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A Thermal Place Preference Test for Discovery of Neuropathic Pain Drugs

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Abstract

Developing potent non-opioid pain medications is an integral part of the battle to conquer both chronic pain and the current opioid crisis. Although most screening approaches use *in vitro* surrogate targets, *in vivo* screening of analgesic candidates is a necessary pre-clinical step in drug discovery. Here, we report the design of a new automated behavioral testing apparatus based on the principle of a thermal place preference test (TPPT). This new design can detect, quantify, and differentiate behavioral responses to cold stimuli between sham and chronic constriction injury (CCI) rodents with up to 12 animals tested simultaneously. At an optimized temperature pair of 12.5°C vs 30.0°C ($\pm 0.5^\circ\text{C}$), the TPPT design has captured the antinociceptive effects of morphine and pregabalin on CCI rats in individual 10-min tests. Moreover, it can differentiate analgesic effects by morphine or pregabalin from anxiolytic effects by diazepam. The results, along with the relatively low cost to construct the apparatus and moderately high throughput, make our TPPT design applicable for behavioral studies of chronic pain in rodents and for high-throughput *in vivo* screening of the next generation of pain medications.

Keywords

pain; neuropathic pain; cold allodynia; thermal place preference test; chronic constriction injury; drug discovery

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Author Contributions

YX and PT designed the project. JC and PT wrote the manuscript with input from KK, MM, TST, and YX. JC along with MM, KK, LJ, and QC collected and analyzed data. JC and LJ performed all animal surgeries. YX, TST, JC, and NB designed and assembled the apparatus. All authors contributed and reviewed the results and approved the final version of the manuscript.

Competing Interests

The authors declare no competing interests.

Chronic pain affects almost one-third of the US population and costs hundreds of billions of dollars annually in combined national healthcare expenses, lost income, and lost productivity.¹ What makes this situation even more devastating is that treatment options are limited and are often compounded by prescription drug abuse – most frequently opioid use disorder.² For many chronic pain indications, the use of opioids proves to be ineffective due to drug tolerance, which often leads to higher doses of medication being prescribed. In the last 20 years, opioid prescriptions have increased tenfold, and opioid addiction, dependence, and overdose are now more prevalent than ever.³ Therefore, discovery and development of potent non-opioid pain medications will take the center stage in the battle against the rapid escalation of the opioid epidemic.

In vivo compound screening in rodents is an essential step in the discovery and development of new analgesics. Von Frey and Hargreaves tests for mechanical and thermal hypersensitivity, respectively, are commonly used for behavioral evaluation of stimulus-evoked pain in rodents.^{4–6} They are also well established for evaluating analgesic effects of compounds and drugs in different rodent pain models.^{7–9} However, both tests require constant and intrusive human interactions with the animals. They are labor intensive and time consuming. Data quality from these tests relies heavily on the experience of individuals conducting the experiments.^{10, 11} These issues limit their application in discovery and development of novel antinociceptive drugs.

The thermal place preference test (TPPT)^{12, 13} enables a quick and noninvasive assessment of thermal sensitivities of rodents under a normal or pathological condition. Because heightened sensitivities to hot and cold stimuli often accompany rodent models of neuropathic pain, an animal's natural response to temperature difference in TPPT can be used to differentiate physiological conditions with or without neuropathic pain, and to allow unbiased assessment of the effects of pharmacological intervention. One of the advantages of using TPPT is that animals can move freely between cold and warm compartments, thereby experiencing less stress and anxiety from human handling.^{6, 14} Less human involvement in animal handling during experiments offers benefits for automated throughput in behavioral phenotyping as well as more objective results. Despite the aforementioned benefits, TPPT has not been well established for automated evaluation of neuropathic pain and *in vivo* drug screening in a moderately high-throughput manner.^{6, 15, 16}

Cold allodynia, pain produced by normally innocuous low-temperature stimuli, is a major characteristic of many neuropathic pain states.¹⁴ In a previous study, the threshold temperature to elicit behavioral pain response to cold (measured by withdrawal latency) was found to be 5°C for naïve rats, but 10°C or 15°C for cold allodynia in rat models of neuropathic pain with spared nerve injury or spinal nerve ligation.¹⁴ Different behavioral responses to cold stimuli between rats in normal and neuropathic pain conditions offer a solid foundation to utilize TPPT for discovery of new analgesics.

