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Icilin-induced wet-dog shakes in rats are dependent on NMDA receptor activation and nitric oxide production

Jennifer Werkheiser [b], Alan Cowan [b,c], Teresa Gomez [a], Craig Henry [a], Shreya Parekh [a], Sony Chau [a], David A. Baron [a], Scott M. Rawls [a,c,*]

[a] Department of Pharmaceutical Sciences, Temple University Health Sciences Center, Philadelphia, PA, USA
[b] Department of Pharmacology, Temple University Health Sciences Center, Philadelphia, PA, USA
[c] Center for Substance Abuse Research, Temple University, Philadelphia, PA, USA

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ABSTRACT

Icilin is a cold channel agonist that produces vigorous wet-dog shaking in rats. The shaking is accompanied by an increase in the level of extracellular glutamate in the brain. Hence, we hypothesized that icilin-induced wet-dog shakes are dependent on increased glutamatergic transmission and nitric oxide (NO) production. Rats injected with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.) displayed a dose-related increase in wet-dog shakes. Pretreatment with LY 235959 (1, 2 mg/kg, i.p.), a NMDA receptor antagonist, or L-NAME (50 mg/kg, i.p.), a NO synthase (NOS) inhibitor, attenuated icilin-induced wet-dog shakes. The shaking was also reduced by intracerebroventricular L-NAME (1 mg/rat, i.c.v.) administration, indicating that the stimulant effect of icilin is dependent on central NO production. Pretreatment with 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) (10, 20 mg/kg, i.p.), an AMPA receptor antagonist, or ceftriaxone (200 mg/kg, i.p. for 5 days), a beta-lactam antibiotic and glutamate transporter subtype 1 (GLT-1) activator, did not alter the incidence of icilin-induced shaking. The present data reveal that icilin produces behavioral stimulation by a mechanism requiring NMDA receptor activation and nitric oxide production and suggest that glutamate and NO signaling play important roles in cold channel pharmacology.

1. Introduction

Icilin activates two transient receptor potential (TRP) channels, TRPM8 and TRPA1, located in the peripheral nervous system (McKemy et al., 2002; Peier et al., 2002; Reid et al., 2002; Nealen et al., 2003; Story et al., 2003; Bandell et al., 2004; Liu et al., 2006). Upon application to the skin or tongue, icilin produces "mild, pleasant sensations of coolness, similar to menthol but discrete and non-irritating" and is 400–600 times more potent than menthol (Wei, 1981; Wei and Seid, 1983; Tse and Wei, 1986; Behrendt et al., 2004). As a result, icilin is a potential alternative to menthol in many over-the-counter medications, and has therapeutic promise as an analgesic, antipruritic and anti-arthritic agent (Biró et al., 2005). The signature overt effect of icilin in rats is the dramatic, stimulant behavior it precipitates following intraperitoneal administration. The behavioral syndrome consists of excessive body grooming, abdominal writhing, ptosis, forepaw tremor, jumping and vigorous wet-dog shakes (Wei, 1976; Cowan, 1981). In addition to its stimulant effects, icilin increases extracellular glutamate in the brain and causes a hyperthermia that is attenuated by NMDA receptor antagonism or nitric oxide synthase inhibition (Werkheiser et al., 2007; Ding et al., 2008). Since icilin produces hyperthermia, shaking and increased extracellular glutamate, and NMDA receptor blockade and NOS inhibition attenuate icilin-induced hyperthermia, we hypothesized that NMDA receptor blockade and NOS inhibition will also attenuate icilin-induced shaking. This hypothesis was tested using two pharmacological agents: (−)-6-[phosphonomethyl-1,2,3,4,4a,5,6,7,8,8a-decahydro-isoquinoline-2-carboxylate] (LY235959), a selective NMDA receptor antagonist; and N(G)-nitro-L-arginine methyl ester hydrochloride (L-NAME), a nonselective NOS inhibitor. Additionally, we investigated the effects of an AMPA receptor antagonist, 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX), and a GLT-1 transporter activator, ceftriaxone, on the frequency of icilin-induced wet-dog shakes (Rothstein et al., 2005).

