TITLE: The Dorsal Raphe Nucleus as a Site of Action of the Antinociceptive and

Behavioral Effects of the Alpha4 Nicotinic Receptor Agonist, Epibatidine.

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1

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The dorsal raphe nucleus and epibatidine

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List of nonstandard abbreviations:

Nicotinic acetylcholine receptor	nAChR
Serotonin	5-HT
Artificial Cerebrospinal Fluid	ACSF
Area Under the curve	AUC
Periaqueductal gray	PAG
Dorsal Raphe	DR

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ABSTRACT

The mechanisms and sites of action of epibatidine-induced antinociception and side effects are poorly understood. The present study tested the hypothesis that the serotonergic dorsal raphe nucleus is a site of action of epibatidine. Behavioral responses of rats to hindpaw formalin injection were compared after direct administration of epibatidine into the dorsal raphe, and after subcutaneous administration. Different groups of rats were injected with formalin into the rear paw after administration of either epibatidine (0.01-0.015-0.03-0.06 µg) in the dorsal raphe or epibatidine (2.5-5 µg/kg) subcutaneously. Assessment of pain related behavior was done evaluating the incidence of favoring, lifting and licking of the injected paw in the different groups. Abnormal behavior (freezing) was also recorded. Epibatidine was at least 100 times more potent when administered into the dorsal raphe nucleus vs. systemically, implicating this nucleus as a site of action of the analgesic effects of epibatidine. Thus, epibatidine (0.015-0.03-0.06 µg) in the dorsal raphe resulted in a significant lower pain score in the second phase of the formalin test compared to control rats and was as effective as subcutaneous epibatidine. The analgesic effects of epibatidine were regionally selective in that administration of epibatidine within the PAG but outside the dorsal raphe area was not analgesic. The highest doses of intra-raphe epibatidine (i.e., 0.03-0.06 µg) also produced "freezing" behavior immediately after injection, which was relatively short-lived compared to the analgesic effect. Together the results implicate the dorsal raphe nucleus as a target for the analgesic and perhaps anxiogenic effects of epibatidine.

Introduction

Nicotine and nicotinic agonists have been known for many years to have analgesic properties. However, the high incidence and severity of side effects associated with these drugs has limited their clinical use. Recent studies on the analgesic effects of epibatidine, a nicotinic acetylcholine receptor (nAChR) ligand, (Qian et al., 1993;Sullivan et al., 1994;Bannon et al., 1998) and other epibatidine derivatives such as ABT-594 (Bannon et al., 1998) have triggered a new interest on the mechanism of antinociception produced by nicotinic agonists. It has been postulated that nicotinic acetylcholine receptor agonists produce their antinociceptive effects predominantly via activation of descending inhibitory pain pathways originating in the brainstem regions including the nucleus raphe magnus (Bitner et al., 1998).

Central modulation of pain involves both the NRM and DR. The NRM can directly control pain transmission in the dorsal horn of the spinal cord via descending projections. The effects of the DR on the spinal cord are most likely mediated by its interconnection with the NRM (Wang and Nakai, 1994). Although there is clear experimental evidence that the nucleus raphe magnus (NRM) mediates the antinociception produced by epibatidine and the nicotinic agonist ABT-594 (Bitner et al., 1998, Curzon et al 1998), the participation of other brain areas has never been investigated. The dorsal raphe nucleus (DR) is another potential site of action, as it is an area where both electric stimulation and morphine application have antinociceptive effects. The DR contains the largest pool of serotonergic neurons in the brain (Dahlstrom and Fuxe, 1964). These neurons express nAChR containing the alpha4 subunit (Cucchiaro and Commons, 2003), which is thought to be a primary receptor site for epibatidine. There are multiple data

4

showing that the systemic administration of nicotine modifies neural activity in the dorsal raphe, with different subgroups of neurons exhibiting different responses. Thus, systemic nicotine inhibits approximately 60-70% of cells recorded in the DR, while increasing the firing of the remaining third of DR neurons (Engberg et al., 2000;Mihailescu et al., 2002). Nicotine can also induce a concentration dependent increase in serotonin release from rat midbrain slices (Mihailescu et al., 1998).

Together these findings suggest that the DR could be an important contributor to the positive effects of nicotinic ligands on antinociception. A few studies have suggested that serotonergic neurons localized in the DR mediate the anxiolytic effects of low doses of nicotine (Cheeta et al., 2001;File, 2000), and this is mediated by increased 5-HT release in the DR (Seth et al., 2002). However, there are no data on the interplay between serotonergic neurons localized in the dorsal raphe, nicotine agonists and antinociception. The purpose of the present study was to test if the DR is a target for epibatidine induced antinociception or side effects. The effect of local administration of epibatidine in the DR on nociceptive response and motor behavior was measured. These data have then been compared with those observed in rats that received systemic epibatidine.

