Published in final edited form as:

Brain Res. 2011 April 12; 1384: 110–117. doi:10.1016/j.brainres.2011.02.002.

Icilin-evoked behavioral stimulation is attenuated by α_2 -adrenoceptor activation

Jae Kim¹, Alan Cowan^{2,3}, Renata Lisek¹, Natalie Raymondi¹, Aaron Rosenthal¹, Daniel D. Hirsch¹, and Scott M. Rawls^{1,2}

¹ Department of Pharmaceutical Science, Temple University Health Science Center, Philadelphia, PA 19140

² Center for Substance Abuse Research, Temple University Health Science Center, Philadelphia, PA 19140

³ Department of Pharmacology, Temple University Health Science Center, Philadelphia, PA 19140

Abstract

Icilin is a transient receptor potential cation channel subfamily M (TRPM8) agonist that produces behavioral activation in rats and mice. Its hallmark overt pharmacological effect is wet-dog shakes (WDS) in rats. The vigorous shaking associated with icilin is dependent on NMDA receptor activation and nitric oxide production, but little else is known about the biological systems that modulate the behavioral phenomenon. The present study investigated the hypothesis that α_2 -adrenoceptor activation inhibits icilin-induced WDS. Rats injected with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.) displayed dose-related WDS that were inhibited by pretreatment with a fixed dose of clonidine (0.15 mg/kg, s.c.). Shaking behavior caused by a fixed dose (2.5 mg/kg) of icilin was also inhibited in a dose-related manner by clonidine pretreatment (0.03–0.15 mg/kg, s.c.) and reduced by clonidine posttreatment (0.15 mg/kg, s.c.). Pretreatment with a peripherally restricted α_2 -adrenoceptor agonist, ST91 (0.075, 0.15 mg/kg), also decreased the incidence of shaking elicited by 2.5 mg/kg of icilin. Pretreatment with yohimbine (2 mg/kg, i.p.) enhanced the shaking induced by a low dose of icilin (0.5 mg/kg). The imidazoline site agonists, agmatine (150 mg/kg, i.p.) and 2-BFI (7 mg/kg, i.p.), did not affect icilin-evoked shaking. These results suggest that α_2 -adrenoceptor activation inhibits shaking induced by icilin and that increases in peripheral, as well as central, α_2 -adrenoceptor signaling oppose the behavioral stimulant effect of icilin.

Keywords

clonidine; icilin; TRPM8; wet-dog shake; α_2 -adrenoceptor; agmatine; ST91; yohimbine

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Corresponding Author: Scott M. Rawls, Ph.D., Associate Professor of Pharmacology, Department of Pharmaceutical Sciences, Temple University Health Sciences Center, 3307 North Broad Street, Philadelphia, PA, 19140, USA Tel: (215) 707-4942, Fax: (215) 707-3678, scott.rawls@temple.edu.

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1. Introduction

Icilin (AG-3-5) activates transient receptor potential cation channel subfamily M member 8 (TRPM8) (McKemy et al., 2002; Peier et al., 2002; Reid et al., 2002; Nealen et al., 2003; Story et al., 2003; Bandell et al., 2004; Biró et al., 2005; Liu et al., 2006). TRPM8 channels are densely expressed in sensory neurons within the dorsal root ganglion, and their activation by icilin and other cooling compounds (*e.g.* menthol, WS-12, and CPS-369) produces sodium and calcium ion influx that results in cellular depolarization and the feeling of cold (Nealen et al., 2003; Babes et al., 2004). Despite the potential of TRPM8 ligands as analgesic, antipruritic and antiarthritic agents, the endogenous and exogenous substances that modulate their pharmacological effects *in vivo* are largely unknown. The trademark overt pharmacological effect of icilin in rats is vigorous wet-dog shakes (WDS) (Wei, 1976, 1981; Werkheiser et al., 2006, 2007, 2009), and this shaking behavior provides a sensitive, reproducible, and quantifiable endpoint to investigate TRPM8 pharmacology *in vivo* and to identify, and exclude, receptor systems that shape the pharmacological response to TRPM8 channel activation.

