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Research report

Amniotic-fluid ingestion enhances central δ-opioid-induced hypoalgesia in rats in the cold-water tail-flick assay in a repeated-measures design

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ABSTRACT

Placental Opioid Enhancing Factor (POEF) is found in amniotic fluid (AF) and placenta. When ingested, it enhances opioid-mediated pain relief. Our laboratory has shown that ingestion of AF specifically enhances the hypoalgesia associated with δ-opioid receptor activation in the brain. The specific biochemical compound in AF responsible for the enhancement of δ-opioid activity is of great interest as an analgesic adjunct for pain but is unknown at this time. Research efforts to isolate and characterize this biochemical compound are hampered by the lack of an algesiometric assay that allows repeated measurement of pain threshold and repeated exposure to δ-opioid receptor activation. The cold water tail-flick assay (CWTF) may be a sensitive and reliable pain threshold test of (a) all species of opioids that is (b) not subject to repeated-testing effects. Therefore the CWTF test is potentially ideal for the study of δ opioid systems in a repeated measures design. Here, we confirm these attributes of the CWTF test, and determined that (a) there are no repeated-exposure effects associated with the CWTF assay; (b) there are no repeated-exposure effects associated with repeated central injections of DPDPE ([D-Pen2,D-Pen5]-Enkephalin, a selective δ-opioid agonist) as measured by the CWTF assay; and (c) ingestion of AF in conjunction with a central injection of DPDPE produced the same hypoalgesic enhancement as previously found using another assay.

1. Introduction

Research over the last 30 years in our laboratory has shown that a molecule or molecules found in placenta and amniotic fluid (AF), when ingested, modifies some of the behavioral effects of central opioids. One of the most important of these modifications is the potentiation of the hypoalgesic property of opioids, whether endogenous (e.g., "analgesia of pregnancy", vaginal-cervical mechanical stimulation) or exogenous (e.g., morphine injection) (Kristal, 1991; Kristal et al., 2012). This hypoalgesia-enhancing substance was eventually termed Placental Opioid-Enhancing Factor (POEF) (Kristal et al., 1986). The enhancing effect of POEF was shown in both male and female subjects, and has been demonstrated in rats, mice, and cows (Gurgel et al., 2000; Kristal et al., 2012; Pinheiro-Machado et al., 1997). POEF must be ingested to be effective, and POEF activity is found in both AF and placenta of all species tested, suggesting that the mechanism for responding to POEF may be ubiquitous among mammals (Abbott et al., 1991; Kristal et al., 2012). DiPirro et al. found that POEF actions are centrally rather than peripherally mediated (DiPirro et al., 1991), and that the effect is to enhance δ- and κ-opioid-induced events (hypoalgesia) while attenuating μ-receptor-mediated events (DiPirro and Kristal, 2004).

Isolation of the molecule and characterization of its structure and full mode of action may allow for the development of a novel approach to the management of pain and addiction.

Opiate-induced hypoalgesia has historically been measured with algesiometric assays using such stimuli as radiant heat (conventional tail-flick test), hot-water tail-immersion (the hot-water tail-flick test), hot plate, and formalin injection (D'Amour and Smith, 1941; Woolfe and Macdonald, 1944). The assays were derived from pain-threshold studies performed on human subjects, and seemed to be sensitive to the analgesics they were interested in testing (e.g., morphine sulfate, codeine sulfate, Demerol). These algesiometric assays are still commonly used to test analgesic drug efficacy, with little variation from the procedures reported initially. However, several studies have also shown that algesiometric tests that rely on heat as a stimulus, such as the hot-water tail-flick, radiant-heat tail-flick, and the hot-plate assays have two main limitations: (a) they are not equally sensitive to all opioid agonist

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species in all routes of administration; and (b) they are not amenable to repeated-testing designs. As to the first limitation, the assays have differential sensitivities across the range of opioid agonists and partial agonists with heat-based assays showing the greatest sensitivity and range of measurement for μ-opioid mediated effects and the least for κ-induced antinociception (Abbott et al., 1986; Adams et al., 1993; Hayes et al., 1987; Leighton et al., 1987; Shaw et al., 1988; Tyers, 1980).

