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Dose-Dependent Effects of Icilin on Thermal Preference in the Hindpaw and Face of Rats

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Abstract

Icilin induces wet dog shakes (WDS) in rodents when injected systemically and activates the cold receptor TRPM8 and putative cold receptor TRPA1. It is assumed that WDS reflect an enhanced cold sensitivity; however, none have examined the relationship between WDS and cutaneous cold sensitivity following systemic icilin. In this study, we sought to characterize the effect of systemic and central icilin administration on WDS and thermal preference with either hindpaw or facial stimulation. It was found that a low dose of icilin (0.025mg), which transiently elevated WDS, decreased preference for cold with hindpaw stimulation (15 and 45°C) when administered i.p. or i.t. Intracisternal administration of this dose produced similar results for facial stimulation (10 and 48°C), but had no effect when administered i.p. In contrast a high dose of icilin (0.25mg), which persistently elevated WDS, strongly increased preference for cold with hindpaw stimulation and had no effect on thermal preference with facial stimulation. These findings indicate that at the low concentration, systemic and central icilin enhances cold sensitivity, likely via TRPM8 and TRPA1 activation. In contrast, systemic icilin at the high concentration produces peripheral and/or central effects that diminish cold sensitivity, while WDS is maintained at a persistent rate.

Perspective—Icilin is a unique compound that produces dissociable effects on an innate behavior (WDS) and on operant behaviors related to thermal perception. This compound could help clarify the relationship between peripheral cold transduction and the central induction of thermogenesis and nocifensive behaviors, as well as alterations that produce pathological pain.

Keywords

icilin; operant; thermal preference; cold; hindpaw; orofacial

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Introduction

The recent characterization of channels activated by cold stimuli has led to increased research regarding peripheral cold signal processing, not only with respect to a role in nociception, but also with regards to regulation of basal body temperature and mechanisms of thermogenesis. Transient receptor potential (TRP) channel family members, including TRP melastatin 8 (TRPM8) and TRP ankyrin 1 (TRPA1) have been identified as molecular mediators of cutaneous sensation 19,23,28. TRPM8 is activated by cold and cooling compounds 19,23, while the capacity for TRPA1 to be activated by cold 28 has been disputed 3,13,21. Both of these channels are activated to some degree by icilin 5,11, a synthetic compound long known to induce “wet dog shakes” in rodents and shivering-like behaviors in other mammals when administered systemically 31,32. Recent work in TRPM8 knock out mice has demonstrated that TRPM8 is required for both icilin-induced WDS when systemically administered and the ability of icilin to enhance avoidance of cold when peripherally administered 6,8. Additionally, intramuscular administration of icilin has been shown to produce hyperthermia without WDS 10

Presumably, systemic icilin treatment mimics a decrease in ambient temperature by widespread activation of cold sensitive primary afferent neurons. However, none have directly examined the relationship between the occurrence of WDS and cutaneous cold sensitivity. Does the presence of WDS indicate an enhanced cold sensitivity? Also, while it has been demonstrated that icilin does not produce WDS when administered to the lateral ventricles 35, the effect of icilin treatment at the spinal or brainstem level has not been investigated. TRPM8 and TRPA1 are expressed in the dorsal horn of the spinal cord and trigeminal nucleus caudalis 7,16, so it is possible that icilin treatment at this level may effect WDS and cold sensitivity in the appropriate dermatomes.

In this study, we characterized the temporal profile of WDS occurrence following systemic (i.p.) and central (intra-cisterna magna, i.c.m.) administration of a range of doses of icilin (0.0025mg-2.5mg). Based on this profile, we chose a low dose (0.025mg) and a high dose (0.25mg) to examine the effect of icilin treatment on thermal preference in either the hindpaw or the face. We used thermal preference rather than reflexive withdrawal because this method directly evaluates cortical processing of sensory input, provides information regarding temporal changes in cold sensitivity, and is also more feasible for shaking animals to perform reliably. Our findings indicated that icilin has dissociable, dose-dependent effects on cold sensitivity and WDS occurrence, suggesting that while these two processes share common mechanisms in the periphery, some independent mechanism maintains WDS. These results indicate that icilin may be a useful tool for evaluating the relationship between somatosensation and homeostatic regulation of body temperature. A preliminary description of this study has been presented at the 37th annual meeting of the Society for Neuroscience (2007, San Diego, CA).