Our studies have traditionally focused on the identification of downstream signaling events that underlie icilin-induced behavioral activation. For example, we have demonstrated that icilin-induced WDS are dependent on NMDA receptor activation and nitric oxide production but not on AMPA receptor or glutamate transporter subtype 1 (GLT-1) activation (Werkheiser et al., 2009). We have also shown that mu and kappa opioid receptor agonists reduce icilin-induced shaking (Werkheiser et al., 2006). The present study tested the overall hypothesis that α_2 -adrenoceptor activation opposes icilin-induced behavioral activation. The hypothesis was based in part on the tenet that increased sympathetic activity contributes to shaking movements in rats and that suppression of sympathetic discharge by an α_2 -adrenoceptor agonist (clonidine) would reduce shaking caused specifically by icilin (Wei, 1983). The hypothesis was also based on a recent finding that α_2 -adrenoceptors and TRPM8 channels are co-expressed on peripherally located sensory neurons and that α_2 -adrenoceptor activation inhibits TRPM8 channel signaling (Bavencoffe et al., 2010). We specifically investigated the effect of: 1) clonidine on the development and maintenance of icilin-induced shaking; 2) N-(2,6-diethylphenyl)-4,5-dihydro-1H-imidazol-2-amine hydrochloride (ST-91), a peripherally restricted α_2 -adrenoceptor agonist, on the development of icilin-induced shaking; and 3) yohimbine, a α_2 -adrenoceptor antagonist, on icilin-induced shaking. We also studied the effects of imidazoline site agonists on icilin-induced shaking behavior.

2. Results

2.1. Clonidine pretreatment reduces icilin-induced shaking

Effects of graded doses of clonidine (0.03, 0.6, 0.15 mg/kg) on WDS produced by a fixed dose of icilin (2.5 mg/kg) are presented in Fig. 1. One-way ANOVA revealed a significant effect [$F(3, 31) = 5.351$, $P = 0.0049$] for the experiment. Injection of icilin (2.5 mg/kg) by itself produced 148 ± 32.5 WDS over 30-min observation interval. Dunnett's post-hoc analysis indicated that the total number of WDS produced by icilin was significantly inhibited by pretreatment with either 0.06 mg/kg ($P < 0.05$) or 0.15 mg/kg ($P < 0.01$) clonidine. Specifically, 0.06 mg/kg and 0.15 mg/kg of clonidine inhibited the incidence of shaking by approximately 41 % and 77%, respectively. The onset of shaking, and excessive grooming, began within 2 min of icilin injection the behaviors persisted for the duration of the 30-min observation period, and were preceded, in all cases, by abdominal writhing. Although clonidine pretreatment (0.06, 0.15 mg/kg) did significantly inhibit the total extent of shaking induced by icilin, it did not significantly delay the onset of shaking. The

administration of clonidine (0.03, 0.06, 0.15 mg/kg) by itself did not elicit abdominal, writhing, wet-dog shaking, excessive grooming, or sedation.

Separate experiments examined the effect of a fixed dose of clonidine (0.06 mg/kg) on WDS elicited by progressively increasing doses of icilin (0.5, 1, 2.5, 5 mg/kg) (fig. 2). Two-way ANOVA (pretreatment, icilin dose) indicated significant effects of pretreatment [$F(1, 48) = 61.37, P < 0.0001$] and dose [$F(3, 48) = 16.92, P < 0.0001$]. Icilin by itself produced dose-related shaking (0.5 mg/kg, 58 ± 16.1 ; 1 mg/kg, 144 ± 19.8 ; 2.5 mg/kg, 189 ± 27.0 ; and 5 mg/kg, 190 ± 18.2). Post-hoc analysis indicated that WDS associated with each icilin dose: (0.5, 1, 2.5, 5 mg/kg) was significantly inhibited by pretreatment with clonidine (0.15 mg/kg). Specifically, clonidine pretreatment (0.15 mg/kg) produced the following effects on icilin-induced shaking (icilin dose, % inhibition, significance): (0.5 mg/kg, 75%, $p < 0.01$); (1 mg/kg, 78%, $p < 0.01$); (2 mg/kg, 60%, $p < 0.01$); and (2.5 mg/kg, 52%, $p < 0.05$).

