPFC and DRN during stressor controllability, the relationship between the mPFC and the mesolimbic reward system has also been extensively studied. Indeed, it is known that ES produces a transient elevation of extracellular dopamine in the nucleus accumbens, although this elevation is similar to that produced by IS (Bland *et al.*, 2003b). ES also initially elevates extracellular levels of dopamine and 5-HT in the mPFC, but these increases are transient and outlasted by IS exposure (Bland *et al.*, 2003a). Since the elevations of extracellular monoamines measured during ES are short-lived, it is unlikely that 5-HT and dopamine directly contribute to behavioral immunization. However, the role of other neurotransmitters and post-translational modifications in reward-related structures following behavioral immunization is an open question.

The behavioral immunization paradigm has previously been investigated using a 7 day interval between ES and IS (Amat *et al.*, 2006; Baratta *et al.*, 2009; Amat *et al.*, 2010). The present experiments reveal that ES produces resistance to IS as soon as 24 hours following ES. Moreover, the stress-buffering effects of ES persist for at least 56 days. Experiment 3 reported here, examined only a 24 hour ES-to-IS interval, and so it cannot be stated with certainty that the long-duration 56-day immunization effect is also mediated by the mPFC. However, given the abundance of evidence reviewed above, activation of the mPFC during the stress experience is a likely mechanism. The necessary biochemical cascade(s) during ES-induced activation of the mPFC have not yet been investigated. However, they must be induced following a relatively short behavioral experience (ES is roughly 90 minutes) and be present 24 hours later. Since behavioral immunization mitigates IS-induced potentiation of morphine CPP for at least 56 days, the plasticity processes involved are likely long lasting. The literature suggests a number of candidates including, PKM ζ (Shema *et al.*, 2009; Richter-Levin & Maroun, 2010; Sacktor, 2011).

Together the results presented above provide additional evidence that an experience with a controllable stressor can protect an organism from the usual behavioral consequences of an uncontrollable stressor. Typically the behavioral outcomes measured following behavioral immunization have been anxiety-related, but here the protective effects of ES have been extended to a drug reward paradigm. Although other studies have examined factors that block IS-induced potentiation of morphine CPP, such as voluntary free-wheel running (Rozeske *et al.*, 2011), the role of the mPFC has been unknown. Certainly, not all factors that blunt the impact of stressors utilize the mPFC (Christianson *et al.*, 2008a), and so involvement of the mPFC in paradigms that blunt the effects of stress continues to be an open issue.

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Abbreviations

5-HT	Serotonin
CPP	Conditioned place preference
DRN	Dorsal raphe nucleus

ES	Escapable tailshock
HC	homecage control
IS	Inescapable tailshock
mPFC	Medial prefrontal cortex

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Figure 1.

Escapable tailshock (ES) 24 hours prior to inescapable tailshock (IS) blocks potentiation of morphine conditioned place preference (CPP). Data are expressed as mean + SEM difference of time (sec) spent in the morphine-paired environment before and after conditioning. Positive scores indicate a shift in preference for the morphine-paired environment. Both a single session of IS (HC/IS) or two sessions of IS (IS/IS) potentiated morphine CPP as compared to behavioral immunization (ES/IS) and non-stressed controls (HC/HC). *Significantly different from HC/IS and IS/IS ($p < 0.05$).

Figure 2.

A. Escapable tailshock (ES) 3 days prior to inescapable tailshock (IS) blocks potentiation of morphine conditioned place preference (CPP). Data are expressed as mean + SEM difference of time (sec) spent in the morphine-paired environment before and after conditioning. Positive scores indicate a shift in preference for the morphine-paired environment. *A single session of IS (HC/IS) potentiated morphine CPP as compared to behavioral immunization (ES/IS) and non-stressed controls (HC/HC) ($p < 0.05$). **B.** ES 14 days prior to IS blocks potentiation of morphine CPP. *A single session of IS (HC/IS) potentiated morphine CPP as compared to behavioral immunization (ES/IS) and non-stressed controls (HC/HC) ($p < 0.05$). **C.** ES 56 days prior to IS blocks potentiation of morphine CPP. *A single session of IS (HC/IS) potentiated morphine CPP as compared to behavioral immunization (ES/IS) and non-stressed controls (HC/HC) ($p < 0.05$).

Figure 3.

A. Cannula placements for rats in Experiment 3. Numerals on the left indicate distance (mm) from Bregma. Medial prefrontal cortex (mPFC) placements were considered successful if they terminated within the infralimbic (IL) and prelimbic (PL) cortices. Not all cannula are shown due to overlapping placements. **B.** Intra-mPFC muscimol blocks behavioral immunization to potentiation of morphine conditioned place preference (CPP) when escapable tailshock (ES) and inescapable tailshock (IS) are separated by 24 hours. Data are expressed as mean + SEM difference of time (sec) spent in the morphine-paired environment before and after conditioning. Positive scores indicate a shift in preference for the morphine-paired environment. #The saline-HC/IS group was significantly different from the saline-ES/IS & saline-HC/HC groups ($p < 0.05$). *Muscimol-ES/IS was significantly different from saline-ES/IS ($p < 0.05$). ^The muscimol-HC/HC group was significantly different from the muscimol-ES/IS & muscimol-HC/IS groups ($p < 0.05$).