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (Ace Laboratories, Boyertown, PA), weighing 100–125 g, were housed in groups of three for five days prior

* Corresponding author. Department of Pharmaceutical Sciences, Temple University School of Pharmacy, 3307 North Broad Street, Philadelphia, PA, 19140, USA. Tel.: +1 215 707 4942; fax: +1 215 707 3678.
E-mail address: scott.rawls@temple.edu (S.M. Rawls).

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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. Each rat was used only once, for a single experiment, and then euthanized immediately.

2.2. Compounds

Icilin, a gift from Delmar Chemicals Ltd. (Montreal, Canada), was suspended in 1% Tween 80/distilled water. LY 235959, L-NAME and DNQX (disodium salt) were purchased from Tocris Laboratories (Ellisville, MO, USA) and dissolved in physiological saline. Ceftriaxone hydrochloride was purchased from Apotex Corporation (Weston, FL, USA) and dissolved in physiological saline. For systemic administration, compounds were administered in a volume of 1 ml/kg and injected intraperitoneally (i.p.). For central administration, L-NAME was administered intracerebroventricularly (i.c.v.) in a volume of 5 µl.

2.3. Experimental design

All 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.

2.3.1. Systemic experiments

Rats were injected with LY 235959 (0.5, 1, 2 mg/kg, i.p.), L-NAME (50 mg/kg, i.p.) or saline 30 min prior to icilin (0.5, 1, 2.5, 5 mg/kg, i.p.). Each compound has been investigated previously in our laboratories, and doses and pretreatment times were selected on the basis of results from those studies (Rawls et al., 2006; Ding et al., 2008). For DNQX experiments, rats were pretreated with DNQX (10, 20 mg/kg, i.p.) or saline and injected with a fixed dose (2.5 mg/kg, i.p.) of icilin 30 min later. For ceftriaxone experiments, rats were injected with ceftriaxone (200 mg/kg, i.p.) or saline once daily for 5 days. On day 6, rats were injected with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.). The ceftriaxone design was based on evidence that the said dosing schedule increases GLT-1 transporter activity and expression in the rat brain (Rothstein et al., 2005; Rawls et al., 2007). The incidence of wet-dog shakes was counted for 30 min after icilin administration in all experiments. 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.

2.3.2. Central experiments

Rats were anesthetized with an i.p. injection of ketamine hydrochloride (150 mg/kg) and acepromazine maleate (0.2 mg/kg). A polyethylene guide cannula was implanted stereotaxically into the right lateral ventricle (Rawls et al., 2006). Coordinates for the lateral ventricle were 0.8 mm posterior from bregma, 1.6 mm lateral from the midline, and 3.5 mm from the skull. Dental acrylic cement was used to secure the cannula to the cranium. The route of L-NAME administration was intracerebroventricular (i.c.v.) and performed by inserting the needle tip of a 10-µl syringe into a polyethylene cannula. The tip of the needle extended 1 mm beyond the tip of the cannula. In behavioral experiments, L-NAME (1 mg/rat, i.c.v.) or an equivalent volume (5 µl, i.c.v.) of saline was injected 30 min before icilin (2.5 mg/kg), after which wet-dog shakes were counted for 30 min. Following i.c.v. experiments, injection sites were verified with an injection of 0.1% Evan's blue (4 µl). The central dose of L-NAME was based on prior results from our laboratory (Rawls et al., 2006).

2.4. Data analysis

Data are expressed as mean wet-dog shakes ± S.E.M. (nonlinear regression, GraphPad Prism). One-way ANOVA was used to evaluate cumulative group means. Bonferroni's *post-hoc* analysis was performed after significance was determined by ANOVA. For the experiment investigating the effects of centrally administered L-NAME on icilin-induced shaking, the two groups were compared using a Students *t*-test. Values of $P<0.05$ were considered statistically significant in all cases.