2.2. Clonidine posttreatment attenuates icilin-induced shaking

Results from the experiment investigating the effect of clonidine posttreatment (0.6 mg/kg) on WDS produced by icilin (2.5 mg/kg) are presented as time-course data in fig. 3. two-way ANOVA (treatment, time) revealed significant effects of treatment [$F(1, 10) = 77.98, P < 0.0001$] and time [$F(6, 60) = 13.97, P < 0.0001$]. Significantly less shaking was observed in icilin (2.5 mg/kg)-exposed rats that were prostrated 5 min later with clonidine (0.06 mg/kg) than in icilin (2.5 mg/kg)-exposed rats that received saline posttreatment. The inhibitory effect of clonidine was both rapid in onset, as a significant attenuation ($P < 0.05$) of icilin-induced shaking was observed 5 min post-clonidine injection, and persistent in duration, as it endured for the entire 35-min observation interval (fig. 3).

2.3. ST-91 pretreatment reduces icilin-induced shaking

Effects of graded doses of the peripherally restricted α_2 -adrenoceptor agonist, ST-91 (0.0375, 0.075, 0.15 mg/kg), on WDS produced by a fixed dose of icilin (2.5 mg/kg) are presented in fig. 4. One-way ANOVA revealed a significant drug effect [$F(3, 28) = 3.298, P = 0.0348$], Dunnett's post-hoc analysis indicated that WDS elicited by icilin were significantly inhibited by pretreatment with either 0.075 or 0.15 mg/kg of ST-91 ($P < 0.05$). Specifically, 0.0375 and 0.15 mg/kg of ST-91 inhibited the incidence of shaking by approximately 42% and 51 %, respectively. The lowest dose of ST-91, 0.0375 mg/kg, reduced the number of WDS induced by icilin by approximately 20% but the effect did not reach statistical significance ($P > 0.05$). The injection of ST-91 (0.15 mg/kg) by itself did not elicit abdominal writhing, wet-dog shaking, excessive grooming, or sedation.

2.4. Yohimbine pretreatment enhances WDS elicited by a low dose of icilin

Effects of graded doses of the α_2 -adrenoceptor antagonist yohimbine (0.5, 1, 2 mg/kg) on WDS elicited by two doses of icilin, 0.5 and 2.5 mg/kg, are presented in fig 5. For experiments with the low dose of icilin, one-way ANOVA revealed a significant drug effect [$F(3, 27) = 1.48, P < 0.0001$] (Fig 5, Panel A). Dunnett's post-hoc analysis indicated that 0.5 mg/kg of icilin produced a significantly greater number of WDS in rats pretreated with 2 mg/kg of yohimbine as compared to rats pretreated with saline ($P < 0.01$). For experiments with the higher dose of icilin (2.5 mg/kg), one-way ANOVA did not reveal a significant drug effect [$F(3, 28) = 0.9647, P > 0.05$] (Fig 5, Panel B).

2.5 Pretreatment with imidiazoline site agonists do not affect icilin-induced shaking

The effects of a fixed dose of agmatine (150 mg/kg) on WDS elicited by icilin (0.5, 1, 2.5, 5 mg/kg) are presented in Fig. 6. Two-way ANOVA (pretreatment, iciline dose) revealed a significant effect of dose [$F(3,46) = 12.58, P < 0.001$] but not a significant effect of

pretreatment [$F(1,46) = 0.07886$, $P = 0.7802$], thus indicating that icilin-induced shaking was not significantly affected by agmatine explore. Lower doses of agmatine (25, 75 mg/kg) were equally ineffective as they failed to significantly alter WDS induced by 2.5 mg/kg of icilin ($P > 0.05$) data not shown). In separate experiments, rat injected with 2.5 mg/kg of icilin following pretreatment with 2-BFI (7 mg/kg) did not display shaking that was significantly different from icilin (2.5 mg/kg)-exposed rats pretreated with saline ($P > 0.05$, Student's t-test) (Fig. 6, box).