METHODS

Male Sprague –Dawley rats (250-300g) were housed in pairs under a 12:12 h light/dark cycle with water and food available *ad-libitum.* For all experiments that used implanted cannulas, rats were singly housed. The protocols were in accordance with the animal care guidelines at the University of Pennsylvania and The Children's Hospital of Philadelphia and followed the Guide for the care and use of laboratory animals as adopted and promulgated by the U.S. National Institute of Health.

Surgical procedure.

Rats were anesthetized with halothane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) with the skull on a horizontal plane. A hole was drilled to accept a skull screw. Coordinates for the placement of the intracranial cannula guides were from intra-aural zero: antero-posterior -0.5 mm; mediolateral +0.27 mm; dorso-ventral –0.49 mm. The entry angle was –25 degrees from the vertical. Coordinates were chosen according to the atlas of Paxinos and Watson (1998). Cannulae guides (26 gauge, Plastics One Inc, Wallingford, CT) were positioned and cranioplastic cement was used to affix the cannula guide to the skull and skull screw. A dummy cannula was inserted into the guide to keep it clear. Rats were allowed to recover for 3 days prior to the behavioral studies.

Peripheral epibatidine injection

Three groups of rats received subcutaneous saline (control group, n=9), epibatidine 2.5 μg/kg (n=9) or epibatidine 5 μg/kg (n=9). The study drugs were injected in the back of the rats, in the lumbar area. Formalin 5% (50 μl) was then injected subcutaneously into

the plantar surface of one rear paw, using a 27-ga needle and an insulin syringe. This group of rats was not implanted with intracranial cannulas.

Intra-DR epibatidine injection

Forty-six rats were implanted with a DR cannula guide. Rats were infused with either ACSF, or epibatidine at different doses: 0.01-0.015-0.03-0.06 μg in 300 nl ACSF. To verify that nAChR alone were responsible of the effects observed after the infusion of epibatidine into the DR, the nAChR channel blocker mecamylamine (1 μg) was infused in the DR 10 minutes prior to the infusion of 0.015 μg epibatidine in a separate group of rats.

Infusions were done by replacing the dummy cannula with an internal cannula (33 gauge) connected to a syringe by PE tubing. The drugs were injected via a syringe pump (Model 11 pluss, Harvard Apparatus Inc., Holliston, MA) over 1 min. At the end of the intracranial infusion of ACSF or epibatidine, formalin 5% (50 μl) was injected subcutaneously into the plantar surface of one rear paw, using a 27-ga needle and insulin syringe.

Behavioral assessment.

To habituate them to the formalin test environment, rats were singly placed in the test chamber for 3 days for 10-15 minutes. The testing room was maintained at 22°C, under normal lighting conditions. The formalin test was carried out in a 60X30X40 clear glass chamber with a mirror under the floor to allow a complete view of the animal and paws. After an initial 20 minutes baseline recording, rats were injected with ACSF or

epibatidine via the DR cannula. The injections were made using a syringe pump, model 11 plus (Harvard Apparatus Inc, Holliston, MA). The volume used was the same in each experiment, 300 nl, and it was infused over 1 minute. Rats were videotaped during the behavioral experiments for later scoring. To score, behavior was rated for 60 minutes after the formalin injection. Using a time-sampling method, rats were scored every 20 sec for pain behavior using four mutually exclusive categories of behavior (Abbott et al., 1999). These consisted of the following:

1) Normal behavior (equal weight bearing on both hindpaws) 2) Favoring (injected paw resting on the floor without pressure on the footpad) 3) Lifting (injected paw elevated without touching the floor) 4) Licking (injected paw licked or bitten).

The observer who evaluated the rats' behavior was not blinded to the type of drug infused or concentration used. However, the evaluation was done before the histological confirmation of the correct placement of the cannulas and the observer did not know whether the study drug was correctly infused into or outside the DR at the time of the behavioral evaluation.

Preliminary observations suggested epibatidine locally administered to the DR influenced motor behavior, therefore offset 20-second intervals and independent from pain behavioral categories, locomotor behavior was also scored using time-sampling method. Behavior was scored as: 1) Normal locomotor, grooming and exploratory behavior, including relaxed stationary postures with natural head and limb movement; 2) Freezing, characterized by complete immobility of all limbs and paws, minimal movement of the head, eyes are open and staring at a specific point with preserved muscle tone (Chung et al., 2000).