In regard to the second limitation – that of repeated-testing effects – assays like the hot plate, radiant heat tail-flick, and the hot-water tail-flick have shown repeated-exposure effects that result in a gradual decrease in baseline latency measurements when retesting the same subjects. Advokat (1989), Advokat and McInnis (1992) and, more recently, Lane and Morgan (2005), showed that repeated exposures to a radiant-heat tail-flick test produced a decrease in response latency over time, regardless of drug injection history (morphine or saline). The data suggest that tail-flick or hot-plate latencies for animals receiving vehicle injections might still be affected by repeated-testing effects, termed by some as "behavioral tolerance" (Advokat, 1980). The decrease in baseline latency due to repeated-measures effects via behavioral tolerance to the antinociceptive assay is likely to skew data in a within-subjects research design and to be interpreted as a drug tolerance effect. This produces a severe limitation on designs that would benefit from having subjects serve as their own controls.

Clearly, the assays originally developed to test the analgesic properties of μ-opioid receptor actions may not be appropriate to measure the actions of the many different opioid receptor agonists, partial agonists, and combined agonist-antagonists that have become available since the assays were developed, nor can they be used for efficient experimental designs utilizing repeated measures. Pizziketti et al. (1985) proposed experiments using a new algesiometric assay called the cold-water tail-flick test. This test followed a protocol similar to that of the hot-water tail-flick assay with the exception of a cold rather than hot noxious stimulus and seems to be responsive to a broader range of opioid agonists (Adams et al., 1993; Chen et al., 2007; Pizziketti et al., 1985; Wang et al., 1995). The data also suggested the absence of a repeated-measures effect (Chen et al., 2007).

Wang et al. (1995) performed experiments on the sensitivity of the cold-water tail-flick test to several opioid agonists, opioid agonist-antagonists, and non-opioid analgesic drugs. The results of the experiments are similar to the findings of Pizziketti et al. (1985) showing that the cold-water tail-flick test is sensitive to opioid-induced hypoalgesia from each of the three opioid receptor sub-types, but not to that produced by non-opioids. More recently, Chen et al. (2007) published data showing that the cold-water tail-flick test is sensitive to a broad range of opioids and may not be vulnerable to the same repeated exposure effects as the hot-water tail-flick test.

The purpose of the present study was two-fold. (1) It was a methodological investigation designed to test the sensitivity and reliability of the cold-water tail-flick assay as a measure of hypoalgesia in a repeated-measures design, to determine if it the impact of repeated measures differs from that described for the hot-plate and hot-water tail-flick assays (Advokat, 1989; Advokat and McInnis, 1992; Hayes et al., 1987; Tyers, 1980). A reliable pain threshold assay that can be utilized in a repeated-measures design would facilitate the search for the mechanisms and biochemical characteristics of POEF, because many more studies must be conducted to further understand the parameters of POEF effects and its actions at specific receptor sites. The use of a repeated-measures design allows for the use of far fewer animals than does a non-repeated-measures design. (2) It is a continuation of the investigation of POEF enhancement on opioid-mediated hypoalgesia, specifically POEF enhancement of δ-opioid-receptor-induced hypoalgesia to a cold noxious stimuli. As mentioned previously, DiPirro and Kristal (2004) found that POEF ingestion enhanced central δ- and κ-opioid receptor induced hypoalgesia, but not μ-opioid receptor induced hypoalgesia. For this reason, the selective δ-opioid receptor agonist, [D-Pen2,D-Pen5]-Enkephalin (DPDPE) was used to determine the degree of POEF-induced enhancement. The design of the following experiments allowed us (a) to determine conclusively whether repeated-exposure effects associated with repeated central DPDPE injections are eliminated by the use of the cold-water tail-flick assay and (b) to confirm whether ingestion of AF in conjunction with a central injection of DPDPE produces enhancement of hypoalgesia produced by DPDPE alone (DiPirro and Kristal, 2004).

2. Results

Of the 30 rats tested, 23 were used in the final data analysis. Before testing began a maximum cut-off time of 60 s for the CWTF assay was assigned based on preliminary pilot data and cut-off times previously used by others (Chen et al., 2007; Pizziketti et al., 1985). Two rats reached the maximum cut-off time during baseline (>3 SD higher than the average baseline value) and their data were not used. Four were removed due to cannula misplacement. One rat was euthanized during the study due to loss of the indwelling cannula during testing.