3. Results

3.1. Effect of a NMDA receptor antagonist on icilin-induced wet-dog shakes

The effect of LY 235959 (0.5, 1, 2 mg/kg, i.p.) pretreatment on icilin-induced wet-dog shakes is presented in Fig. 1. One-way ANOVA revealed a significant main effect [$F(15, 176) = 10.34$, $P<0.0001$]. When given by itself, icilin (0.5, 1, 2.5 and 5 mg/kg, i.p.) produced a dose-dependent increase in the incidence of wet-dog shakes over the 30-min observation interval. Onset of shaking occurred within 2 min of icilin administration. In all cases, icilin-induced abdominal writhing, which preceded the onset of shaking and excessive body grooming that was evident for the duration of the experiment. The administration of LY 235959 (0.5, 1, 2 mg/kg, i.p.) by itself did not elicit abdominal writhing, wet-dog shakes or excessive grooming. When LY 235959 (1 mg/kg, i.p.) was given in combination with 2.5 or 5 mg/kg of icilin, the incidence of wet-dog shaking was reduced by about 60% and 46%, respectively ($P<0.01$). A higher dose of LY 235959 (2 mg/kg, i.p.) also antagonized wet-dog shaking induced by 2.5 or 5 mg/kg of icilin by about 50% and 56%, respectively ($P<0.01$). The lowest dose of LY 235959, 0.5 mg/kg, did not affect the number of wet-dog shakes produced by any of the doses (0.5, 1, 2.5, 5 mg/kg, i.p.) of icilin ($P>0.05$).

3.2. Effect of a NOS inhibitor on icilin-induced wet-dog shakes

The effect of systemically injected L-NAME (50 mg/kg, i.p.) on icilin-induced wet-dog shakes is presented in Fig. 2A. One-way ANOVA revealed a significant main effect [$F(7, 88) = 28.03$, $P<0.0001$]. Administration of

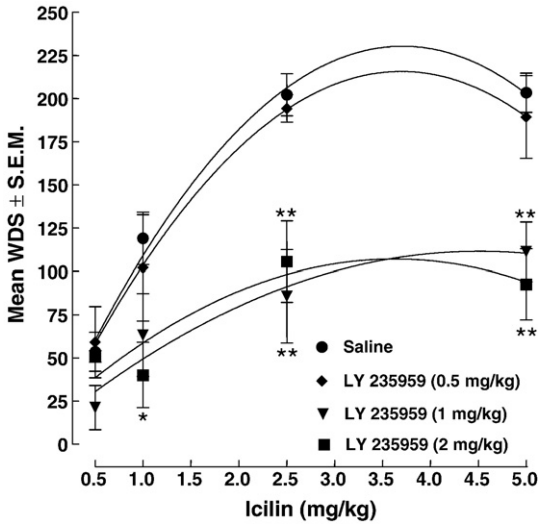

Fig. 1. Effect of LY 235959 on icilin-induced wet-dog shakes. Rats pretreated with LY 235959 (0.5, 1, 2 mg/kg, i.p.) or saline were injected 30 min later with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.). Data from 12 rats per group are expressed as mean wet-dog shakes (WDS) ± S.E.M. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ compared to rats pretreated with saline.