8

Histology

At the end of the experiment, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and perfused via the ascending aorta with saline for 2 minutes followed by a 5 min perfusion with 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.6). The brains were then removed, cut in three blocks and left in 4% paraformaldehyde for 24 hours and then left in 25% sucrose overnight. Forty-micron-thick sections were cut on a freezing microtome. Sections including the DR were then stained with neutral red and examined under light microscope to identify the correct placement of the intracranial cannula. Only rats in which the tip of the cannula was located in the DR as defined by the presence of large 5-HT-like cell bodies were considered for the final analysis.

Data Analysis

For each of the pain behavioral data a single composite pain score was derived using the weighted score technique (CPS-WST) described by Dubuisson. For analysis, scores were binned into 5-minute epochs. For each epoch, a pain score was calculated by multiplying the number of observations by a weighted value. Weights by behavioral category were: normal behavior = 0; favoring = 1; lifting = 2; licking/biting = 3 (Abbott et al., 1999;Dubuisson and Dennis, 1977;Watson et al., 1997).

The pain score was compared among groups at different time points using analysis of variance (ANOVA). The area under the curve (AUC) was calculated as a measure of global pain score during phase 2 (time point 10 to 60). Once the analysis of variance for pain score or global pain score showed a highly significant result, the Duncan's multiple range test was further used to perform one-way layout with mean comparisons. Duncan's

test is a result-guided test that compares the group means while controlling the comparison-wise error rate.

RESULTS

Intracerebral cannulas were placed in 46 rats. We could histologically confirm the correct placement of the cannulas within the DR in 31 rats, and we considered the other 15 rats with the cannula outside the DR as control group (OOA group). The placement of the cannulas is shown in Figure 1.

Pain behavior: peripheral epibatidine

Intraplantar administration of formalin induces a biphasic pattern of pain-related behavior. An early acute period (phase 1; 0-9 min), which correspond to an acute pain response, a brief quiescent period, and a second phase of sustained `tonic' pain behavior (phase 2; 10-60 min) which represents a chronic inflammatory (Abbott et al., 1995).

Rats that received subcutaneous saline (control group) exhibited this typical biphasic time course (Figure 2). Pain behavior decreased after the initial 5 minutes peak (phase 1), to rise again after about 10 minutes and peak at 25-30 minutes (phase 2). The pain score of rats that received subcutaneous epibatidine (2.5 and 5 μg/kg) showed an initial peak, which was significantly lower than that observed in the control group ($P<0.001$) (Figure 2). During Phase 2, the pain score after the formalin injection was also attenuated from minute 15 through 40 compared to control rats ($p<0.001$) (Figure 2). The pain behavior equalized to that of control rats after 40 minutes. The global pain score during phase 2 (AUC), was significantly lower in rats that received 2.5 and 5 μg/kg epibatidine compared to the AUC observed in control rats ($p<0.02$) (Figure 3).

Pain behavior: intra-DR epibatidine

Differences in pain scores in animals that received intra-DR epibatidine were analyzed at every time point after the formalin injection. However, in all time points before 30 minutes there is a possible interference of the "freezing" behavior, which was seen after higher doses of epibatidine (see below), on the pain response. Consistent with the appearance of freezing, the initial response to the formalin administration (phase 1) was significantly lower after intracranial administration of 0.01-0.015-0.03-0.06 μg epibatidine (Figure 4) compared to that observed after intracranial ACSF (p=0.0002). When analyzing the phase 2, we observed that the intra-DR administration of 0.01 μg epibatidine (n=5) resulted in similar pain behaviors compared to those observed after ACSF (n=6) (Figure 3), with similar AUC (Figure 3). However, the AUC after intra-DR administration of higher doses epibatidine, 0.015 μg (n=6) - 0.03 μg (n=7) - 0.06 μg (n=7), was significantly lower compared to that seen after the intra-DR administration of ACSF or 0.01 μg epibatidine (p<0.02) (Figure 3). The analysis of pain scores at individual time points after 30 minutes, when freezing scores are at baseline values, we found significant differences in pain scores between rats injected with ACSF and 0.01 μg epibatidine versus rats injected with the higher doses of epibatidine (0.015-0.03-0.06 μg). At this time point the curves of rats treated with higher doses of epibatidine plateau (Figure 4) while those of rats injected with ACSF and 0.01 μg epibatidine continued to rise showing a typical second phase of sustained `tonic' pain behavior. Pain scores were still significantly different at 35 and 40 minutes after administration of 0.015-0.03-0.06

µg epibatidine and became similar to those of rats injected with ACSF and 0.01 µg epibatidine 45 minutes after the drug administration.