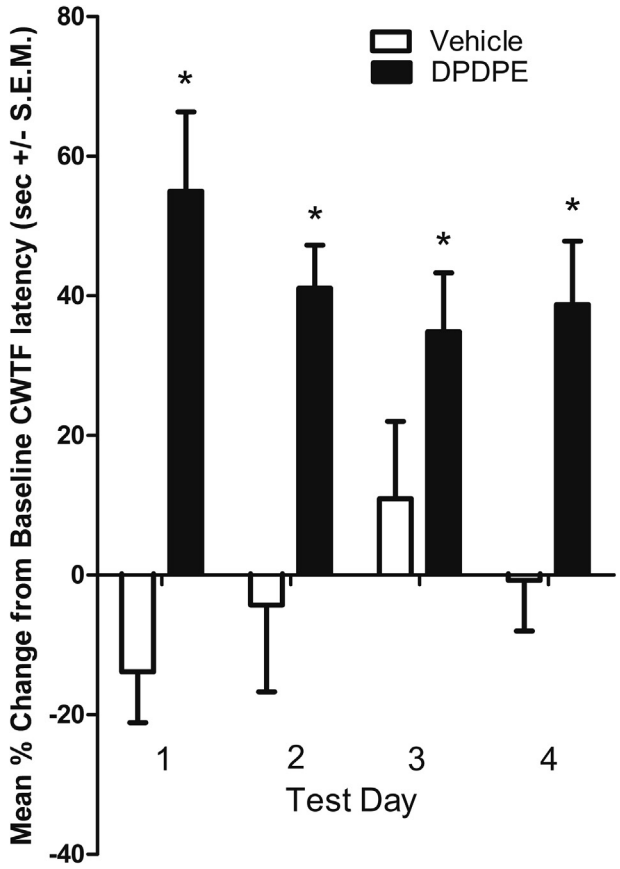

Fig. 1. Results from Experiment A. The Mean percent change from baseline in cold-water tail-flick latency of rats (±S.E.M.) in sec after a 5-μl i.c.v. microinjection of either VEH or 50 ng of DPDPE across 4 days of testing. Test days were separated by 3-day intervals. * = significantly different from VEH within that Day ($p < 0.0001$). No significant differences between Days were found in either DPDPE or VEH treated rats.

2.1. Experiment A

The results of this experiment are represented in Fig. 1. Baseline CWTF latencies ranged from 7.22 ± 0.90 s to 9.17 ± 1.25 s and did not differ significantly by Drug Treatment ($F_{1,21} = 1.33$, $p < 0.26$), Day of testing ($F_{2,48} < 1$), or individual group (i.e., Drug Treatment X Day ($F_{2,48} < 1$). A 2-way ANOVA on Day (1; 2; 3; 4) X Drug Treatment (VEH; DPDPE), with repeated measures on Day, was conducted on percent change from baseline CWTF latency. The results of the ANOVA revealed a significant Drug Treatment effect ($F_{1,21} = 26.46$, $p < 0.0001$), but no significant 2-way interaction ($F_{3,55} = 2.58$, $p = 0.07$) or main effect of Day ($F_{3,55} < 1$).

These results indicate that the DPDPE- and VEH-treated groups had similar baseline CWTF latencies throughout testing, suggesting that there were no underlying differences between the drug-treatment groups that could confound the results of the experiments. Further, and as expected, an i.c.v. injection of DPDPE (50 n g/5 μl) induced a significant increase in CWTF latency, suggesting an increase in pain threshold. No significant differences in CWTF response after VEH or DPDPE injections were observed over different days of testing, indicating that there was no effect of repeated exposure to the CWTF assay and no effect of repeated exposure on DPDPE-induced change from baseline CWTF latency. This indicates that (a) there were no repeated testing effects due to the assay; and (b) that the repeated exposure to DPDPE (50 ng i.c.v. 4 times at 72 h intervals) does not alter the rats' CWTF response to DPDPE at a later exposure (see Fig. 1).

2.2. Experiment B

The results of Experiment B (Days 5 and 6) are illustrated in Fig. 2. A 3-way ANCOVA on Drug Treatment (DPDPE; VEH; i.c.v.) X Enhancer (AF; SAL; p.o.) X Test (Baseline, Post-Drug) with repeated measures on Enhancer and Test and using Order of Enhancer (AF-SAL; SAL-AF) and Drug Treatment History (DPDPE or VEH in Experiment A) as covariates, was conducted on Mean CWTF latency. It revealed a significant 3-way interaction ($F_{1,19} = 4.93$, $p = 0.039$); main effects of Drug Treatment ($F_{1,15} = 61.71$; $p < 0.0001$) and Enhancer ($F_{1,15} = 13.89$; $p = 0.002$); and a significant 2-way interaction of Drug Treatment X Enhancer ($F_{1,15} = 8.81$; $p = 0.01$). There were no effects of the Order of Enhancer presentation and no effect of the Drug Treatment History.