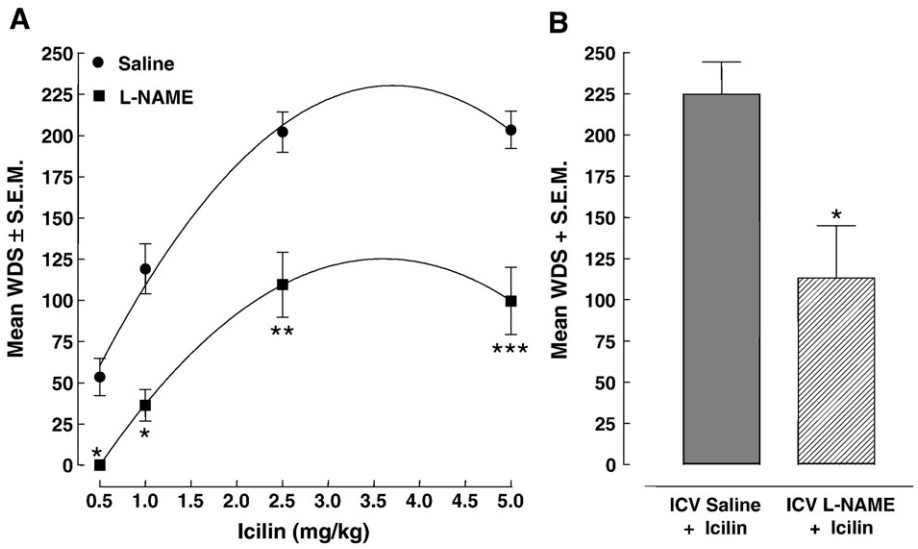

Fig. 2. Effect of systemically or centrally administered L-NAME on icilin-induced wet-dog shakes. A) Rats pretreated with L-NAME (50 mg/kg, i.p.) or saline were injected 30 min later with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.). Data from 12 rats per group are expressed as mean wet-dog shakes (WDS) ± S.E.M. *P<0.05, **P<0.01 and ***P<0.001 compared to rats pretreated with saline. B) Rats pretreated with L-NAME (1 mg, i.c.v.) or saline were injected with a fixed dose of icilin (2.5 mg/kg, i.p.) 30 min later. Data from 8 rats per group are expressed as mean wet-dog shakes (WDS) + S.E.M. *P<0.05 compared to ICV saline + icilin group.

L-NAME (50 mg/kg, i.p.) by itself did not produce abdominal writhing, wet-dog shakes or excessive grooming. When given with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.), L-NAME (50 mg/kg, i.p.) significantly reduced the number of wet-dog shakes. L-NAME (50 mg/kg, i.p.) completely blocked wet-dog shaking evoked by the lowest dose, 0.5 mg/kg, of icilin (P<0.05) (Fig. 2A). When administered with higher doses of icilin, L-NAME (50 mg/kg, i.p.) produced about a 70% inhibition of shaking evoked by 1 mg/kg of icilin (P<0.05); 46% inhibition of shaking evoked by 2.5 mg/kg of icilin (P<0.01); and a 52% inhibition of shaking evoked by 5 mg/kg of icilin (P<0.001) (Fig. 2A). Central administration of L-NAME (1 mg/rat, i.c.v.) caused approximately a 50% inhibition of wet-dog shakes induced by systemically injected icilin (2.5 mg/kg, i.p.) (P<0.05, Student's t-test) (Fig. 2B).

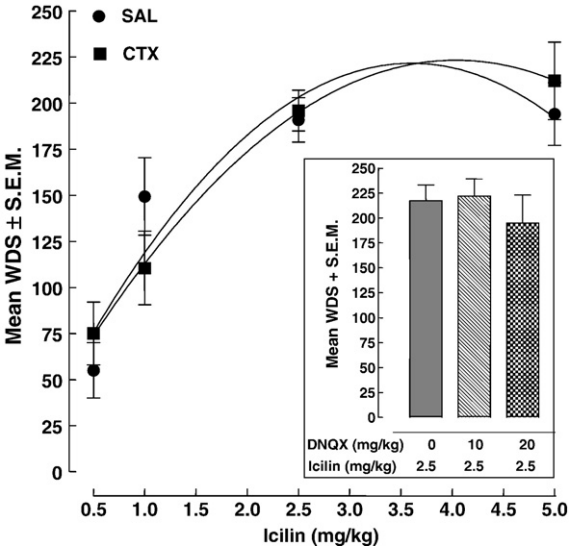

Fig. 3. Effect of ceftriaxone (CTX) or DNQX on icilin-induced wet-dog shakes. CTX experiment) Rats were injected for 5 days with CTX (200 mg/kg, i.p.) or saline. One day later, rats from both groups were injected with icilin (0.5, 1, 2.5, 5 mg/kg, i.p.). Data from 6–8 rats per group are expressed as mean wet-dog shakes (WDS) ± S.E.M. Inset) Rats pretreated with DNQX (10, 20 mg/kg, i.p.) or saline were injected with icilin 30 min later. Data from 7–9 rats per group are expressed as mean wet-dog shakes (WDS) + S.E.M.

3.3. Effect of GLT-1 activation or AMPA receptor antagonism on icilin-induced shaking

The effect of ceftriaxone (200 mg/kg, i.p.) on icilin-induced wet-dog shakes is presented in Fig. 3. One-way ANOVA revealed a significant main effect [F (7, 40) = 12.51, P<0.0001], but Bonferroni's *post-hoc* analysis indicated that ceftriaxone pretreatment (200 mg/kg, i.p. for 5 days) did not alter the shaking produced by any of the doses of icilin (0.5, 1, 2.5, 5 mg/kg, i.p.) (P>0.05). In a separate set of experiments, pretreatment of rats with DNQX (10, 20 mg/kg, i.p.) did not alter the number of wet-dog shakes produced by a fixed dose (2.5 mg/kg, i.p.) of icilin [F (2, 22) = 0.4783, P>0.05] (Fig. 3, inset).