3. Discussion

Icilin is a cold-inducing compound that produces a behavioral stimulant effect comprised of WDS, hyperthermia, writhing, and excessive grooming in rats following intraperitoneal injection (Collier, 1974; Wei, 1976, 1981; Collier et al., 1981; Cowan and Watson, 1978; Cowan, 1981; Werkheiser et al., 2006, 2007, 2009; Ding et al., 2008). Consistent with prior work, icilin elicited a dose-dependent increase in shaking behavior in rats in the present experiments. Although the dramatic stimulant effect of icilin was identified 38 years ago (Burford and Chappel, 1972), its cellular sites of action of icilin have only recently been identified. The TRPM8 channel is one site of action (McKemy et al., 2002; Peier et al., 2002; Story et al., 2003; Liu et al., 2006). A second possible site of action is the TRPA1 channel (Bandell et al., 2004), although this finding was contradicted by another study which demonstrated that TRPA1 channels are insensitive to icilin and cold (Jordt et al., 2004). TRPM8 and TRPA1 channels are located in the periphery on dorsal root ganglia and trigeminal neurons and are part of the TRP superfamily of ion channels that share significant sequence homology (Moran et al., 2004). Mechanisms underlying the behavioral stimulant effect of icilin, including potential roles for TRPM8 and TRPA1 channels, remain poorly understood. Wei and Seid (1983) originally proposed that icilin exerts its stimulant actions through a peripheral mechanism, an interpretation now supported by evidence that direct administration of icilin into the lateral ventricle or dorsal striatum fails to cause WDS or hyperthermia (Werkheiser et al., 2006; Ding et al., 2008). These observations are also supported by molecular evidence that TRPM8 and TRPA1 channels are located primarily, if not exclusively, in the periphery (Fonfria et al., 2006; Voets et al., 2007). Furthermore, pretreatment of rats with a small molecule TRPM8 channel antagonist (JNJ-39267631) inhibits shaking produced by intraperitoneal icilin injection (Colburn et al., 2008). Taken together, these data suggest that activation of TRPM8 channels located outside of the CNS is responsible for the development of icilin-induced shaking.

The first significant finding in the present study was that clonidine pretreatment and posttreatment reduced icilin-evoked WDS. These outcomes suggest that systemic clonidine administration inhibits both the development and maintenance of icilin-evoked shaking. One clue to the mechanism by which clonidine inhibited icilin-evoked shaking in our experiments may reside in its wide-ranging efficacy against WDS induced by a panoply of pharmacological manipulations, including kainic acid, naloxone-precipitated morphine withdrawal, carbachol, benzyldeneaminoxyacetic acid derivatives, and thyrotropin (TRH)-releasing hormone analogues (Jahn and Mixich, 1976; Byrska et al., 1980; Turski et al., 1981; Wei, 1983; Ohno et al., 1987; Velíšek et al., 1994). The fact that dissimilar pharmacological manipulations evoke a similar behavioral stimulant effect, WDS, suggests that a common downstream signaling pathway underlies the phenomenon and that α_2 -adrenoceptor activation by clonidine inhibits the normal activation or recruitment of the said pathway.

One possibility is that clonidine disrupts the central glutamate component of icilin-induced shaking. The full stimulant effect of icilin is dependent on increased glutamate transmission in the brain, as the shaking response is accompanied by an elevation in extracellular striatal

glutamate and inhibited by NMDA receptor antagonism (Werkheiser et al., 2007, 2009). Neurochemical data obtained from conscious rats reveal that clonidine reduces extracellular glutamate and inhibits, through α_2 -adrenoceptor activation, presynaptic glutamate release in the brain and spinal cord (Tingley et al., 1990; Kamisaki et al., 1992). Thus, when administered with clonidine, icilin still activates TRPM8 channels, but the ensuing downstream increase in glutamate transmission that underlies a significant proportion of the behavioral stimulant effect is suppressed because of α_2 -adrenoceptor activation by clonidine, leading to an overall reduction in icilin efficacy. The behavioral stimulant effect of icilin is also dependent on a non-glutamate component, and it is possible that clonidine targets substrates within this component to inhibit the shaking response, perhaps through indirect opioid receptor activation or inhibition of nitric oxide production (Werkheiser et al., 2006, 2009). A role for 5-HT_{2A} receptors in the pharmacological effects of icilin has not been identified, but it is documented that 5-HT_{2A} receptor activation elicits WDS (Kitamura et al., 2002; Umeda et al., 2007). *In vivo* microdialysis studies demonstrating that clonidine reduces extracellular serotonin in brain regions that trigger stimulant activity suggest clonidine efficacy may have been partly dependent on its ability to reduce serotonergic transmission at the 5-HT_{2A} receptor (Pudovkina et al., 2003).