Materials and Methods

General

For evaluation of WDS, Sprague-Dawley rats (n = 10, 200-250g, Harlan) were housed in pairs. For hindpaw thermal preference testing, male Hooded Long Evans rats (n = 8, 200-225g, Harlan) were housed in pairs, with enrichment (PVC tunnels and toys for gnawing). For facial thermal preference testing, male hairless Sprague-Dawley rats (n = 17, seven weeks old, Charles River, Raleigh, NC) were housed in groups of five in enriched housing. All rats were maintained in a standard 12-hour light/dark cycle and were allowed access to food and water *ad libitum* when not being tested. Rats' weights were recorded every week to monitor general health. Animal testing procedures and general handling complied with the ethical guidelines

and standards established by the Institutional Animal Care & Use Committee at the University of Florida (Council 1996).

Icilin and DMSO Treatment

Based on WDS quantification (see below), Icilin (0.25 or 0.025mg in DMSO) or DMSO was administered to rats 45 minutes prior to thermal preference testing, either intraperitoneally (i.p., 100µl), intrathecally (i.t., 10µl), or intra-cisterna magna (i.c.m., 10µl). For a summary of drug administration schedule for each behavioral assessment described below, see Table 1. Intrathecal and intra-cisterna magna injections were performed under isoflurane anesthesia (inhalation, 2.5 %). Intracisternal injection was performed by dorsally flexing the rat's head at a 45 degree angle, inserting a needle near the base of the occipital bone, and stepping the needle down until the atlanto-occipital membrane was breeched. A small amount of cerebrospinal fluid (CSF) was aspirated to confirm needle placement before drug administration (see Fig. 1). Intrathecal injections were performed by inserting an 18 gauge needle between the 4th and 5th lumbar vertebrae into the intrathecal space. PE10 tubing was inserted through the needle and a small amount of CSF was withdrawn to confirm placement. The drug was injected through the tubing over approximately two minutes.

Evaluation of Wet Dog Shaking Induced by Icilin

Dose selection and timing of thermal testing was based on quantification of wet dog shakes (WDS). Sprague Dawley rats (n = 4-5 each dose) were injected with icilin i.p. (100µl total volume) or i.c.m. (10µl total volume) and observed in holding cages for up to two hours following injection. WDS were counted in thirty minute intervals and averaged across rats for each interval and for the total two-hour observation period. The rate of WDS frequency was calculated for each rat by dividing the counts by the appropriate observation time, averaging across rats. The following doses were used: 0.0025, 0.025, 0.25, and 2.5 mg of icilin (in DMSO). DMSO treated rats did not receive icilin. The other rats received icilin treatments, with at least 2 wash-out days between doses. The lowest dose of icilin, 0.0025mg, was found to be no different from DMSO treatment, and the highest dose of icilin, 2.5mg, was not significantly different from 0.25mg icilin. Thus, 0.025 and 0.25mg were used for to test the effects of icilin on thermal preference.

Hindpaw Thermal Preference Testing

Thermal preference testing was carried out as previously described³⁰. Briefly, male Long-Evans rats (n =8) were initially placed in a single compartment with a floor temperature of 36° C for 15 minutes, then were transferred to the thermal preference apparatus where they remained for a 15-minute recording period. The thermal preference apparatus consisted of an enclosure with two 15.2 cm by 20.3 cm compartments. The floor of one compartment was cold (10-15°C) and the other was hot (45-48°C). Rats had unrestricted access to each compartment through an opening in a dividing wall and learned to alternate between the hot and cold compartments. Rats were placed on the cold side at the beginning of each testing session. Animals underwent 2 weeks of baseline testing to discriminate between different ranges of cold (first compartment) and heat (second compartment). Based on these responses, a combination of 15 and 45°C was used to evaluate the effects of icilin on hind paw thermal preference. The time spent in each compartment was recorded by custom software that detected light beam interruptions on each side of the dividing wall. The software tracked the sequence of beam interruptions and did not register a change in occupancy until the animal completely crossed the barrier.