4. Discussion

Our prior work has demonstrated a positive correlation between extracellular glutamate and icilin-evoked wet-dog shakes (Werkheiser et al., 2006, 2007). Doses of icilin that produce wet-dog shakes cause a dose- and time-dependent elevation in extracellular glutamate in the rat brain. The onset of shaking also coincides with the rise in glutamate levels. This evidence suggested that increased glutamatergic transmission mediated the expression of icilin-induced wet-dog shakes, but the components (e.g., receptors, transporters) of the glutamate system involved in the process were unknown. We now report that LY 235959, a highly selective NMDA antagonist that blocks the glutamate recognition site on the NMDA receptor complex (Benveniste and Mayer, 1991), reduces the incidence of icilin-induced wet-dog shakes. This finding identifies the NMDA receptor as a key target in icilin-induced shaking and suggests that NMDA receptor activation is necessary for icilin to precipitate behavioral stimulation in rats.

L-NAME, a NOS inhibitor, also reduced icilin-induced wet-dog shakes. This finding reveals that icilin-induced shaking is dependent on NO production. L-NAME pretreatment completely blocked shaking evoked by the lowest dose (0.5 mg/kg) of icilin and significantly reduced the shaking induced by all doses of icilin. Doses of L-NAME greater than 50 mg/kg were not tested because they antagonize muscarinic receptors and produce hypothermia, cognitive impairment, and hyperactivity in animals (Buxton et al., 1993; Scammell et al., 1996; Prendergast et al., 1997; Pechánová et al., 2006; Sakae et al., 2008). Due to the extent to which systemically administered L-NAME inhibited shaking, follow-up experiments were conducted to

determine whether the stimulant effects of icilin were dependent on NO production in the brain and/or periphery. Regardless of its injection route, L-NAME produced about a 50% reduction in icilin-evoked wet-dog shakes. The finding that L-NAME produces a similar degree of inhibition following its systemic and central administration suggests that the behavioral stimulant action of icilin is more dependent on NO production in the brain than in the periphery. A role for central NO synthesis in the expression of wet-dog shakes evoked by other stimuli, including physiological stimulation of limbic structures and getting wet, has been reported previously (Xu and Miller, 1998; Hara et al., 2000; Koylu et al., 2002).

Our data identify NMDA receptors and NOS as key mediators of icilin-induced shaking, but the exact mechanism remains elusive 37 years after the phenomenon was first documented (Burford and Chappel, 1972). One reason is that a cellular site of action for icilin was discovered only recently (Behrendt et al., 2004). Icilin is thought to activate two cold channels, TRPM8 and TRPA1 (Peier et al., 2002; Story et al., 2003; Dhaka et al., 2006; Fajardo et al., 2008), although some research proposes that TRPA1 is not activated by icilin and should not be classified as a cold channel receptor (Babes et al., 2004; Jordt et al., 2004). Development of selective TRPM8 and TRPA1 antagonists would help us to identify which cold channel, if either, mediates icilin-evoked wet-dog shaking. A key role for TRPM8 channels in the process is supported by recent evidence that a small molecule TRPM8 channel antagonist (JNJ-39267631) attenuates the incidence of icilin-induced wet-dog shaking (Colburn et al., 2008). When selective cold channel antagonists become commercially available, we will discriminate between a TRPM8 and TRPA1 site of action in our assay. It is known that icilin administered into the lateral ventricle or striatum does not elicit shaking behavior in rats, suggesting that the parent compound (i.e., icilin) acts directly on cold channels within the periphery to initiate behavioral stimulation (Werkheiser et al., 2007). In keeping with this idea, we believe that downstream signaling following initial stimulation of peripheral cold channels leads to the expression of icilin-induced shaking. One explanation for our results is that peripheral cold channel activation by icilin initiates a downstream increase in extracellular glutamate and NMDA/NO signaling within brain regions that mediate stimulant activity (Umeda et al., 2007). When administered with a NMDA receptor antagonist, icilin still increases extracellular glutamate but the ensuing receptor block prevents NMDA receptor activation, leading to an inhibition of the NMDA receptor-dependent component of icilin-induced wet-dog shakes. Similarly, when icilin is administered with a NOS inhibitor, the enzyme block reduces NO production and disrupts NMDA/NO signaling, leading to an overall reduction in shaking.