The positive results with clonidine suggest that α_2 -adrenoceptor activation suppresses the behavioral stimulant effect of icilin, but they do not discriminate between peripheral and central α_2 -adrenoceptor mechanisms. Because clonidine is a lipophilic compound that readily penetrates the CNS, its systemic administration activates α_2 -adrenoceptors located in the peripheral and central nervous systems. Thus, we conducted experiments to determine the effect of a peripherally restricted α_2 -adrenoceptor agonist (ST91) on icilin-induced shaking. What we found was that pretreatment of rats with ST91 dose-dependently inhibited shaking induced by icilin. The degree of inhibition by ST91 was significant but still less than that of clonidine, an observation which suggests that both peripheral and central α_2 -adrenoceptor activation inhibits icilin-induced shaking. Support for this interpretation is provided by *in vivo* studies in rats which demonstrated that the potency of ST91 is about ten times less than that of clonidine following systemic injection, whereas ST91 displays the same degree of potency as clonidine after injections into the cerebral ventricles (Chariot et al., 1988; Taylor et al., 1988; Eshel et al., 1990). Furthermore, ST91, when administered by the same route and within the same dose range (0.075 – 0.15 mg/kg) as in our study, did not alter the extent of shaking precipitated by naloxone in morphine-dependent rats (Taylor et al., 1988). It is equally important to note that administration of ST91 into the midbrain almost completely inhibits WDS produced by naloxone-precipitated morphine withdrawal (Taylor et al., 1988). The combined findings with ST91 suggest that its effects against icilin are mediated by peripherally located α_2 -adrenoceptors and that activation of peripheral α_2 -adrenoceptors inhibits icilin-evoked behavioral activation. In fact, α_2 -adrenoceptors and TRPM8 channels are co-expressed by sensory neurons in the dorsal root ganglia and activation of these α_2 -adrenoceptors is postulated to reduce signaling events initiated by TRPM8 channel activation (Bavencoffe et al., 2010). Thus, enhanced activity at the subpopulation of peripheral α_2 -adrenoceptors, through a reduction in TRPM8 channel signaling, may be one mechanism that counters the normal behavioral activation produced by icilin.

Another interesting finding was that rats pretreated with the α_2 -adrenoceptor antagonist, yohimbine, displayed an increased incidence of WDS following icilin administration. Yohimbine did not produce shaking per se. These results suggest that endogenous adrenergic tone exerted at α_2 -adrenoceptors inhibits icilin-induced shaking. Since yohimbine was administered systemically and penetrates the CNS, it is unclear whether peripheral or central α_2 -adrenoceptors are involved in the effect. The ability of yohimbine to enhance icilin-induced shaking was, however, dependent on the dose of icilin.

Yohimbine enhanced the shaking produced by a low dose (0.5 mg/kg) of icilin but not by a higher dose (2.5 mg/kg). These data suggest that tonic α_2 -adrenoceptor activation counters the shaking elicited by a low level of TRPM8, or perhaps TRPA1, channel activity. The ineffectiveness of an α_2 -adrenoceptor block on WDS induced by a higher dose of icilin may be due to a “ceiling effect”. In this case, a greater level of TRPM8 channel activity resulting from the administration of higher icilin doses may cause a maximum degree of shaking that cannot be further enhanced. Alternatively, the endogenous tone exerted at α_2 -adrenoceptors may be simply incapable of countering the greater degree of shaking (*i.e.*, the shaking overwhelms the compensatory adrenergic response).