Pain behavior score of rats in which epibatidine was injected in areas outside the DR was analyzed as a placement control. Two of these rats received 0.01 µg epibatidine, three 0.015 µg epibatidine, seven 0.03 µg epibatidine and three 0.06 µg epibatidine. The pain scores in this group of rats were similar to those observed in rats injected with intra-DR ACSF or epibatidine 0.01 µg (Figure 5) and significantly higher than those observed in rats in which the cannulas were correctly placed and that received higher doses of epibatidine (p<0.01). The difference was significant at time 30-35-40 after administration of epibatidine (Figure 5).

When analyzing the pain behavior in rats that received mecamylamine prior to the infusion of the study drug (n=7), we did not observed any analgesic effect of epibatidine and the pain score in this group of rats was similar to that of rats infused with ACSF (Figure 6). In addition, freezing behavior was not observed (data not shown).

Freezing Behavior.

The administration of subcutaneous epibatidine (2.5 and 5 µg /kg) did not affect motor behavior. However, injection of epibatidine directly into the DR led to substantial freezing. The duration and intensity of freezing was dose-dependent. Post-injection freezing was greater in rats that received 0.03 and 0.06 µg epibatidine, compared to rats injected with the lower doses (0.015-0.01 µg) as well as rats injected with subcutaneous epibatidine or saline (p<0.001) (Figure 7). Freezing extinguished within 15 minutes in the lower dose experiments and within 25-30 minutes after 0.03 and 0.06 µg epibatidine

respectively (Figure 7), when there was a rapid transition to a normal motor behavior. During this period of time rats were immobile, with an increased muscular tone, and fixed gaze. No evident signs of tonic and/or clonic convulsion were noticed.

DISCUSSION

The original studies on the antinociceptive properties of nicotine implicated the midbrain and descending antinociceptive pathways in mediating nicotine induced antinociception (Iwamoto, 1991). More recent data showed that the direct administration into the nucleus raphe magnus of epibatidine and ABT-594, a novel nAChR agonist, produces antinociception in acute pain models (Curzon et al., 1998). In the present study the administration of epibatidine directly into the DR produced antinociception in a dose-dependent manner. Moreover, epibatidine was at least 100 times more potent when administered into the DR compared to systemically. The minimum effective dose was 0.015 µg/kg. The administration of higher doses (0.03 and 0.06 µg/kg) did not increase the intensity of antinociception. To confirm the regional specificity of epibatidine we quantified the pain score of rats in which the intracranial cannula was located outside of the DR. In contrast to intra-DR infusions, epibatidine administration in sites located outside the DR, in the PAG or areas adjacent to the DR, had no antinociceptive effects even at the highest doses studied. Together, these findings support the conclusion that epibatidine acts specifically in the DR to produce the antinociceptive effects, at least in a chronic pain model.

Our data can not be compared to those of previous studies where epibatidine was infused into the nucleus raphe magnus (Curzon et al 1998). These studies were done using acute pain models (i.e. hot box test) and even though the formalin test consists of two different phases (initial acute phase followed by a tonic response) we could not properly analyze rats' behavior in the initial phase because of freezing. Freezing probably

interfered with the rats' ability to respond to the acute nociceptive stimulus. However, we could determine that antinociception was still present at 30 minutes, when the freezing behavior was no longer evident.

Motor inhibition (freezing) was observed after infusion of epibatidine in the DR. The duration of the freezing behavior was dependent on the dose administered and was significantly prolonged in rats injected with the highest dose. Freezing behavior could reflect anxiogenic activity. Consistent with this possibility, other studies have shown that the electrical stimulation of the DR induces a transient inhibitory reaction similar to that observed in our study (Graeff and Silveira Filho, 1978) and the administration of high dose nicotine in the DR has anxiogenic effects (Cheeta et al., 2001). These effects seem to be mediated by serotonin because they can be blocked by the selective 5HT1A receptor antagonist WAY 100635 (Cheeta et al., 2000(Kenny et al., 2000). Previously, we have shown that serotonergic cells in the DR express α4 nicotinic receptors (Cucchiaro and Commons, 2003). Therefore, it is conceivable that the activation of DR serotonergic cells by epibatidine induces this specific behavior.