The difference between the effects of L-NAME and LY 235959 on icilin-induced shaking may provide insight into the mechanism of action of icilin. The two agents produced qualitatively similar effects, but the magnitude of inhibition by L-NAME was much greater. For example, shaking induced by the lowest dose of icilin was completely blocked in rats pretreated with L-NAME but not reduced in rats pretreated with LY 235959. Furthermore, L-NAME reduced a significant proportion of shaking evoked by all doses of icilin whereas LY 235959 was only effective against the two highest doses. These data might indicate that NO signaling is more important to the production of wet-dog shakes than NMDA receptor activation. One possibility is that the activation of NO signaling is temporally closer to peripheral cold channel activation than glutamate release and NMDA receptor activation, which could be recruited later. Although data regarding the effects of icilin on the actual production of NO are not yet available, a comparison of the temporal profiles of icilin-induced wet-dog shakes and glutamate levels in the dorsal striatum indicates that the maximal increase in extracellular glutamate coincides with peak shaking (Werkheiser et al., 2007). It has also been shown that NO production within the striatum produces an increase in extracellular glutamate levels through cyclic GMP formation (Trabace and Kendrick, 2000). Hence, one explanation for icilin-induced shaking is that peripheral cold channel activation activates NO signaling which, in turn, causes glutamate release and NMDA receptor activation within the striatum. Future experiments will examine this question at both the neurochemical and molecular levels.

Neither NMDA receptor antagonism nor NOS inhibition completely inhibited icilin-induced wet-dog shakes in our experiments. This suggests that endogenous targets other than NMDA receptors, and NOS, contribute to icilin-evoked shaking. Glutamate, in addition to activating NMDA receptors, activates AMPA and kainate receptors (Monaghan et al., 1988; Seeburg, 1993). Because AMPA or kainate receptor agonists produce wet-dog shakes in rats (Turski et al., 1981; Yamashita et al., 2004), we determined if icilin-induced shaking was dependent on AMPA and kainate receptor activation. Experiments revealed that DNQX, an AMPA/kainate receptor antagonist, did not alter icilin-induced wet-dog shakes, suggesting that neither of the receptors contributes to the stimulant effect of icilin. Glutamate also activates at least eight metabotropic glutamate receptors (mGluRs) that are subdivided into three groups: Group I (mGluR1 and 5), Group II (mGluR2 and 3) and Group III (mGluR4, 6, 7 and 8) (Nakanishi, 1992; Conn, 2003). A possible role for mGluRs in the stimulant effect of icilin will be the subject of a future investigation. It is known that icilin-induced wet-dog shakes are reduced by mu opioid, kappa opioid, muscarinic and alpha$_2$-adrenergic receptor agonists (Wei, 1981, 1983; Werkheiser et al., 2006). Based on those data and the current results, multiple neurotransmitter systems, activated downstream of cold channel stimulation, most likely interact to shape the incidence and duration of wet-dog shaking. It is probable that the stimulant activity of icilin is enhanced by the activation of some targets, such as NMDA receptors and NOS, and inhibited by the activation of other systems, such as kappa and mu opioid.

While it is now apparent that NMDA receptor activation and NO production are required for icilin to produce shaking and hyperthermia, the relation between the two responses remains unclear (Wei, 1976; Ding et al., 2008). One question is whether the shaking is a result of the hyperthermia, or vice versa. We do know that both effects are influenced by the route of administration. Significant hyperthermia is produced following intraperitoneal or intramuscular injection whereas significant shaking occurs only after intraperitoneal administration (Wei, 1976; Werkheiser et al., 2006; Ding et al., 2008). Hence, it is evident that the hyperthermia can occur in the absence of significant shaking but that robust shaking occurs only in the presence of hyperthermia. This suggests that hyperthermia triggers the shaking and supports a previous hypothesis that hyperthermia induced by intraperitoneal administration of icilin causes wet-dog shakes through a “heat gain” or shivering response (Wei, 1976). Another possibility is that a metabolite of the parent compound, formed following the intraperitoneal administration of icilin, causes the shaking whereas icilin itself produces the hyperthermia. Alternatively, the shaking could be the result of a local effect of icilin within the peritoneum. Future studies will better delineate the relationship between icilin-induced shaking and hyperthermia and determine how NMDA/NOS systems contribute to this association.