Thermal preference is plotted cumulatively as time spent in the heated compartment minus time in the cold compartment during 15 min. trials. Response 1 is the duration of the initial time on the hot floor minus the initial time on the cold floor; response 2 is the duration of the

first plus the second time on the hot floor minus the first plus the second time on the cold floor, and so on. The last response pair represents the cumulative time on the hot floor minus the cumulative time on the cold floor for the trial. Each point on the graphs in Fig. 2 is an average of the responses for 8 rats. Heat preference is represented by positive values and cold preference by negative values in Figure 2. All baseline responses represent the average of two or more baseline sessions for the eight rats collected within a week of the icilin or DMSO treatment. Preferences were reassessed between repeated treatments, and at least one week passed between treatments (Table 1).

Facial Thermal Preference Testing

Facial testing was conducted using a reward-conflict operant testing paradigm as described previously 22. Briefly, rats were trained to drink sweetened condensed milk while making facial contact with a single thermode and lick-tube. During the training period (approximately 2 weeks) baseline intake was recorded, and rats were considered ready for experimental testing once their average baseline intake was 10g or greater at 37°C. The facial testing region for each animal was depilated under light isoflurane anesthesia (inhalation, 2.5 %) once a week to maximize thermal stimulus contact. Rats were fasted for 13-15 hrs prior to thermal preference testing.

For facial stimulation, the thermal preference of the rats was recorded as previously described 26. Rats (n = 16) trained in the single stimulus condition were initially placed in the thermal preference apparatus with two thermodes and lick tubes. The thermode temperatures were set at 37°C to allow the animals to become accustomed to this new task. A second session was recorded to ensure that the rats did not demonstrate a side preference. Rats were able to move freely from one side of the compartment to the other and explore both thermodes. During thermal preference testing, the hot and cold sides were alternated. A combination of 10°C and 48°C was used to evaluate the effects of icilin on thermal preference for stimulation of the face, which was assed by the number of licks achieved in contact with the hot and cold thermodes.

One group of rats (n =9) was used initially with a cross-over design to test the effects of low dose icilin and DMSO on 10°C versus 48°C preference for facial stimulation. A second group (n = 6) was used to test the effect of high dose icilin on 10 versus 48°C preference for facial stimulation (Table 1). Both groups of rats exhibited similar baseline preferences; thus baseline data are pooled for the two groups (Fig. 3).

Statistical Analysis

For the quantification of WDS and facial thermal preference, data were entered into a box-plot using SPSS (v.16), and outliers were identified. Outliers two times greater or less than the interquartile range were excluded from statistical analysis. For WDS, one outlier was identified following 0.25 i.c.m. icilin and one following i.c.m. DMSO. For facial preference testing there were three baseline outliers and one from low dose icilin. Kruskal-Wallis tests were used to assess the effect of icilin dose on WDS and facial thermal preference, because the variance was not homogeneous for these data (determined by Levene's test). Mann-Whitney U tests were used to determine significance between treatment groups. For the hind paw thermal preference tests, the effect of icilin treatment compared to baseline and the effect of icilin treatment compared to DMSO vehicle were evaluated using repeated-measures ANOVA. Significance was set at $p < 0.05$ for all statistical tests.

Results

Evaluation of Wet Dog Shaking Induced by Icilin

Wet Dog Shaking rate increased in a dose dependent manner (Table 2). The bodily location and intensity of WDS differed depending on the icilin delivery method. When administered i.p., the classic head-to-tail WDS were produced, sometimes so intense at the high dose (0.25mg) that the rat was briefly unbalanced. When administered i.c.m., the shakes tended to be focused in the upper body, beginning in the head but usually ending in the upper abdominal region.