Here, we report a new TPPT design suitable for behavioral studies of pain in rodents and for discovering the next generation of pain medications. This TPPT design (Figure 1) possesses not only temperature-control elements as in conventional TPPT, but also the throughput

capability of data collection from 12 rodents simultaneously. The setup can be easily scaled for more animals. The uniformly black color of the walls and floors (both warm and cold plates) provides an environment for rodents to explore freely without distractive points of interest. The mirrored temperature layout allows users to swap rodents between front and back chambers in different experiments to counterbalance warm and cold placement to prevent place learning by rodents. Because rodents are more willing to explore the environment under low-light or dark conditions,¹⁷ our TPPT is conducted under dim red light that is invisible to rodents. Two low-light sensitive cameras are positioned above the apparatus to record the full view of 12 chambers during experiments using the Any-Maze behavioral tracking software (Stoelting Co., Wood Dale, IL, USA), which tracks locations and movement of rats in individual chambers between cold and warm plates and provides video data for post analysis. More details about the TPPT set up is provided in the Methods section.

To determine whether there exists inherent bias in the TPPT apparatus, we performed background tests on two groups of rats. One group of rats had chronic constriction injury (CCI), a well-developed neuropathic pain model.¹⁸ Rats with sham operation served as the control group. We placed both CCI and sham rats randomly into individual chambers with both plates at room temperature and collected baseline data of their occupancy on each plate (Figure 2). During the habituation, neither group of rats showed preference for one plate over another. These results assure that rats have no inherent bias towards either plate.

Observation of hyperalgesia or allodynia in CCI rats is a precondition for screening analgesic candidates using TPPT. In order to identify temperature pairs that can sensitively detect cold allodynia in CCI rats and differentiate them from sham controls, we fixed the warm plate at 30°C¹⁹ (a margin of error $\pm 0.5^\circ\text{C}$ in both the warm and cold plates) and varied the temperature of the cold plate to optimize the differential condition, under which the CCI rats would spend significantly less time on the cold plate than the sham control. At a temperature 10°C, water condensation was observed on the surface of the cold plate that prohibited a reliable behavioral test. At 11°C, both groups of rats were deterred from exploring the cold plate, whereas a temperature 15°C reduced the behavior separation between CCI and sham rats (Figure 3). In pairing with the warm plate control temperature at 30°C, a temperature of 12°C, 13°C or 14°C ($\pm 0.5^\circ\text{C}$) on the cold plate emerged as appropriate noxious stimuli to induce cold allodynia in the CCI rats that significantly separated behavior responses between the CCI and sham rats (Figure 3) based on two-way ANOVA with the Fisher's LSD post-hoc analysis. The additional t-tests provided the temperature effect sizes (η^2) of 0.229, 0.172, and 0.179 between sham and CCI rats at 12, 13, and 14°C, respectively. These temperatures are within the range of 10–15°C reported previously¹⁴ for detecting cold allodynia. The small temperature discrepancy may result from different experimental settings: the previous study measured paw withdrawal latency when rats were trapped on a single temperature plate¹⁴, whereas in our experiments, rats traveled freely between plates of two different temperatures, allowing the animals to willingly escape from the uncomfortable plate.

With an optimized temperature pair, such as 12.5 vs. 30°C, how soon can one observe cold allodynia in the CCI rats that differentiates them from the sham control rats? To find an

answer, we extended the data collection time up to 2 hours and processed the data in conjunction with a sliding time window. Interestingly, a statistical difference of percentage time on the cold plate between the sham and CCI rats was observed as early as 5 mins ($p < 0.01$) after the experiment began and further improved at 10 mins ($p < 0.0001$) (Figure 4). Although both groups of rats show reduced occupancy on the cold plate in a time-dependent manner, the CCI rats consistently spent much less time on the cold plate than the sham control. The statistically significant differences between the sham and CCI rats were sustained for over an hour of the experiment. Overall, 10–20 mins of data collection were a good time window for carrying out the most efficient *in vivo* screening of candidate analgesics.

