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Research Report

Contribution of the rostral ventromedial medulla to post-anxiety induced hyperalgesia

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ABSTRACT

Rats exposed to an elevated plus maze (EPM) with four open arms display antinociception while on the maze and hyperalgesia immediately upon removal. Little is known about the neural mechanisms underlying EPM-induced antinociception and the subsequent hyperalgesia except that the antinociception is not mediated by endogenous opioids. The objective of the present study was to test the hypothesis that endogenous cannabinoids and/or the rostral ventromedial medulla (RVM) contributes to EPM-induced antinociception. Administration of the CB1 receptor antagonist AM251 (1 mg/kg, i.p.) had no effect on baseline nociception to formalin administration into the hindpaw or on the antinociception produced by placing a rat on the open EPM. Likewise, inactivation of the RVM by microinjecting the $GABA_A$ receptor agonist muscimol (10 ng/0.5 μL) had no effect on the antinociceptive effect of placing a rat in the EPM. However, RVM inactivation blocked the hyperalgesia produced upon removal from the EPM. Although distinct classes of RVM neurons inhibit and facilitate nociception, the present data demonstrate that the antinociception induced by the EPM and the subsequent hyperalgesia is mediated by distinct neural pathways.

1. Introduction

A variety of threatening and/or stressful stimuli have been shown to produce antinociception (Fanselow, 1991; Fardin et al., 1984; Kelly, 1982; Terman, et al., 1984; Watkins and Mayer, 1982). Exposure to the standard elevated plus-maze (EPM, two enclosed and two open arms), a test originally validated to study anxiety-like behaviors in rats and mice (e.g., Lister, 1987; Pellow et al., 1985; Stephens et al., 1986), also has been shown to produce antinociception (Lee and Rodgers, 1990, 1991; Rodgers et al., 1992). This antinociception was relatively mild, but persisted for up to 30 min following removal from the EPM (Lee and Rodgers, 1990, 1991). On the other hand, it has been shown that removal of the walls (so all four arms of the maze are open) enhances the antinociceptive effects (Cornélio and Nunes-de-Souza, 2009; Mendes-Gomes and Nunes-de-Souza, 2005, 2009). Unlike many aversive stimuli (e.g., footshock) (Terman et al., 1984) and studies with standard EPM (Lee and Rodgers, 1990, 1991), EPM-induced antinociception is short lived: Removing rats from the maze causes an immediate shift from antinociception to hyperalgesia (Cornélio et al., 2011). Little is known about the

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Abbreviations: EPM, elevated plus maze; RVM, rostral ventromedial medulla; PAG, periaqueductal gray

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neural mechanisms underlying EPM-induced antinociception or the subsequent hyperalgesia.

Previous research has shown that endogenous opioids do not contribute to EPM-induced antinociception. This antinociception is not reversed by the opioid receptor antagonist naltrexone and does not produce cross-tolerance to morphine antinociception (Cornélio and Nunes-de-Souza, 2009). Many other transmitter systems could underlie EPM induced antinociception. Endogenous cannabinoids are a likely candidate because both cannabinoids and exposure to the EPM are associated with anxiety (Ruehle et al., 2012) and produce antinociception (Hohmann et al., 2005; Mendes-Gomes and Nunes-de-Souza, 2005; Pertwee, 2001).

Endogenous cannabinoids and cannabinoid receptors are present at several levels of the pain pathway, from peripheral sensory nerve endings to spinal cord and supraspinal centers (Iversen, 2003). Synthetic and endogenous cannabinoids have antinociceptive and anti-hyperalgesic effects in a variety of animal models of acute and tonic pain when administered orally, systemically or directly into brain or spinal cord (for review see Pertwee, 2001). In addition, endocannabinoids have been shown to contribute to some forms of stress-induced analgesia such as that elicited by brief and continuous electric foot shock to rats (Hohmann et al., 2005).

The antinociceptive effects of opioids and cannabinoids are known to be mediated in part by the nociceptive modulatory system that runs through the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) (Hohmann et al., 2005; Lane et al., 2005; Meng et al., 1998; Proudfit and Anderson, 1975; Yaksh et al., 1977). The RVM is of particular interest because RVM on- and off-cells (Fields et al., 1983) have been shown to facilitate and inhibit nociception, respectively (Heinricher et al., 1994; Neubert et al., 2004). These findings suggest that endogenous cannabinoids could mediate EPM-induced antinociception, and the RVM could contribute to both antinociception and post-EPM hyperalgesia. These hypotheses were tested by exposing rats to the EPM following systemic administration of the CB1 receptor antagonists AM251 or inactivation of the RVM with the $GABA_A$ receptor agonist muscimol.