There were dose-related and administration-related differences in the rate of WDS across the two hour observation period (Table 2). The two highest doses of icilin, 0.25 and 2.5mg, produced a regular rate of WDS that persisted throughout the two hour observation period, but the rate of WDS was not significantly different between these doses (i.c.m.). For 0.25mg of icilin, the rate was significantly higher for i.p. administration (two to three WDS/minute) than for i.c.m. (one to two WDS/minute), particularly in the middle hour of the observation period (Table 2, 30-90 minutes). In contrast, 0.025mg of icilin transiently elevated WDS in the first 30 minutes for i.c.m. administration (roughly one WDS every two minutes) and in the second 30 minutes for i.p. administration (roughly one WDS every three minutes). After one hour, WDS did not occur with significantly greater frequency following 0.025mg of icilin than after DMSO (i.p. or i.c.m.). The lowest dose of icilin, 0.0025mg, and DMSO (i.c.m.) did not illicit regular WDS. In the entire two hour testing period rats exhibited an average of three WDS for both administration routes of DMSO.

Of the doses tested, 0.025mg was the highest dose of icilin for both administration routes that elevated WDS but did not produce a persistent and regular rate of WDS, as was the case for 0.25mg of icilin. Thus, 0.025 and 0.25mg of icilin were subsequently used for preference testing (hereafter the “low” and “high” doses respectively). WDS remained elevated between 30 and 60 minutes for both high and low doses, so thermal preference testing was performed within this interval.

Effects of Icilin on Thermal Preference with Hindpaw Stimulation

Thermal preference for stimulation of the hindpaws was evaluated cumulatively over 15 sequential occupancies of the hot and cold compartments within 15-minute trials (each rat began trials in the cold compartment). Each sequential occupancy of both compartments is referred to as a “response pair” (see x-axis on all graphs in Fig. 2). Response pair 1 is presented as the duration of initial time on the hot plate minus the initial time on the cold plate; response pair two is the succeeding (second) duration on the hot plate minus the cold plate, etc. The differences scores from successive response pairs are accumulated, so that heat preference is represented by progressively more positive values and cold preference by negative values (see y-axis on all graphs in Fig. 2). The accumulation of difference scores over 15 response pairs reveals the time course of thermal reference within trials, and the 15th sum of difference scores approximates the overall preference in 15 minutes of testing. The average number of response pairs per animal was 14, accounting for the plateau in total preference over 15 pairs.

At baseline and following DMSO treatment, rats spent slightly more time on the cold floor (~58% of the total testing time), indicating a slight cold preference for stimulation of the hindpaws with a 45°C and 15°C combination of temperatures in the two compartments. Following i.p. administration of the low dose of icilin (0.025mg, squares), initial durations in each compartment were similar to DMSO (Fig. 2A). However, beginning with the sixth response pair, rats spent increasingly more time on the heated floor than the cold floor, indicating an increased preference for heat and increased avoidance of cold hindpaw

stimulation (Fig. 2A, squares). Systemic administration of a high dose of icilin (0.25mg) had an effect on thermal preference opposite to the low i.p. dose. Beginning with the first response pair, rats spent more time on the cold compartment, as indicated by increasingly negative values, with the maximal difference achieved by the sixth response pair and maintained to the 15th response pair. This indicates a preference for cold stimulation and a reciprocal avoidance of heat stimulation of the hindpaws following the high dose of i.p. icilin (Fig. 2A, triangles). Intrathecal administration of the low dose had a similar effect to i.p. administration on thermal preference for hindpaw stimulation (Fig. 2B).