Probes of the Drug Treatment X Enhancer interaction revealed a significantly greater change from baseline CWTF latencies in rats receiving DPDPE than in rats receiving vehicle, regardless of orogastrically infused substance: SAL ($F_{1,27} = 21.6$; $p < 0.0001$); AF ($F_{1,27} = 67.2$; $p < 0.0001$). There was a significantly greater increase from baseline CWTF latencies in rats receiving i.c.v. DPDPE coupled with an AF infusion than in rats receiving i.c.v. DPDPE coupled with a SAL infusion ($F_{1,15} = 25.23$; $p = 0.0002$). There was no significant effect of orogastric AF or SAL in the i.c.v. VEH groups; those groups did not differ significantly from each other ($F_{1,15} < 1$]. These results indicate that a central injection of DPDPE given in conjunction with an orogastric infusion of the control enhancer (SAL) showed increased CWTF latencies, similar to the DPDPE-induced hypoalgesia found in Experiment A. However, as is the case using other algesiometric assays (DiPirro and Kristal, 2004), a central injection of DPDPE in conjunction with AF ingestion caused a greater increase in pain thresholds than did DPDPE in conjunction with SAL.

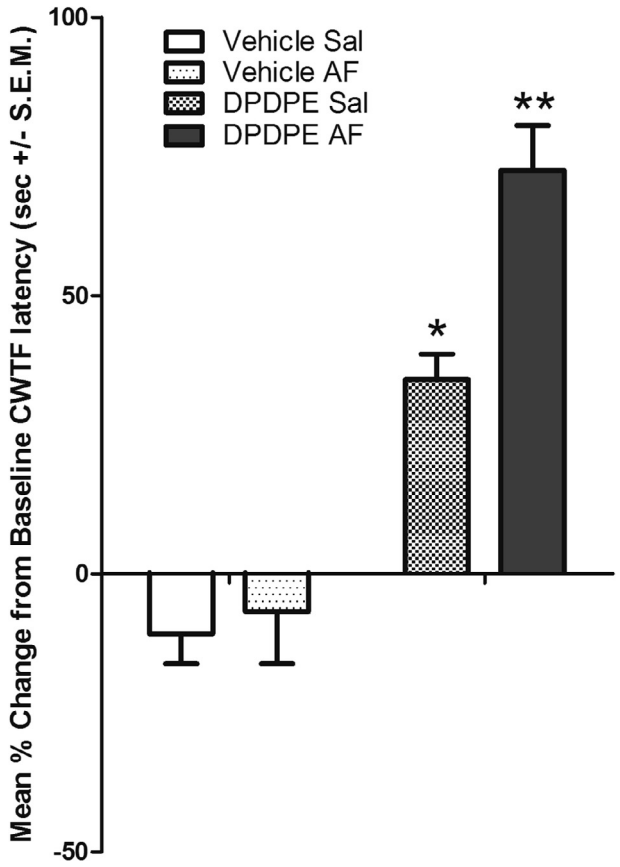

Fig. 2. Results from Experiment B. The Mean percent change (±S.E.M.) from baseline cold-water tail-flick latency of groups in which rats were given an orogastric infusion of 0.25 ml of either saline (SAL) or amniotic fluid (AF) immediately followed by a 5-μl i.c.v. microinjection of either vehicle (VEH) or DPDPE. The groups were divided as follows: VEH/SAL, VEH/AF, DPDPE/SAL, and DPDPE/AF. * = significantly different from VEH/SAL and VEH/AF groups ($p < 0.0001$); ** = significantly different from all other groups ($p = 0.0002$).

3. Discussion

The purpose of these experiments was two-fold: to verify the reliability of the cold-water tail-flick assay to be used to assess pain thresholds, as suggested by CWTF latencies, in a repeated measures design; and to determine whether DPDPE-induced hypoalgesia is enhanced by AF ingestion in the CWTF assay.