2. Results

2.1. *Experiment 1: endogenous cannabinoids*

Systemic administration of the CB1 receptor antagonist AM251 had no effect on baseline nociception assessed during the first phase of the formalin test [t (26) = 0.41; $p > 0.05$]. The average time spent licking the hindpaw following formalin administration was similar whether rats were pretreated with AM251 (52.9 ± 5.5 s) or vehicle (56.4 ± 6.4).

As reported previously (Cornélio and Nunes-de-Souza, 2009), exposure to the open EPM during the second phase of the formalin test produced a significant antinociception compared to rats in the enclosed EPM [Fig. 1; $F(1,24) = 52.75$, $p < 0.05$]. This antinociception was not reversed by AM251 administration as indicated by the lack of an interaction between rats pretreated with AM251 or vehicle ($F(1,24) = 0.28$, $p = 0.60$). Although administration of AM251 did not produce a statistically significant difference in the amount of time spent licking the hindpaw compared to vehicle treated rats ($F(1,24) = 2.68$, $p = 0.11$), there was a trend toward greater antinociception in rats treated with AM251. Enhanced antinociception on the EPM would be consistent with the anxiogenic effects of AM251 (Ruehle et al., 2012; Sink et al., 2010).

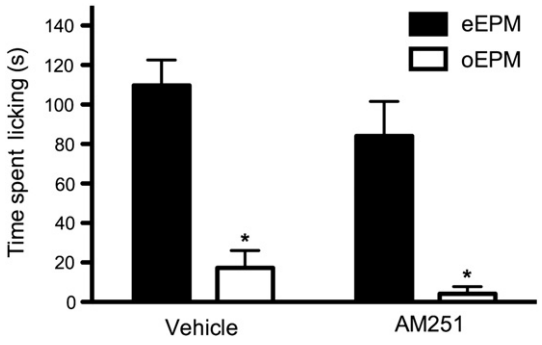

Fig. 1 – Endogenous cannabinoids do not contribute to EPM-induced antinociception. Time (in seconds) spent licking the paw during the second phase (n = 7/group) of the formalin test in rats treated with vehicle or AM251 (1 mg/kg, i.p.) and exposed to the enclosed EPM or open EPM. Although exposing rats to the open EPM produced antinociception compared to rats in the enclosed EPM (* denotes a significant difference, $p < 0.05$), administration of AM251 had no effect. Note: animals were exposed to the enclosed EPM or open EPM only during the second phase of the formalin test.

2.2. *Experiment 2: rostral ventromedial medulla*

A total of 39 rats had cannula placements in or along the border of the RVM (Fig. 2). The placements were similar whether rats were injected with saline into the RVM and placed in the open (N = 8) or enclosed EPM (N = 9), or injected with muscimol and placed in the open (N = 12) or enclosed EPM (N = 10).

The first phase of the formalin test was assessed prior to the RVM microinjection. Thus, the groups did not differ at this point and there was no difference in the amount of time spent licking the hindpaw between the saline (51.8 ± 3.2 s) and muscimol (47.5 ± 4.5 s) treated groups during this phase [t (37) = 0.74; $p > 0.05$].

Rats were placed in the EPM 15 min after saline or muscimol was microinjected into the RVM. Rats exposed to the open EPM spent significantly less time licking the hindpaw during the second phase of the formalin test compared to rats in the enclosed EPM [$F(1,35) = 6.84$, $p < 0.05$]. Inactivation of the RVM by microinjecting muscimol into the RVM had no effect on this antinociception [$F(1,35) = 0.00$, $p > 0.05$; Fig. 3] indicating that the RVM does not contribute to EPM-induced antinociception.