Effects of Icilin on Thermal Preference with Facial Stimulation

At baseline, rats strongly preferred 10°C over 48°C, licking more than 90% on that thermode (Fig. 3). Following i.c.m. administration of DMSO, although the baseline preference for 10°C was not altered, there was a significant increase in the licks achieved in contact with the 48°C thermode. This indicates that DMSO may block some of the heat pain experience with 48°C stimulation, decreasing the rats' natural avoidance of this temperature. Following i.c.m. administration of a low dose of icilin (0.025mg), the strong preference for 10°C exhibited at baseline and following DMSO administration was abolished. Roughly 65% of total licks occurred with 48°C stimulation, in contrast to about 5% at baseline or 20% with DMSO. Thus, a low dose of icilin strongly decreased preference for 10°C facial stimulation when administered i.c.m. Following high dose icilin (0.25mg), a preference for 10°C was maintained (about 60% of licks) and was not significantly different from DMSO or baseline (Fig. 3). Taken together, these data show that the two doses of icilin had different effects on thermal preference for facial stimulation. Intraperitoneal administration of DMSO and low dose icilin had no significant effects on thermal preference with facial stimulation (data not shown).

Discussion

The recent characterization of TRPM8 as a cold-activated channel has led to an increased interest in cold signal transduction *in vivo*. Icilin is a potent agonist of TRPM8 6 and a less potent agonist of TRPA1 28, but its effects on cold mediated behaviors have not been extensively characterized, nor is it clear what relationship exists between WDS and cold perception. In the present study we examined the effect of icilin on WDS induction and thermal preference for stimulation of the hindpaw and face (Table 3).

Effects of Icilin on WDS

We first characterized the WDS produced by different doses of icilin when administered either i.p. or i.c.m. to Sprague Dawley rats. High dose icilin produced a persistent rate of WDS with either administration route. WDS rate was greater with i.p. administration than i.c.m. spanning 30-90 minutes post-injection. In contrast, low dose icilin only transiently induced WDS, in the first 30 minutes following i.c.m. injection and the second 30 minutes following i.p. injection. This difference is likely related to the different local environment and distance of the icilin agonist. Intraperitoneal injection delivers the agonist to the cutaneous ends of primary afferent neurons, which are diffusely distributed in heterogeneous tissues. Once the agonist is in the vicinity of a primary afferent, it must activate a sufficient number of channels to depolarize the cell. In contrast, i.c.m. injection delivers the icilin directly to the synapses between the primary afferent neurons and the second order neurons, increasing the chance that the agonist will interact with enough channels to alter the neuron's membrane potential.

Although not quantified to the same extent as for Sprague Dawley rats, the Long Evans rats exhibited frequent WDS at the high dose, and infrequent WDS at the low dose. Also, we observed that Sprague Dawley rats will WDS spontaneously, in mildly stressful contexts. This

was evident in rats treated with DMSO (see Table 2), who shake several times within the first 15 minutes of being injected and placed alone in an observation cage.

Effect of Icilin on Thermal Preference with Hindpaw and Facial Stimulation

Based on the quantification of icilin-induced WDS, we chose two doses of icilin to manipulate thermal preference: a low dose that elevated WDS somewhat but did not produce a persistent rate of shaking, and a high dose that produced a persistent rate of one to three WDS/minute. With hindpaw stimulation, a low i.p dose of icilin decreased preference for the cold stimulus (i.e., increased cold sensitivity) beginning with the fifth (i.p.) or seventh response pair (i.t.). Intracisternal administration of low dose icilin produced similar results with facial stimulation, while i.p. administration had no effect on facial preference.