Ceftriaxone, a GLT-1 transporter activator and beta-lactam antibiotic, did not affect icilin-induced shaking. GLT-1 transporters are expressed in rats and humans (excitatory amino acid transporter 2, EAAT2) and control glutamatergic transmission by mediating 90% of cellular glutamate uptake in the mammalian brain (Rothstein, 1996; Mitani and Tanaka, 2003). The only practical drugs known to increase GLT-1 transporter activity and expression are the beta-lactam antibiotics (Rothstein et al., 2005). Through this mechanism, ceftriaxone protects against ischemic injury and motor neuron degeneration *in vitro*; delays loss of neurons and muscle strength in a mouse model of ALS; protects against neurotoxicity of human immunodeficiency virus proteins; blocks symptoms of Huntington's disease; and modulates kappa and mu opioid activity (Rothstein et al., 2005;

Rumbaugh et al., 2007; Mineur et al., 2007; Lipski et al., 2007; Miller et al., 2008; Rawls et al., 2007, 2008). In our experiments, ceftriaxone did not reduce the behavioral stimulant effect of icilin. We had hypothesized that ceftriaxone, by stimulating glutamate uptake and inhibiting glutamatergic transmission, would inhibit the glutamate-dependent component of icilin-induced shaking. The ineffectiveness of ceftriaxone may be related to temporal differences between the processes of glutamate uptake and synaptic glutamate release. Transport of a glutamate molecule from the extracellular compartment into a cell is significantly slower than the time course for the synaptic release of glutamate (Wadiche et al., 1995; Sheldon and Robinson, 2007). Therefore, even in cases such as the present in which glutamate uptake is presumably increased, the rate of glutamate clearance from the extracellular compartment may be too slow to counter pharmacological effects mediated by synaptically released glutamate. Although the extent to which synaptic or extrasynaptic activity mediates icilin-evoked shaking is unknown, microdialysis data from the rat striatum suggest that synaptic glutamate release plays a role (Werkheiser et al., 2007). The ineffectiveness of ceftriaxone may also be related to its downstream effects on glutamate receptor function. For example, one potential consequence of enhanced glutamate uptake by ceftriaxone is a reduction in the level of basal extracellular glutamate, an effect that could lead to sensitization of glutamate receptors (Sah et al., 1989). If glutamate receptor sensitization is a consequence of ceftriaxone administration, then an actual increase in glutamatergic transmission (e.g. at NMDA receptors) might occur in ceftriaxone-exposed rats injected with icilin due to the ability of the stimulant to increase extracellular glutamate (Werkheiser et al., 2007). In this case, one in which extracellular glutamate levels are elevated and glutamate receptors are sensitized, it is unlikely that a glutamate-dependent behavioral effect such as icilin-induced shaking would be decreased. It is unlikely that ceftriaxone was ineffective because of the cellular location of GLT-1 transporters, which are located mostly on astrocytes rather than neurons (Rothstein et al., 1994). This is because electron microscopic analysis shows that GLT-1 is enriched on astrocytic processes near synaptic termini, suggesting that they are specifically targeted to portions of the membrane which release glutamate (Chaudhry et al., 1995).

In summary, the current study is one of only a limited number of investigations into the *in vivo* effects of icilin, likely related to its ability to activate cold channel receptors and turn on multiple downstream neurotransmitter systems (Peier et al., 2002; McKemy et al., 2002; Werkheiser et al., 2006, 2007; Colburn et al., 2007; Dhaka et al., 2006; Ding et al., 2008; Brignell et al., 2008). Highly relevant to our study is the stimulant activity of icilin in rats (Burford and Chappel, 1972; Wei, 1976; Cowan, 1981; Werkheiser et al., 2007). We demonstrate specifically that icilin produces behavioral stimulation by a mechanism requiring NMDA receptor activation and NO production. By doing so, we extend the *in vivo* pharmacological profile of icilin, a therapeutically beneficial cold-inducing agent, beyond its role as an agonist at TRPM8 and TRPA1 receptors within the periphery.

Acknowledgements

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