Assessment of nociception using the hot plate test immediately after removing the rat from the open EPM produced a hot plate latency of 10.4 ± 1.0 s (Fig. 4). Microinjection of muscimol into the RVM reversed this hyperalgesia as is evident by a significant increase in hot plate latency [t (11) = 2.275, $p < 0.05$]. Closer analysis of this effect revealed a bimodal effect of muscimol. Six rats injected with muscimol and removed from the open EPM had hot plate latencies greater than 18 s and 6 had

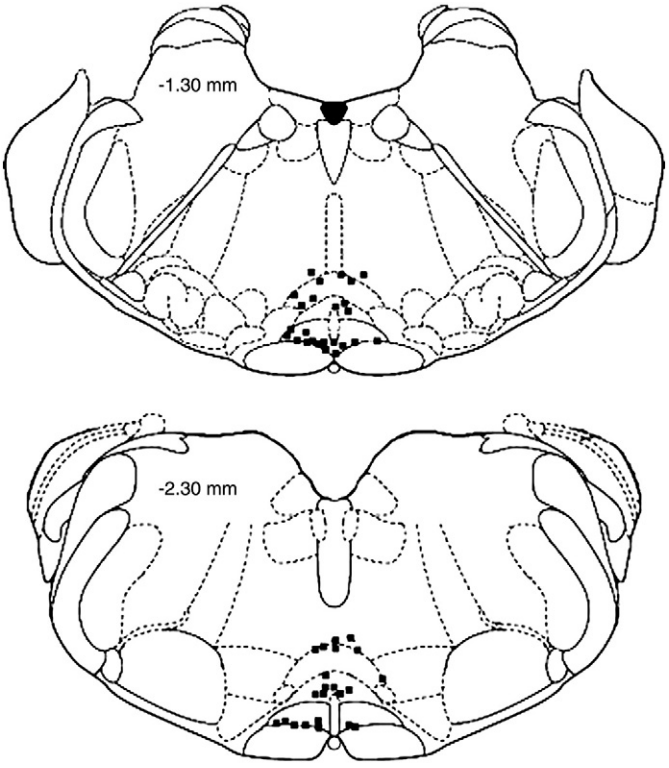

Fig. 2 – Schematic representation of location of microinjection sites in the RVM. Black squares indicate saline or muscimol administration into the RVM. Coronal sections are taken from the Atlas of Paxinos and Watson (2005). The number in the top left corner refers to the distance from the interaural line.

latencies less than 11 s. This distribution was significantly different from the eight rats receiving RVM saline, all of which had hot plate latencies less than 15 s (Chi square = 5.71, $p < 0.05$).

Muscimol treated rats showing antinociception on the hot plate test also tended to be the same rats showing antinociception while on the EPM. Complete antinociception (no hindpaw licks) on the EPM was evident in 7 of the 12 rats treated with muscimol. This antinociception was maintained in 5 rats when removed from the open EPM and tested on the hot plate. The lack of antinociception on the hot plate in any of the saline pretreated rats suggests that RVM muscimol blocked the post-maze hyperalgesia so that the antinociception induced by the open EPM persisted. This antinociception

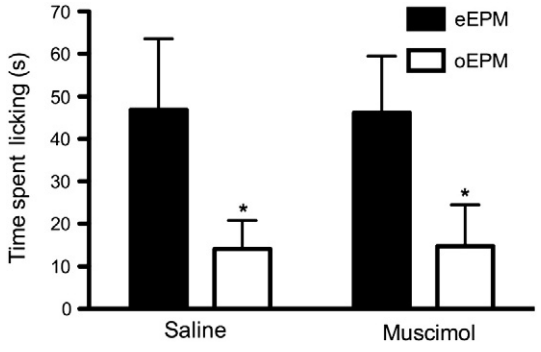

Fig. 3 – Inactivation of the RVM had no effect on EPM-induced antinociception. Time (in seconds) spent licking the right hind paw during the second phase (n = 8–12/group) of the formalin test. Exposure to the open EPM produced antinociception (* denotes a significant difference with rats exposed to the enclosed EPM, $p < 0.05$) that was not altered by prior microinjection of the $GABA_A$ receptor agonist muscimol (10 ng/0.5 μL) into the RVM.

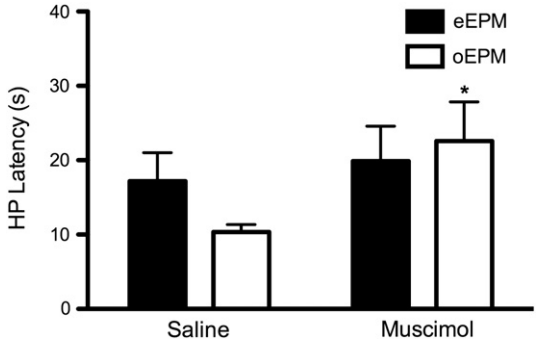

Fig. 4 – Inactivation of the RVM blocks post-EPM induced hyperalgesia. Hot plate latency (n = 8–12/group) of rats previously injected with formalin into the right hindpaw and exposed to the enclosed or open EPM for 10 min. Although hyperalgesia was evident in rats removed from the open-EPM, inactivation of the RVM by microinjecting muscimol reversed the hyperalgesia (* denotes a significant difference between muscimol and saline pretreated rats exposed to the open EPM, $p < 0.05$).