A reduced locomotor activity has been reported after the systemic administration and direct infusion into the nucleus raphe magnus of both epibatidine and ABT-594. The motor effects of systemic epibatidine have been attributed in the past to the affinity of epibatidine for neuromuscular nicotinic receptors. ABT-594 has the theoretical advantage of inducing fewer side effects relative to epibatidine because of a preferential selectivity for neuronal α4β2 nAChR. This explanation seems to be an over simplification since a reduced locomotor activity has also been observed after infusion of ABT-594 directly

into the nucleus raphe magnus and in our studies where epibatidine was infused into the DR and not systemically.

It should be noted that we observed freezing only in rats in which epibatidine was infused in the DR and not in rats where the cannula was located in the PAG, even at the highest doses. Freezing is a behavior commonly found also after electrical manipulations of the dorsal PAG area of the midbrain (Borelli et al., 2004). This difference can be explained by the fact that none of the injections made in the PAG area in our study were localized in the dorsal PAG (see Figure 1), which is the area leading to defensive reactions and freezing (Vianna et al., 2003;Brandao et al., 1999).

The results of this study suggest, as it has been previously shown for the PAG (Helmstetter and Landeira-Fernandez, 1990), that the DR could integrate the mechanisms of fear-anxiety and analgesia. However, the sensitivity to epibatidine of the two neuronal processes is different because the antinociceptive effect could be dissociated from freezing behavior at a low dose (0.015 µg) of epibatidine.

The analgesic and motor effects of epibatidine were blocked by the prior administration of mecamylamine into the DR, suggesting that both effects were secondary to stimulation of nAChR and specific for activation of DR neurons.

In conclusion, data from the present study provide evidence consistent with the possibility that the antinociceptive action of epibatidine and henceforth other nicotinic agonists may in part be mediated by activation of the dorsal raphe. The major limitation to the clinical use of nicotinic agonists is their toxicity, convulsions being one of the most severe. We have shown that the intra-DR administration of antinociceptive doses of

epibatidine does not cause tonic-clonic convulsions. However, it does result in behavior

manifestations that resemble a fear or anxiogenic response.

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Footnote to the title:

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Legends

Figure 1 Coronal sections of the midbrain showing the localization of the intracranial

cannulas. Sections are arranged in a caudal to rostral order.

A-D) Dorsal raphe nucleus.

B-E) Dorsal and Ventro-Lateral sections of the dorsal raphe.

C-F) Caudal sections of the dorsal raphe.

G) Rostral sections of the periaqueductal gray area.

PAG: periaqueductal gray area.

DR: dorsal raphe

AQ: aqueduct

EW: Edinger Westphal nucleus.

LVT: lateral dorsal tegmental nucleus

VTN: ventral tegmental nucleus

A-C: Numbers indicate the location of the cannula for each drug dose as follows:

1) ACSF; 2) epibatidine 0.01 µg; 3) epibatidine 0.015 µg; 4) epibatidine 0.03 µg; 5)

epibatidine 0.06 µg

D-G: Location of injection sites where cannula miss the dorsal raphe nucleus, primarily

located in the PAG. Numbers indicate the drug dose as in A-C.

Figure 2

Pain behavior score in rats injected subcutaneously with saline, epibatidine 2.5 µg/kg and

epibatidine 5 µg/kg. **Phase 1:** the pain score was significantly different between rats

injected with saline and those that received 2.5 and 5 µg/kg epibatidine (**p<0.001).

Phase 2: the pain score was significantly lower at 15-25-30-35 and 40 minutes both in rats treated with epibatidine 2.5 and 5 µg/kg compared to control rats. (p<0.001) (mean±SE; ANOVA test ± Duncan's multiple range test)

Figure 3

Area under the curve (AUC) during Phase 2 after administration into the dorsal raphe (DR) of ACSF, epibatidine 0.01-0.015-0.03-0.06 mcg or after the administration of saline, 2.5 and 5 mcg/kg epibatidine subcutaneously. OOA represents rats that received epibatidine in the PAG areas, outside the DR. *p<0.02 epibatidine 0.015-0.03-0.06 mcg versus intra-DR ACSF, epibatidine 0.01 mcg or OOA infusions. +p<0.02 epibatidine 2.5 and 5 mcg/kg sc versus saline sc (mean±SE).