In summary, centrally injected DPDPE (50 ng/5 μl) produced a significant elevation of cold-water tail-flick (CWTF) latency (increased hypoalgesia); this replicates previously results found with the hot-plate assay (DiPirro and Kristal, 2004). The DPDPE-induced increase in CWTF latency was further enhanced when an orogastric infusion of AF was administered in conjunction with the central injection of DPDPE. There was no evidence of a repeated-measures effect associated with repeated exposure to either the CWTF assay or to DPDPE, as baseline measurements and drug-induced increases in CWTF latency across sessions did not differ throughout Experiments A and B. There was also no effect of drug-treatment history on the enhancing effect of orogastric AF on i.c.v. DPDPE in the second experiment.

The results of Experiment A and B indicate that there are no significant repeated-measures effects associated with this assay or associated with repeated DPDPE exposure. Repeated exposure to central DPDPE has been shown to induce analgesic tolerance in some studies (e.g., Kovács et al., 1988), but not others (e.g., Bartok and Craft, 1997). The expression of tolerance to the analgesic effects of opioids, largely studied for morphine tolerance, is influenced by a number of variables including the interval between doses and the assay used. Our study employed a 72-h test-retest interval, which may be long enough to prevent the development of longer lasting analgesic tolerance, and of course, used an assay

that we found did not produce a repeated-measures effect, which, as mentioned above, might be misinterpreted as the formation of tolerance. This should be regarded as an additional advantage of the paradigm we tested.

The results demonstrate the assay's adaptability to a repeated-measures design and suggests that it can be employed multiple times both within short (i.e., within a day) and long (over days) inter-test intervals. This result is consistent with other published results (Advokat, 1989; Advokat and McInnis, 1992; Chen et al., 2007; Lane and Morgan, 2005). The one condition not tested for in the repeated-measures procedure in Experiment A was the condition in which DPDPE was administered in conjunction with an orogastric infusion of AF.

The mean CWTF-latency baseline (8.28 s ± 0.67 s) was within a reasonable time range for a baseline measurement, as only 2 of 30 rats even approached the maximum cut-off time of 60 s. In addition, baseline latencies within this range allowed for detection of DPDPE-induced hypoalgesia (35.11% ± 4.12% increase from baseline), as well as for AF enhancement of DPDPE-induced hypoalgesia (71.87% ± 9.65% increase from baseline). This evidence supports the idea that the CWTF assay can be used in our research in preference to the hot-water tail-flick and hot-plate assays. The attributes of the cold-water tail-flick latency assay, namely its rapid response relative to the cutoff, which allows for the measurement of both drug-induced hypoalgesia and enhanced hypoalgesia, its sensitivity to central δ-opioid activity (perhaps the most difficult receptor activity to measure), and its ability to produce stable results across repeated measurement, supports the idea that it may be perhaps the most desirable algesiometric assay for use in this type of investigation. Repeated-measures designs allow for the most data to be collected from the fewest subjects, thereby achieving the goal of minimizing animal use.

A goal in Experiments A and B was to replicate the findings of DPDPE-induced hypoalgesia and its enhancement by ingested AF in the CWTF assay. We expected that DPDPE-produced hypoalgesia would be enhanced by POEF ingestion (in AF or placenta) as has been shown previously in our laboratory using a hot-plate assay to measure pain or discomfort threshold (DiPirro and Kristal, 2004). These results extend previous research by demonstrating that the effect of POEF ingestion on opioid-induced hypoalgesia include cold-stimulus-induced pain or discomfort.

Our ultimate goal in studying the POEF effect is to determine how to utilize the modulatory effect of POEF with specific opioid receptor ligands to create a drug therapy designed to avoid the negative side effects of repeated opioid drug treatments (tolerance and sensitization to the effects of chronic morphine treatment, for example), and be able to consistently provide pain relief to manage pain in humans and other animals.

4. Conclusions

a) The cold-water tail-flick assay is sensitive to the hypoalgesic effects of a centrally administered δ-opioid agonist.
b) The cold-water tail-flick assay can be used effectively in repeated-measures designs, even those spanning days, and can therefore be useful in significantly reducing the number of subjects needed.
c) Repeated i.c.v doses of DPDPE do not produce noticeable physiological/behavioral tolerance in the cold-water tail-flick assay using a 72-h test-retest interval.
d) The effect of combining amniotic fluid (and therefore POEF) ingestion with i.c.v. DPDPE was to enhance the increase in CWTF latency produced by DPDPE alone in response to a noxious cold stimulus. The effect of combining amniotic fluid (and therefore POEF) ingestion with i.c.v. DPDPE was only performed once, so induction of tolerance in this combination was not examined.