was not evident in rats not exposed to the EPM. Seven rats treated with formalin and RVM muscimol, but not exposed to the EPM, had a mean hot plate latency of 22.04±5.13 s and only 2 of these rats had a hot plate latency greater than 18 s (vs. 50% of the rats exposed to the open EPM).

3. Discussion

Although the primary objective of these experiments were to determine the neural mechanisms underlying antinociception produced by exposure to the open EPM, neither endogenous cannabinoids nor the nociceptive modulatory system that includes the RVM appears to be involved. However, the present data indicate that the hyperalgesia produced when rats are removed from the EPM is mediated by the RVM. That is, inactivation of the RVM reversed the hyperalgesia and allowed the EPM-induced antinociception to persist.

Experiment 1 showed that blockade of cannabinoid CB_1 receptors had no effect on baseline nociception or the antinociception induced by exposure of rats to the open EPM. That is, administration of the CB1 receptor antagonist AM251 did not alter hindpaw licking during the first phase of the formalin test prior to exposure to the EPM. Administration of AM251 also had no effect on nociception during the second phase of formalin test when rats were on the EPM. Antinociception, measured as a reduction in hindpaw licking, was evident in rats exposed to the open EPM whether pretreated with vehicle or AM251. The greater antinociception in rats exposed to the open as opposed to the enclosed EPM is in agreement with previous results (Cornélio and Nunes-de-Souza, 2009; Mendes-Gomes and Nunes-de-Souza, 2005, 2009). The inability of AM251 to attenuate this antinociception indicates that the mechanism mediating EPM-induced antinociception is different from footshock-induced antinociception which is mediated, in part, by PAG cannabinoids (Hohmann et al., 2005).

Both opioids and cannabinoids produce antinociception in part by activation of the nociceptive modulatory system that runs through the PAG and RVM. Given that neither opioids (Cornélio and Nunes-de-Souza, 2009) nor cannabinoids appear to contribute to EPM-induced antinociception, it was not surprising that microinjection of muscimol to inactivate the RVM did not disrupt EPM-induced antinociception. Muscimol is a $GABA_A$ receptor agonist that has been shown to inactivate RVM neurons (Martenson et al., 2009) and attenuate PAG mediated antinociception when microinjected into the RVM (Lane et al., 2005). In the present study, microinjection of muscimol had no effect on EPM-induced antinociception measured with the formalin test. In contrast, the RVM has been implicated in other forms of stress-induced antinociception (Morgan and Fields, 1993; Watkins and Mayer, 1982).

The antinociception produced by exposure to the open, but not the enclosed EPM is consistent with previous studies (Cornélio and Nunes-de-Souza, 2009; Mendes-Gomes and Nunes-de-Souza, 2005, 2009). This antinociception appears to be adaptive in that it reduces behavioral competition from noxious stimuli that could disrupt appropriate defensive behavior (Fanselow, 1991). Although the neural mechanisms for EPM-induced antinociception remain unknown, structures throughout the CNS have been implicated in stress-induced antinociception (Butler and Finn, 2009) and could contribute.

In contrast, inactivation of the RVM reversed the hyperalgesia that occurs when rats are removed from the EPM (Cornélio et al., 2011). This finding is consistent with previous studies showing that RVM on- and off-cells can facilitate and inhibit nociception, respectively (Heinricher et al., 1994; Neubert et al., 2004). The finding that on- and off-cells appear to be mutually inhibitory (Barbaro et al., 1989) provides a model by which hyperalgesia can rapidly replace antinociception as the environmental situation changes. The RVM has been shown to contribute to several forms of hyperalgesia such as that produced by activation of the dorsomedial nucleus of the hypothalamus (Martenson et al., 2009), microinjection of low doses of neurotensin into the RVM (Neubert et al., 2004), and neuropathic pain (Carlson et al., 2007; Pertovaara et al., 1996).