The decreased cold preference following low dose icilin is likely the consequence of increased sensitivity to cold due to icilin's action on TRPM8 channels and perhaps TRPA1 channels as well. Icilin potentiates activation of TRPM8 by cold stimuli 5 and also activates TRPA1, but with less potency 11. The capacity for TRPA1 to be directly activated by cold stimulation is debated 3,13,17,28, but a lack of direct activation by cold does not necessarily exclude TRPA1 from contributing to sensitivity. TRPA1 is highly co-expressed with TRPV1, a heat activated channel 15. Icilin-induced activation of TRPA1 could be perceived as a burning sensation that may occur simultaneous with activity in cold-sensitive afferents. Icilin could also enhance calcium mediated activation of TRPA1 initiated by another unidentified cold receptor 2,20 expressed within the same cell. Alternatively, TRPA1 has been linked to mechanical processing 4,14,17, and may mediate pricking sensations induced by icilin. Finally, TRPA1 has also been shown to facilitate excitatory synaptic transmission in the substantia gelatinosa 16, so central application of icilin could enhance transmission of cold input from peripheral afferents by its actions on TRPA1. Thus, both TRPM8 and TRPA1 may be involved in icilin-induced cold sensitization.

In contrast to low dose icilin, the high i.p dose of icilin increased preference for cold stimulation of the hindpaws. There are several factors that could contribute this effect. Repeated agonist application results in desensitization of TRPM8 via calcium-dependent PKC activity 1. A sufficient concentration of icilin, coupled with repeated cold stimulation could desensitize TRPM8 receptors *in vivo*. The higher dose of icilin may also activate more TRPA1-expressing cells than the lower dose, which could increase the burning character of the signal. Thus, the global activation pattern of TRPM8- and TRPA1-expressing cell populations could result in cold desensitization and a reciprocal sensitization to heat. The coincidence of these factors could lead to a pattern of cold seeking and heat avoidance behavior that would account for our findings in the hindpaw following high dose icilin.

Another factor that could contribute to an increased cold preference after high dose icilin is the upstream activation of basal thermoregulatory mechanisms mediated by the hypothalamus. An intense activation of peripheral afferents could lead to activation of hypothalamic thermoregulatory nuclei that can exert descending inhibitory control on peripheral afferents 24,27 and/or alter basal body temperature 9,25. Intramuscular administration of icilin (minimum of 2.5 mg/kg) has been shown to produce a long lasting hyperthermia within 30 minutes of treatment 10. Thus, induction of hyperthermia with high dose icilin could contribute to cold seeking behavior.

While there was no significant effect of DMSO on hindpaw preference, there was a significant effect on facial preference. Thus a potential interaction between DMSO and icilin must be considered. DMSO is commonly used as a solvent for application of agonists in electrophysiological and calcium imaging studies. It has also been used *in vivo* to sensitize A

delta fibers^{29,37} and has also been shown to block C fiber conduction¹² (likely a consequence of long term depression induced by the sensitized A delta fibers¹⁸).

In the current study, DMSO increased the licking and time spent at 48°C, but did not have a significant effect on 10°C, indicating a possible reduction of heat pain. The inhibition of heat pain conducted by C fibers may allow rats to recover from 48°C stimulation more readily. This would enable them to linger on the hot thermode. When DMSO is combined with a low dose of icilin that increases cold sensitivity, the effects of these compounds are complementary, and rats are more successful licking at the 48°C thermode. Following low dose icilin administration, the activity of A delta fibers containing TRPM8 is likely enhanced by icilin's actions on TRPM8. In contrast, high dose icilin's capacity to sensitize TRPA1-expressing C fibers may be blocked by DMSO. Conversely, any sensitizing effect that DMSO may have on TRPM8-expressing A delta fibers could potentially be inhibited by desensitization of TRPM8 that may occur in the presence of high dose icilin.

WDS behavior and thermoregulation

The WDS behavior produced by icilin has been equated with shivering³³. However, we demonstrate that a low i.p dose of icilin decreased cold preference but did not produce a persistent rate of shaking. In contrast the high i.p. dose of icilin increased cold preference, even as WDS persisted. This suggests that icilin can maintain the WDS reflex even after the peripheral responsiveness to cold stimulation has been reduced. Therefore, reflexive WDS behavior cannot be used to make inferences regarding icilin's effect on cold sensitivity.