A final series of experiments tested the effect of agmatine on icilin-evoked shaking. Agmatine is a biogenic amine that is produced in the brain from arginine by the catalytic activity of arginine decarboxylase (Reis and Regunathan, 2000). Perhaps the most notable pharmacological effect of agmatine is its biphasic modulation of mu opioid receptor function, namely enhancement of acute morphine analgesia and inhibition of morphine-induced analgesic tolerance, physical dependence, and behavioral sensitization (Su et al., 2003; Regunathan, 2006; Wei et al., 2007). Agmatine is a pharmacologically diverse substance. It acts through multiple mechanisms, including imidazoline site activation, NMDA receptor antagonism, nitric oxide synthesis inhibition, and peptide hormone release (Loring, 1990; Li et al., 1994; Piletz et al., 1995; Gilad et al., 1996; Fairbanks et al., 2000; Reis and Regunathan, 2000). Agmatine also interacts with the target of interest in our experiments, α_2 -adrenoceptors, but its actual effects at these receptors remain unclear. Some studies suggest that a number of the pharmacological effects of agmatine are dependent on active α_2 -adrenoceptors whereas other reports suggest that agmatine binds to α_2 -adrenoceptors but neither inhibits nor activates these receptors (Li et al., 1994; Pinthong et al., 1995; Kotagale et al., 2010). In the present study, we found that agmatine, even when administered at doses as great as 150 mg/kg, failed to affect icilin-induced shaking. Moreover, 2-BFI, a selective imidazoline site agonist, also lacked efficacy against icilin-induced shaking (Macinnes and Duty, 2004). The ineffectiveness of agmatine and 2-BFI do not support a major role for imidazoline sites in the mechanism of icilin-induced shaking, especially since other mechanisms of action of agmatine (*i.e.*, NMDA receptor antagonism and inhibition of nitric oxide production), do significantly reduce behavioral activation produced by icilin (Ding et al., 2008; Werkheiser et al., 2009). Although it is unclear why the NMDA receptor antagonist LY 235959 attenuates icilin-induced shaking and agmatine is ineffective, it may be related to the mechanisms of the compounds. LY 235959 is a selective NMDA antagonist that competitively blocks the glutamate recognition site at the NMDA receptor complex whereas agmatine displays properties that are similar to the pharmacological profiles of NMDA receptor channel blockers such as phencyclidine and ketamine (Yang and Reis, 1999). Evidence also suggests that compounds targeting non-NMDA receptors, including AMPA receptor antagonists and GLT-1 activators, do not affect icilin-induced shaking (Werkheiser et al., 2009).

In conclusion, our results confirm and expand the original observation by Wei (1976, 1983) that shaking movements in rats are dependent on the level of adrenergic activity. Specifically, we demonstrate that the α_2 -adrenoceptor agonist clonidine reduces both the development and maintenance of icilin-induced shaking. We also demonstrate that a peripherally restricted α_2 -adrenoceptor agonist reduces icilin-induced shaking. Taken with the recent finding by Bavencoffe et al. (2010) that sensory neurons co-express α_2 -adrenoceptors and TRPM8 channels, our results suggest that activation of α_2 -adrenoceptors located in both the peripheral and central nervous systems reduce the behavioural stimulation elicited by icilin.

4. Experimental Procedures

4.1. Animals and compounds

Male Sprague-Dawley rats (Ace Laboratories, Boyertown, PA), weighing 100–125 g, were housed in groups of three for five days prior to use, with food and water available *ad libitum*. The temperature in the room was 23 ± 1 °C and a standard light–dark cycle was maintained with a timer-regulated light period from 0700 to 1900 h. The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Temple University. Icilin, a gift from Delmar Chemicals Ltd. (Montreal, Canada), was suspended in 1% Tween 80/distilled water and injected interapertioneally (i.p.). Clonidine hydrochloride, ST91, yohimbine hydrochloride, agmatine sulfate, and 2-(2-benzofuranyl)-2-imidazoline hydrochloride (2-BFI) were purchased from Tocris Laboratories (Ellisville, MO, USA), dissolved in physiological saline, and injected i.p. All compounds were injected in a volume of 1 ml/kg.

4.2. Experimental design

Experiments were performed during the light phase between noon and 5 p.m. Each rat was weighed and acclimated in a Plexiglas observation box (22 cm long; 18 cm wide; 25 cm high) 90 min before administration of test compounds as described previously (Werkheiser et al., 2006, 2009; Ding et al., 2008). For the clonidine studies, three separate experiments were performed in which rats were injected with: (1) clonidine (0.03, 0.06, 0.15 mg/kg) or saline 15 min prior to a fixed dose of icilin (2.5 mg/kg); (2) clonidine (0.15 mg/kg) or saline 15 min prior to one progressively increasing icilin dose (0.5, 1, 2.5, 5 mg/kg); or (3) clonidine (0.15 mg/kg) or saline 5 min following injection of icilin (2.5 mg/kg). For the ST91/icilin experiments, rats were pretreated with ST91 (0.0375, 0.075, 0.15 mg/kg) or saline 15 min prior to the injection of a fixed dose of icilin (2.5 mg/kg). For the yohimbine/icilin experiments, rats were pretreated with yohimbine (0.5, 1, 2 mg/kg) or saline 15 min prior to the injection of icilin (0.5, 2.5 mg/kg). For the agmatine/icilin and 2-BFI/icilin experiments, rats were injected with agmatine (150 mg/kg) 15 min prior to icilin (0.5, 1, 2.5, 5 mg/kg), and with 2-BFI (7 mg/kg) 15 min prior to icilin (2.5 mg/kg). The incidence of WDS was counted for 30 min after icilin administration in all experiments except the clonidine posttreatment experiment, in which the number of WDS were counted for 35 min. Following experimentation, each rat was placed in the prone position on the Perspex lid of the observation chamber, and tested for sedation. If the rat moved freely off the lid and on to the bench top, it was considered “not behaviorally depressed.” None of the rats in our experiment were observed to be behaviorally depressed following experimentation. Rats were used only once for each experiment and euthanized immediately following experimentation. Doses of icilin and the test compounds, as well as pretreatment intervals, were based on previous *in vivo* studies in our, and other, laboratories (Taylor et al., 1988; Werkheiser et al., 2006, 2009; Aggarwal et al., 2009).