Figure 4

Pain behavior score in rats injected into the dorsal raphe with different doses of epibatidine or ACSF. **Phase 1**: a significant difference in rats pain score was detected 5 minutes after administration of 0.01-0.015-0.03-0.06 epibatidine and after ACSF (**p=0.0002, ACSF versus 0.01-0.015-0.03 and 0.06 epibatidine). **Phase 2:** The curve showing the pain score in rats treated with 0.015-0.03 and 0.06 µg plateau 30 minutes after the administration of epibatidine, while continued to rise in rats injected with ACSF or epibatidine 0.01 µg. The difference between rats that received either 0.015-0.03 or 0.06 and rats that received either ACSF or 0.01 epibatidine became significant at time 30-35 and 40 minutes. * p=0.001 (mean±SE; ANOVA test ± Duncan's multiple range test)

Figure 5

Pain behavior score in rats injected into the dorsal raphe with epibatidine 0.01 and 0.06 µg or ACSF and rats injected with different doses of epibatidine (0.01-0.015-0.03-0.06 µg) in areas outside the dorsal raphe. The difference between the pain score of rats injected with epibatidine 0.06 µg in the dorsal raphe versus those injected outside the dorsal raphe area is significantly different at time 5 (**Phase 1**) and 30-35 and 40 minutes (**Phase 2**). * $p<0.0001$ + $p<0.05$ (mean±SE; ANOVA test ± Duncan's multiple range test)

Figure 6

Pain behavior score in rats injected into the dorsal raphe with ACSF, 0.015 µg epibatidine and mecamylamine 1 µg followed by 0.015 µg epibatidine. The pain score in rats injected with mecamylamine prior to the infusion of epibatidine was similar to that of rats injected with ACSF and significantly higher than that of rats infused with epibatidine alone at time 5 **(Phase 1)** and 30-35 and 40 minutes **(Phase 2)**.

** $p<0.0002$ * $p<0.001$ (mean±SE; ANOVA test ± Duncan's multiple range test)

Figure 7

Freezing behavior: Rats injected with epibatidine intra-DR experienced freezing for a significantly higher percent of the observation time compared to rats injected with subcutaneous epibatidine. Rats injected intra-DR with higher doses of epibatidine (0.03-0.06 mcg) experienced freezing for a significantly higher percent of the observation time

and longer period of time compared to rats that were injected with lower doses (0.01-0.015 µg).