These findings and recent work with TRPM8 knockout mice^{6,8} suggest that while activation of TRPM8 receptors on peripheral afferents may be necessary for the initiation of WDS, other central factors may be responsible for maintenance of persistent shaking. This idea is supported by the fact that icilin-induced WDS are blocked by central administration of opioids^{31,33,35,36}, but not by ICI 20448, a peripherally directed kappa opioid agonists³⁴. Glutamatergic signaling originating in the periphery is required for i.m. icilin induction of hyperthermia¹⁰, which may also mediate the initiation and maintenance of WDS.

The WDS reflex is not restricted to cold contexts. We have observed WDS in other nociceptive contexts such as the capsaicin eye wipe test, which does not elicit a cold sensation. This may be due to the activation of a small subpopulation of TRPM8 and TRPV1 co-expressing receptors which has been reported in rats^{15,38,39}. WDS may also occur spontaneously and may be modulated by stress or anxiety. Further study of icilin's effects on WDS, perhaps in the context of hypothalamic lesions, could provide further insights as to how WDS are generated and maintained.

Conclusion

The effects of icilin on thermal preference are complex and indicate that peripheral encoding of temperature may be the consequence of activation and desensitization of populations of cells expressing different receptor patterns. WDS occurrence reflects TRPM8 activation but not the perception of cold. The finding that a high dose of icilin produces persistent WDS while causing a shift from activation of TRPM8 receptors (by low doses) to desensitization, suggests that maintenance of WDS by central pathways can be maintained independent of TRPM8 activity. Further work with icilin could help to elucidate the relationship between peripheral thermoception and central control of thermogenesis and nocifensive behavior, as well as what occurs during pathological pain states.

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Figure 1. Illustration of intracisternal (i.c.m.) injection. Picture shows the flow of liquid, depicted in white, delivered by blind intracisternal injection (photo courtesy of Dr. Andrew Mannes, NIDCR, and the NIH Mouse Imaging Facility (MIF), NIH, Bethesda, MD). White arrow indicates needle path.

Figure 2.

Effects of two doses of icilin and DMSO on thermal preference with hindpaw stimulation. For each graph, the cumulative difference in occupancy duration for the two compartments (hot - cold) is shown over successive response pairs (error bars = one standard deviation). Over 25 baseline sessions, the animals spent more time on the cold side during each response pair, resulting in a cumulative cold preference of 150-200 sec. in 900 sec. trials. DMSO had no significant effect on thermal preference compared to baseline ($F_{14} = 0.71$, $p = 0.75$). (A) There was a delayed but significant reversal toward heat preference following 0.025 mg i.p. icilin compared to i.p. DMSO ($F_{14} = 8.23$, $p < 0.001$) and compared to baseline ($F_{14} = 8.49$, $p < 0.001$). In contrast, cold preference was significantly increased following 0.25mg i.p. icilin compared to DMSO ($F_{14} = 3.46$, $p < 0.001$). (B) Similar to i.p. administration, i.t. administration of 0.025mg icilin progressively attenuated cold preference compared to baseline ($F_{14} = 3.27$, $p < 0.001$). Significant interactions determined by repeated-measures ANOVA.

Figure 3.

Effect of two doses of icilin and DMSO (i.c.m.) on thermal preference with facial stimulation at 10 and 48°C. The number of licks achieved in contact with either the 10°C thermode (white bars) or the 48°C thermode (black bars) are shown for baseline (n = 12) and following DMSO (n = 6), 0.025mg (n = 8), or 0.25mg icilin (n = 6). There was a significant effect of treatment on licking at 10°C (Kruskal-Wallis test, chi squared = 16.55, p = 0.001) and 48°C (chi squared = 20.93, p < 0.001). Post-hoc comparisons between treatments were made using the Mann-Whitney U test. * indicates a significant effect of low dose icilin treatment as compared to all other treatments (baseline, DMSO, and high dose icilin). + indicates a significant difference between DMSO and baseline only. For all statistical tests p < 0.05. Data are represented as mean ± SEM.