More relevant to our data is the role of the RVM in antianalgesia. Cues that signal safety produce a rapid reversal of both stress and morphine-induced antinociception (Watkins et al., 1997; Wiertelak et al., 1992). Removing rats from the EPM and placing them in an enclosed hot plate could provide similar safety cues. The rapid shift from antinociception to hyperalgesia reported here and in our previous work (Cornélio et al., 2011) is consistent with the rapid onset of antianalgesia. This hyperalgesia is not caused by testing rats on the hot-plate following administration of formalin. Hyperalgesia occurs upon removal from the EPM whether rats are injected with formalin or not, is evident in mice tested with formalin upon removal from the EPM, and does not occur when rats are injected with formalin and tested on the hot plate, but not exposed to the EPM (Cornélio et al., 2011). Hyperalgesia is not caused by a change in how nociception is assessed (i.e., formalin to hot-plate) because control rats did not show hyperalgesia when tested on the hot plate, and our previous study showed a similar enhancement of nociception when mice were assessed with the formalin test after removal from the maze (Cornélio et al., 2011). However, assessment of tail-flick latency revealed a mild antinociception in mice following exposure to the standard EPM (Lee and Rodgers, 1990, 1991). This antinociception could be caused by differences in stress induced by the open and standard EPM's or from restraint stress when moving the mouse from the EPM to the tail flick test.

Finally, inactivation of the RVM attenuates antianalgesia (Watkins et al., 1998) just like muscimol inactivation of the RVM attenuated hyperalgesia following removal from the EPM in the present study. Removing this hyperalgesic input from the RVM allowed the EPM-induced antinociception to persist after the rats were removed from the EPM. This finding suggests that the antinociceptive and hyperalgesic effects are mediated by distinct and competing systems. The RVM contributes to the hyperalgesic effect, but the neural basis for EPM-induced antinociception remains unknown. Opioid (Cornélio and Nunes-de-Souza, 2009), cannabinoid, and descending modulation from the RVM do not appear to contribute. Thus, additional studies are needed to investigate the role of other nociceptive modulatory systems and transmitters. In this context, the GABA/benzodiazepine receptor complex located within the amygdala seems to be a strong candidate for mediation of this

type of environmentally-induced antinociception. Microinjection of midazolam into this limbic forebrain structure attenuated pain inhibition induced by EPM-open arm confinement in mice (Baptista et al., 2009; Nunes-de-Souza et al., 2000).

4. Experimental procedure

4.1. Subjects

Adult male Sprague–Dawley rats (213–290 g; Harlan, Kent WA, USA) were housed in pairs in a polycarbonate cage (33×21×20 cm) under a reverse light/dark schedule (lights off at 07:00) in a temperature-controlled environment (22 °C). Food and water were freely available except during the brief test period. Rats were handled daily for at least 4 days prior to the beginning of the experiment to reduce stress associated with handling. All animals were experimentally naïve and all testing was conducted during the dark phase when rats are awake and active. Rats were transported to the dimly light experimental room and left undisturbed for at least 1 h prior to testing. Testing was conducted between 10:00 and 16:00. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Washington State University. Efforts were made to minimize the number and potential suffering of subjects.

4.2. Drugs

The CB_1 receptor antagonist AM 251 [(N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide); Tocris Cookson Inc., USA], was dissolved in 60% DMSO and saline (Wilson et al., 2008). The $GABA_A$ receptor agonist muscimol (Sigma-Aldrich; St. Louis, MO, USA) was dissolved in saline. AM251 (1 mg/kg, i.p.) and muscimol (10 ng/0.5 μL) doses and concentrations were selected based on previous studies (Lane et al., 2005; Wilson et al., 2008).

4.3. Enclosed and open elevated plus maze

The enclosed and open EPM are modifications of the standard elevated plus maze previously described (Pellow et al., 1985). They consist of four arms (50×10 cm) connected to a common central platform (10×10 cm) covered in gray plastic. All four walls were either enclosed with Plexiglas (40 cm high) or open except for a 0.25 cm high edge. The arms of the maze were 50 cm above floor level.

4.4. Nociceptive tests

Nociception was assessed using the formalin and hot plate tests. The formalin test consists of injecting 50 μL of formalin (2.5% formaldehyde) into the plantar surface of the right hind paw and measuring the amount of time spent licking the paw over two phases of responding (Abbott et al., 1999; Dubuisson and Dennis, 1977). The first phase begins immediately following formalin injection and lasts approximately 5 min. Rats were placed in a clear Plexiglas cage to record the amount of time spent licking the hind paw during the first phase. The second phase begins approximately 20 min after the formalin injection and lasts approximately 40 min (Bon et al., 2002). The amount of time spent licking the hind paw during the second phase of the formalin test was evaluated for 10 min (25–35 min after formalin injection) while rats were on the enclosed or open EPM.