5. Methods

5.1. Animals and housing

Subjects were 30 experimentally naïve, virgin female Long-Evans, hooded, rats. Rats were 2–3 months old, weighing 200–300 g. The rats were purchased from Harlan Laboratories (Harlan Sprague Dawley, Indianapolis, IN). All procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee and are consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8/e, revised 2011).

Rats were singly housed in standard plastic cages and maintained in a controlled environment of 22° ± 1 °C, 40–60% relative humidity, on a 14 h-on/10 h-off light/dark cycle (lights on at 0700 EST) and received *ad libitum* access to standard lab rat chow (Teklad Rodent Diet 2018, Harlan Teklad, Madison, WI) and water, except as noted.

5.2. Stereotaxic implantation

After at least one week of acclimation to the laboratory, rats underwent stereotaxic surgery to implant a single, permanent, indwelling guide cannula (22-ga, Plastics One, Roanoke, VA) through which the δ-opioid agonist, [D-Pen2,D-Pen5]-Enkephalin (DPDPE, American Peptide Company, Sunnyvale CA) could be injected directly into the right lateral ventricle. Anesthesia was induced and maintained with isoflurane gas (1–3%) during surgery; carprofen (5 mg/kg, s.c.) was administered immediately after, and again 24 h after, surgery. Coordinates were determined using the stereotaxic atlas of Pellegrino et al. (1979): AP: 0.0 mm (bregma); ML: −1.7 mm (from midsagittal suture); DV: −2.9 mm (from dura), with the incisor bar set to 5 mm above interaural line. The guide cannula was anchored to the skull with dental cement and supported by 4 stainless steel jeweler's screws. A stainless steel obturator was inserted into the guide cannula. It filled the guide cannula, but did not extend beyond the tip of the guide cannula. Rats were given 5 days to recover in their home cage.

5.3. Microinjections

Microinjections were performed using a BASi microinjection pump (Bioanalytical Systems, West Lafayette, IN) at a rate of 1 μl/min over a 5-min period, for a total injection volume of 5 μl for 50 ng of DPDPE. DPDPE was mixed in a sterile saline vehicle. Microinjections were performed in a separate room from either the testing room or colony room. Rats were freely moving in their home cage with the wire cage top removed during the microinjection. After the microinjection was complete, the injector was kept in the guide cannula for an additional 30 s to allow the drug to disperse from the tip of the cannula. The obturator was replaced immediately after the injector was removed to prevent backflow of the drug up the guide cannula. The 50 ng dose of DPDPE was the same as that used by Chen et al. (2007) in their cold-water tail flick assay study. Sham microinjections were performed by attaching tubing with a screw-on cap to the cannula for 5 min, with the syringe pump running.

5.4. Amniotic fluid collection

AF was collected on Day 21 of pregnancy from female rats euthanized with CO_2 as described previously (Kristal et al., 1986) and

immediately stored at −40 °C for later use. AF was heated for 15 min using a heating block set to 37 °C immediately before orogastric infusion.

5.5. Orogastric intubation

AF or saline control was infused directly into the stomach via orogastric intubation. As one experimenter gently restrained the rat and held a plastic speculum in its mouth, a second experimenter guided tubing attached to a syringe down the throat of the rat until the tubing reached the stomach (DiPirro et al., 1991). Sham infusions (intubation without infusion) were performed the same way, but with an empty syringe.

5.6. Cold-water tail-flick assay

5.6.1. Apparatus

A Neslab Digital Plus Refrigerated Bath (Thermo Scientific) was used to circulate a 40% ethylene glycol in distilled water solution and maintained at −10 °C.

5.6.2. Procedure

Each rat was gently restrained in a black sock and held firmly over the water bath. The distal quarter of its tail was dipped into the circulating ethylene glycol solution. The latency to a complete withdrawal of the tail from the water served as the dependent measure, with a maximum latency allowed of 60 s.