*p<0.001 epibatidine 0.03 µg DR and 0.06 µg DR versus ACSF DR, epibatidine 0.01 µg DR, epibatidine 0.015 µg DR, saline sc, epibatidine 2.5 µg/kg sc and epibatidine 5 µg/kg sc; +p<0.03 epibatidine 0.01 µg DR and epibatidine 0.015 µg DR versus ACSF DR, saline sc, epibatidine 2.5 µg/kg sc and epibatidine 5 µg/kg sc (mean±SE; ANOVA test ± Duncan's multiple range test)
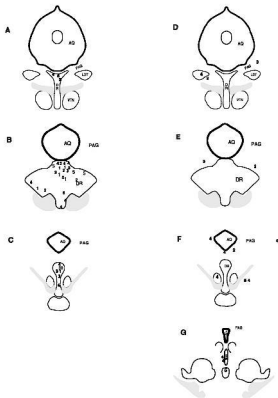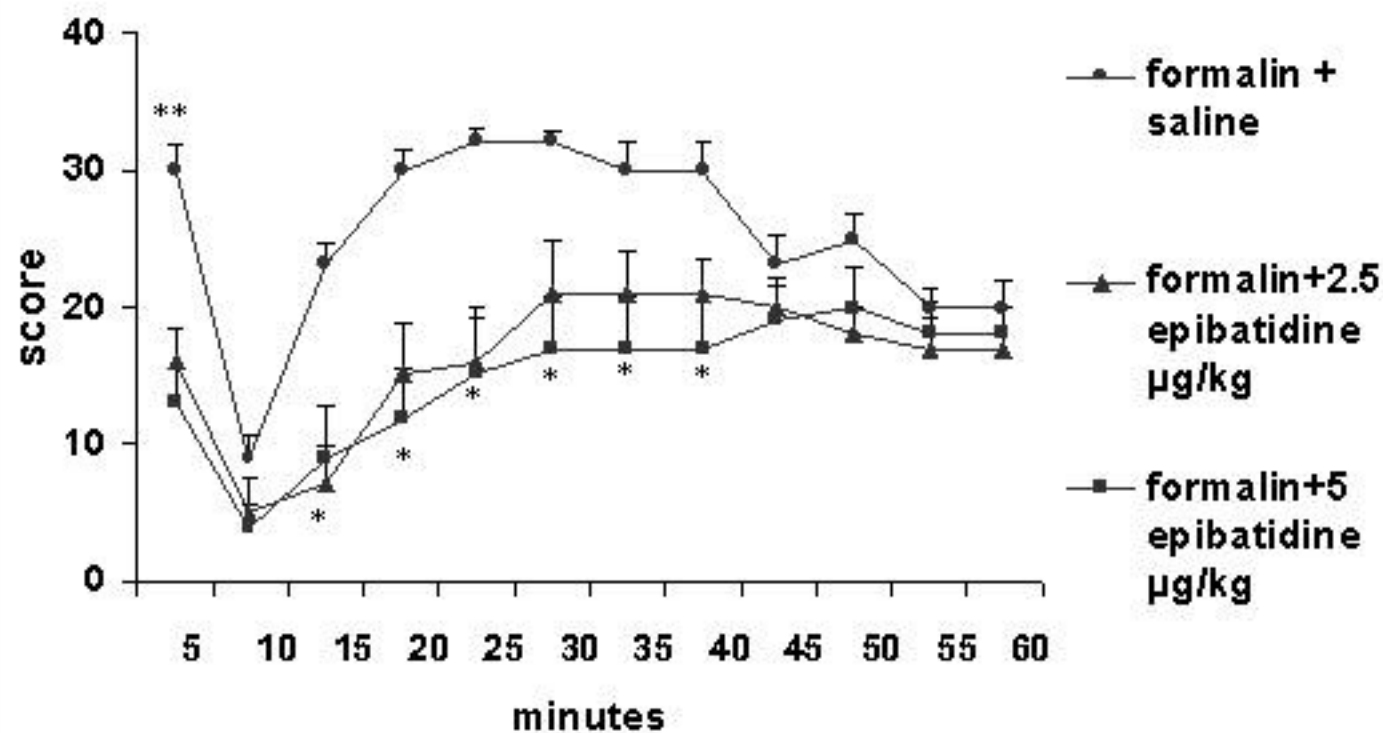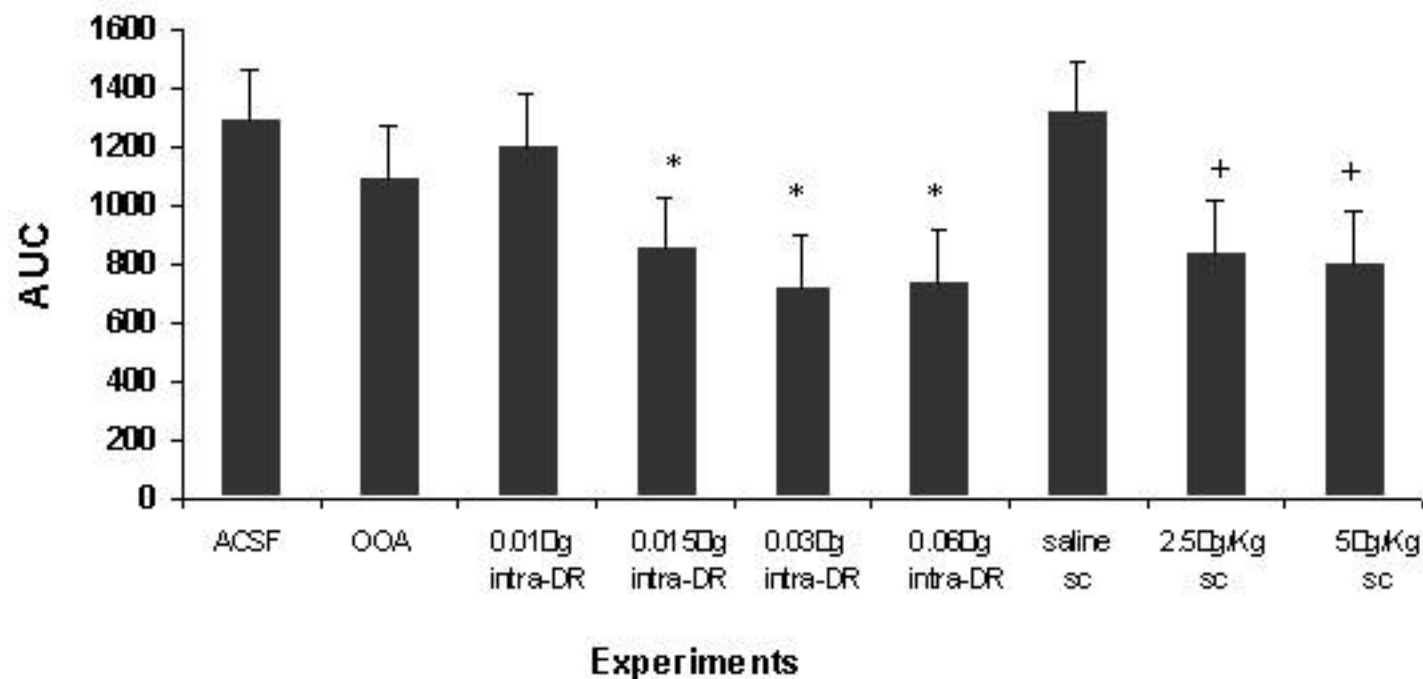
FIGURE 1
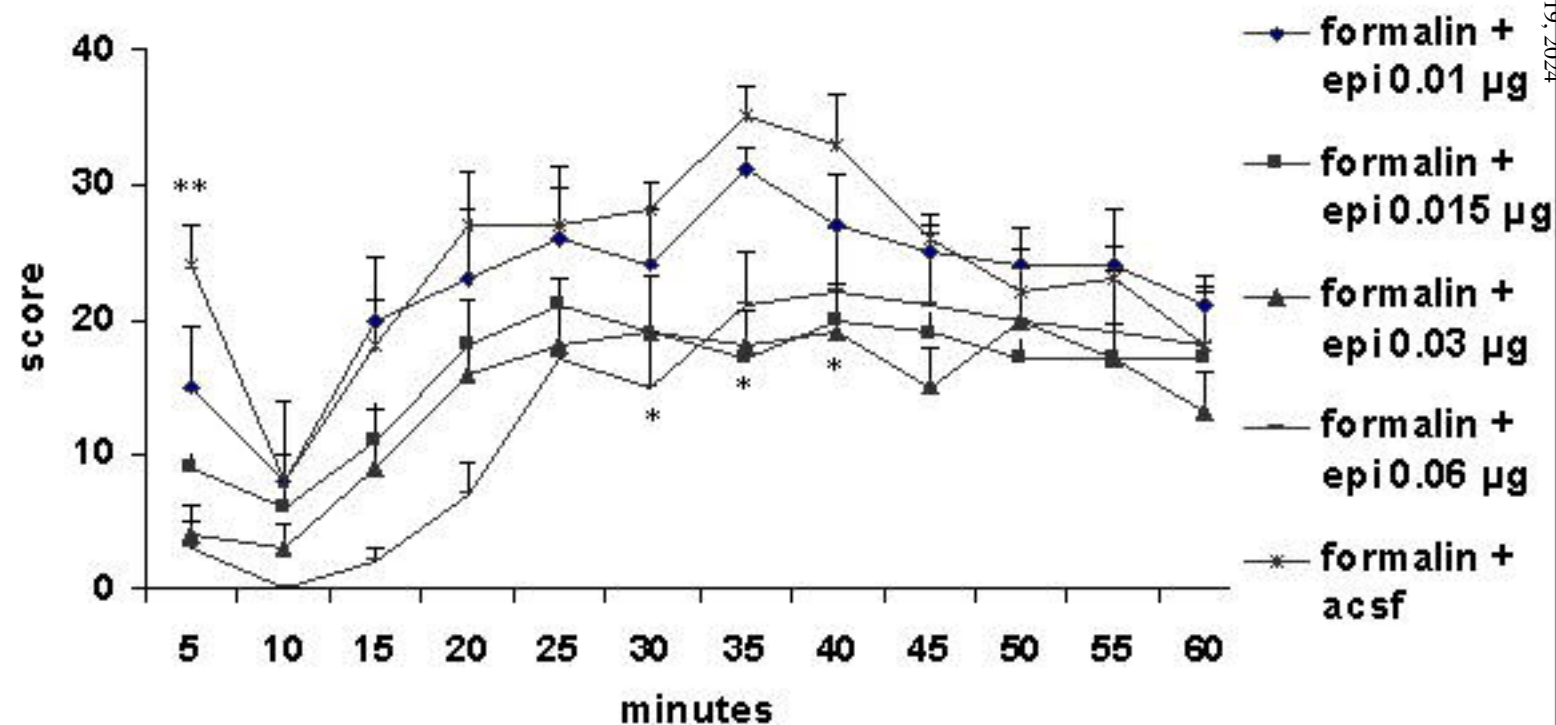
FIGURE 2

FIGURE 3

FIGURE 4

Legend:
- formalin + epi 0.01 µg
- formalin + epi 0.015 µg
- formalin + epi 0.03 µg
- formalin + epi 0.06 µg
- formalin + acsf

(y-axis: score; x-axis: minutes)

FIGURE 5

FIGURE 6

FIGURE 7