4.3. Data analysis

Data are expressed as mean wet-dog shakes $\pm$ S.E.M. (GraphPad Prism). For experiments testing a fixed dose of clonidine or agmatine against multiple doses of icilin, two-way ANOVA (pretreatment, treatment) was used to evaluate overall differences between groups followed by Bonferroni's *post-hoc* analysis to compare individual groups. For experiments testing multiple doses of clonidine, ST91, or yohimbine against a fixed dose of icilin, one-way ANOVA was used to determine a significant main effect followed by a Dunnett's *post-hoc* analysis to compare individual groups to the icilin-treated group. For the 2-BFI experiments, two groups were compared using a Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Acknowledgments

This study was supported by NIDA grants P30DA01342 and T32 DA07237-17.

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Fig. 1. Clonidine pretreatment reduces WDS induced by a fixed dose of icilin. Rats pretreated with clonidine (0.03, 0.06, 0.15 mg/kg) or saline were injected 15 min later with icilin (2.5 mg/kg). Data from 6–8 rats per group are expressed as mean WDS + S.E.M. * $P < 0.5$ or ** $P < 0.01$ compared to rats pretreated with saline.

Fig. 2.

Pretreatment with a fixed dose of clonidine (0.15 mg/kg) reduces WDS evoked by graded doses of icilin. Rats pretreated with clonidine (0.15 mg/kg) or saline were injected 15 min later with icilin (0.5, 1, 2.5, 5 mg/kg). Data from 6–8 rats per group are expressed as mean WDS + S.E.M. * $P < 0.05$ or ** $P < 0.01$ compared to respective icilin group pretreated with saline.

Fig. 3.

Clonidine posttreatment reduces WDS produced by a fixed dose of icilin. Rats injected with icilin (2.5 mg/kg) were injected 5 min later with clonidine (0.15 mg/kg) or saline. Time-course data from 6 rats per group are expressed as mean WDS + S.E.M post-clonidine or post-saline treatment. * $P < 0.05$ or ** $P < 0.01$ compared to icilin + vehicle group.

Fig. 4. ST-91 pretreatment reduces WDS induced by a fixed dose of icilin. Rats pretreated with ST-91 (0.0375, 0.075, 0.15 mg/kg) or saline were injected 15 min later with icilin (2.5 mg/kg). Data from 6–8 rats per group are expressed as mean WDS + S.E.M. * $P < 0.05$ compared to rats pretreated with saline.

Fig. 5.

Yohimbine enhances WDS induced by a low dose of icilin. Rats pretreated with yohimbine (YOH) (0.5, 1, 2 mg/kg) or saline were injected 15 min later with icilin (0.5, 2.5 mg/kg). Data from 6–8 rats per group are expressed as mean WDS + S.E.M. * $P < 0.05$ compared to rats pretreated with saline.

Fig. 6.

Imidazoline site agonists, agmatine and 2-BFI, do not reduce WDS elicited by icilin. Rats pretreated with a fixed dose of agmatine (150 mg/kg) or saline were injected 15 min later with icilin (0.5, 1, 2.5, 5 mg/kg). Data from 6–8 rats per group are expressed as mean WDS + S.E.M. **Box)** Rats pretreated with 2-BFI (7 mg/kg) or saline were injected 15 min later with icilin (2.5 mg/kg). Data from 6–8 rats per group are expressed as mean WDS + S.E.M.