The hot plate test was used to assess nociception immediately following exposure to the EPM. This test consists of placing a rat on an enclosed square plate heated to 52.5 °C. The latency for the rat to lick a hind paw was measured. Rats were removed from the hot plate if no response occurred within 50 s.

4.5. Procedure

4.5.1. Experiment 1: role of endogenous cannabinoids

The objective of this experiment was to test the hypothesis that endogenous cannabinoids contribute to antinociception produced by exposure to the open EPM. Rats (n=7/group) were treated with vehicle or the CB1 receptor antagonist AM251 (1 mg/kg, i.p.). This dose was selected based on its ability to block cannabinoid effects in our previous study (Wilson et al., 2008). Fifteen minutes later, formalin was injected into the plantar surface of the right hind paw. Twenty-five minutes after formalin injection, rats were placed in the enclosed or open EPM for 10 min and nociception was assessed by measuring the time spent licking the paw.

4.5.2. Experiment 2: role of the RVM in EPM-induced antinociception

The objective of this experiment was to test the hypothesis that the nociceptive modulatory system that includes the RVM contributes to antinociception produced by exposure to the open EPM. Rats were anesthetized with equithesin (60 mg/kg, i.p.) and stereotaxically implanted with a 23-gauge stainless steel guide cannula (13 mm long) aimed at the RVM (anterior: −2.3 mm; ventral: −7.9 mm; lateral: 0 mm from lambda) (Paxinos and Watson, 2005). The guide cannula was held in place with dental cement affixed to two screws in the skull. Following surgery, a removable stainless steel stylet was inserted into the guide cannula. No postoperative medication was administered to avoid possible cross-tolerance to environment-induced antinociception. Rats were housed individually for 1 day following surgery before being returned to a cage with two rats.

Rats were allowed to recover for 5–8 days prior to testing. One day before testing, each animal received a sham injection, in which an injector (31 gauge) extending 2 mm beyond the tip of a 13 mm guide cannula was inserted into the guide cannula but no drug was administered. This procedure habituates the rat to the microinjection procedure and reduces confounds resulting from mechanical damage to neurons on the test day.

On the test day, formalin was injected into the right hind paw, and the time spent licking the paw was recorded for 5 min while the rat was in a Plexiglas cage. Ten minutes after formalin administration saline or muscimol (10 ng/0.5 μL) was microinjected into the RVM using an injection cannula that extended 2 mm beyond the guide cannula. This dose

of muscimol was selected to inactivate the RVM as has been shown previously (Lane et al., 2005; Martenson et al., 2009). The injection cannula was connected to a 1 μL syringe (Hamilton Co., Reno, NV) via PE20 tubing filled with sterile water. Drugs were administered in a volume of 0.5 μL at a rate of 0.5 μL/60 s while the rat was gently restrained by hand. The injection cannula remained in place an additional 40 s to minimize backflow of the drug up the cannula track. The stylet was replaced following the injection.

Animals were exposed to the enclosed or open EPM 15 min after the microinjection, and the time spent licking the paw was measured for 10 min. Immediately after exposure to the EPM, rats were tested on the hot plate. A control group (n=7) injected with formalin in the hind paw and muscimol in the RVM, but not placed in the EPM was included to assess whether these factors influence hot plate latency independent of the EPM. At the end of the experiment rats were given a lethal dose of Halothane and their brains were removed and placed in formalin. At least two days later, 100 μm coronal sections were made and the location of the injection site was plotted on an atlas (Paxinos and Watson, 2005). Data from animals with injection sites outside the RVM were not included in data analysis.

4.6. *Statistics*

Data were initially submitted to a test of homogeneity of variances. Groups were compared by submitting data to analysis of variance (ANOVA) or *t*-test. Data from the first phase of the formalin test were analyzed by *t*-test for independent samples. Data from the second phase of the formalin test were submitted to two-way ANOVA (factor 1: type of maze; factor 2: treatment). Hot plate data were analyzed by *t*-test for independent samples. Chi squared was used to analyze the number of rats displaying antinociception. In all cases, a p value$\leq$0.05 was required for significance.

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