Table 1

Schedule of drug administration and time between repeated treatments for Wet Dog Shaking assessment and for the hindpaw and facial thermal preference tasks

Testing Procedure	Rats (n)	Administration schedule	Time Between Treatments
Wet Dog Shaking	4-5	2.5 - 0.0025mg icilin	2 days minimum
Assessment	4-5	DMSO vehicle	N/A
Hindpaw Thermal Preference	8	Baseline, 0.25mg i.p., 0.025mg i.t., 0.025mg i.p., DMSO	2 weeks minimum *
Facial Thermal Preference	9	Baseline, 0.025mg i.c.m. icilin, DMSO (crossed over across two sessions)	One month *
	6	Baseline, 0.25mg i.c.m. icilin	N/A

* Baseline preferences were reassessed before the next treatment and found to be no different than the first baseline.

Table 2

Rate of Wet Dog Shaking (WDS per minute, mean $\pm$ SEM) calculated from observed counts over thirty-minute intervals following two doses of icilin or DMSO administered intraperitoneally (i.p.) or intracisternally (i.c.m.)

Route of Delivery	Dose Icilin (mg)	Rats (n)	Rate of WDS within each Interval				Total Observation
			0-30	30-60	60-90	90-120	
i.p.	0.25	4	3 $\pm$ 0.6*	3.4 $\pm$ 0.3 [†]	2.8 $\pm$ 0.3 [†]	1.5 $\pm$ 0.5*	2.7 $\pm$ 0.2 [†]
	0.025	4	0.1 $\pm$ 0.05	0.3 $\pm$ 0.04*	0.1 $\pm$ 0.03	0	0.1 $\pm$ 0.01*
	DMSO	5	0.08 $\pm$ 0.02	0.01 $\pm$ 0.01	0	0	0.02 $\pm$ 0.01
i.c.m.	2.5	5	1.9 $\pm$ 0.4*	3.3 $\pm$ 0.2*	2.7 $\pm$ 0.2*	1.6 $\pm$ 0.3*	2.4 $\pm$ 0.2*
	0.25	4	2.2 $\pm$ 0.3*	1.9 $\pm$ 0.3*	1.2 $\pm$ 0.3*	0.6 $\pm$ 0.1*	1.5 $\pm$ 0.2*
	0.025	5	0.6 $\pm$ 0.1 [†]	0.2 $\pm$ 0.06	0.1 $\pm$ 0.02	0.1 $\pm$ 0.02	0.2 $\pm$ 0.06*
	0.0025	5	0.01 $\pm$ 0.01	0.04 $\pm$ 0.03	0.01 $\pm$ 0.01	0	0.02 $\pm$ 0.01
	DMSO	4	0.03 $\pm$ 0	0.03 $\pm$ 0	0	0	0.04 $\pm$ 0.004
Total			Chi squared values (Kruskal-Wallis test, df = 5, p $\leq$ 0.001)				
			26	22.03	23.27	20.43	20.37
							22.99

* indicates significant difference between icilin dose and DMSO vehicle value of same delivery type.

[†] indicates significant difference between delivery type at the same dose. Significance is defined by p<0.05 (Mann-Whitney U test).

Table 3

Summary of icilin's effects on wet dog shaking (WDS) and thermal preference for hindpaw stimulation (15 vs. 45°C), and facial stimulation (10 vs. 48°C)

Behavioral Test	Baseline	DMSO	Low Icilin (0.025mg)	High Icilin (0.25mg)
WDS	N/A	Sporadic, no effect	Transient elevation of WDS	Persistent occurrence of WDS (2-3 WDS/minute)
Hindpaw* Preference (15 vs. 45°C)	Slight Cold	No Effect	Decreased Cold	Increased cold
Face* Preference (10 vs. 48°C)	Very Strong Cold	Strong cold (heat analgesia)	Decreased Cold	Slight cold

* Effects for hindpaw stimulation are for i.p. delivery and i.t. delivery, while the effects for facial stimulation are for i.c.m. delivery only (there was no effect of i.p. administration on preference for facial stimulation).