Each cold-water tail flick (CWTF) test consisted of three trials. After each, the distal quarter of the rat's tail was dipped into room temperature water for 15 s, and wiped once with a tissue. The rat was held for 30 s until the next trial began. The dependent variable, latency to withdraw the tail, was calculated by averaging the second and third trials of each CWTF test.

On testing days, each rat was tested in the CWTF assay twice, once to obtain a pre-injection baseline measurement, and a second time to obtain a post-injection measurement (after receiving a drug or vehicle by i.c.v. microinjection).

5.7. Habituation procedures

All subjects were handled and habituated to all testing procedures: orogastric intubation, microinjection, and the cold-water tail flick assay, for the 3 days preceding stereotaxic surgery. On habituation days each rat was given a sham orogastric intubation (with no infusion), followed by exposure to the handling (i.e., gentle restraint of the head) and sounds of the microinjection procedure, and then was restrained in a sock and held over the running, but empty, cold-water tank for approximately 3 min. During habituation to the cold-water tank, the rat's tail was wiped once every minute with a tissue.

5.8. Experiment A (Days 1–4)

Rats received a sham orogastric infusion (intubation only) immediately before receiving a sham i.c.v. microinjection and were then returned to the colony room. After 10 min in the colony room, rats were brought into the CWTF testing area and a baseline tail-flick latency measurement was obtained. Sham procedures were used to control for any stress associated with the intubation or microinjection procedure that could have an impact on the outcome of the CWTF measurement. One hour later, rats were given another sham infusion and then an i.c.v. microinjection of DPDPE or VEH, and returned to the colony room. After 10 min in the colony room, rats were brought into the CWTF testing area to obtain a post-injection CWTF measurement. The experimenter performing the CWTF procedure was blind to the experimental condition of the rats.

5.9. Experiment B (Days 5–6)

All procedures remained the same for Days 5 and 6 except that the sham infusion before the microinjection of drug (DPDPE or VEH) was replaced with an orogastric infusion of 0.25 ml AF or SAL.

5.10. Design

The general approach was to administer DPDPE or VEH i.c.v. to rats every 4 days, for 4 times, and then randomly divide these two groups in half and reassign one half of each group to the other drug condition. This created 4 groups: (a) one that received DPDPE on every test day; (b) one that received VEH every test day; (c) one that received i.c.v. DPDPE on the first 4 test days and received VEH on Days 5 and 6; and (d) one that received VEH on the first 4 test days and received DPDPE on Days 5 and 6. This was done to test the possibility that repeated exposure to DPDPE would alter later performance in the CWTF. On Days 1–4, all rats received sham infusions before each i.c.v. microinjection, but on Days 5 and 6, all rats received an orogastric infusion of the enhancer, either AF or saline, before they were microinjected. This was done to compare the analgesic effect of DPDPE with and without an enhancer, as well as verify that there is no effect of the enhancer alone. Presentation of enhancer type (AF or saline) was randomly balanced across days by rat and counterbalanced for DPDPE history. Rats receiving an orogastric infusion with one enhancer type on Day 5 received the other enhancer type on Day 6 (i.e., half the rats received AF on Day 5 and saline on Day 6 and vice versa).

5.11. Statistical analysis

Parametric statistical analyses were conducted using the IBM SPSS statistical package (v.24, IBM Inc.). The dependent variable analyzed, for expository reasons, was the percent change from baseline latency (after analysis of baseline data showed that there were no systematic group differences in latencies at the start of each session).

For Experiment A, the data were analyzed using a repeated-measures ANOVA with a Greenhouse-Geisser probability correction for violations of sphericity in a repeated-measures design. For Experiment B, the data were analyzed by a repeated measures ANCOVA using Drug history, DPDPE or Veh exposure during Experiment A, and order of AF exposure as covariates. In both experiments, post-hoc probes of significant interactions used adjusted MS_{error} from the overall analyses. The alpha level for all experiments was set at $p \leq 0.05$.

5.12. Histology

At the end of the study, each rat was euthanized with CO_2. Methyl blue dye (0.5 μl) was then injected through the cannula, the brains were extracted and frozen at −20 °C until cut into sections on a cryostat at 30 μm, mounted onto slides, and examined under a microscope to determine accurate placement. A placement was considered accurate if the dye was present in the ventricle and if the cannula track led into but not beyond the ventricle. The examiner was blind to the experimental conditions of the rats.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.brainres.2018.06.012.

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