Pregabalin and morphine are commonly used analgesics for treating neuropathic pain.²⁰ We chose them to validate the TPPT assay and to evaluate whether the cold allodynia observed in the CCI rats can be alleviated by the pharmacological intervention. The drugs were administered subcutaneously (s.c.) to the CCI rats 30 minutes prior to TPPT. Sham rats served as a positive control and the CCI rats treated with vehicle (PBS) served as a negative control. Using a 10-min time window and the 12.5 ± 0.5 °C vs. 30 ± 0.5 °C temperature pair, we observed statistically significant improvement of cold allodynia in the CCI rats treated with 30 mg/kg pregabalin [$F(2,33)=4.74$, $p < 0.05$, $\eta^2=0.223$] in comparison to the CCI rats injected with vehicle (Figure 5A), but 1 mg/kg pregabalin did not show a significant effect. To further test whether the TPPT setup can capture a dose-dependent behavior response, we varied the morphine dosage from 1 µg/kg to 20 mg/kg (Figure 5A) and observed significant improvement of cold allodynia starting at a dose of 5 mg/kg [$F(6,80)=5.41$, $p < 0.0001$, $\eta^2=0.289$]. The estimated EC_{50} of ~3 mg/kg from the morphine data is close to the low end of EC_{50} values demonstrated previously using Hargreaves and von Frey measurements on a similar rat model.²¹ To test whether the TPPT setup can differentiate analgesic effects from other factors that potentially influence animal behavior and produce a false-positive analgesic-like effect, we treated CCI rats with diazepam, an anxiolytic drug.⁸ A dose of 5 mg/kg diazepam did not alleviate cold allodynia of CCI rats and 10 mg/kg significantly lowered the occupancy of CCI rats on the cold plate [$F(2,36)=5.38$, $p < 0.01$, $\eta^2=0.23$] (Figure 5A). The lack of analgesic effect of diazepam observed in our test seems to be in conflict with a previous study²², in which diazepam at 5 mg/kg (i.p.) was reported to produce analgesic effects on CCI rats in von Frey and Hargreaves tests. It is highly possible that the perceived diazepam analgesic effects²² actually resulted from inhibition of withdrawal reflexes due to diazepam's anxiolytic and sedative effects. Indeed, diazepam (5 mg/kg and 10 mg/kg, s.c.) significantly impaired rodents' locomotion as reflected in the drastically shorter total travel distances of CCI rats treated with diazepam than those injected with vehicle during TPPT [$F(2,36)=54.55$, $p < 0.0001$, $\eta^2=0.75$] (Figure 5B). This observation agrees with the previously reported sedative effect of diazepam that could significantly attenuate explorative mobility of rats.²³

In contrast to diazepam, pregabalin showed no effect on the total distance traveled [$F(2, 33)=1.32$, $p > 0.05$, $\eta^2=0.074$] (Figure 5B). Interestingly, relative to the CCI rats injected with vehicle, the morphine-treated CCI rats traveled significantly longer distances at low doses [1 mg/kg; $F(3, 56)=7.92$, $p < 0.001$, $\eta^2=0.298$] but significantly shorter distances at high doses [5 mg/kg; $F(3, 56)=25.74$, $p < 0.0001$, $\eta^2=0.579$] (Figure 5B). These results are consistent

with previous reports that pregabalin at a dose <100 mg/kg has negligible locomotor effects,²⁴ whereas morphine can produce either hyperactivity or sedative effects at a low or high dose, respectively.^{25–27} Thus, by analyzing the tolerance to the cold plate and the total distance traveled, our device can differentiate analgesic from anxiolytic effects of drugs without conflating locomotor side effects with analgesia, avoiding false positive analgesia as observed in the sedation by diazepam in animal reflex measurements.

All the results presented here demonstrate that our TPPT can meet the requirement for *in vivo* screening of new analgesic drugs for neuropathic pain. Although we used only the CCI rats and sham control in the current study, the reported TPPT can be used in principle for rodents in different pain models. In the absence of pharmacological intervention, the TPPT revealed statistical differences between the control group and the CCI rats that showed significantly higher avoidance (~20%) to cold stimulations (Figure 3). Note that we fixed one side of the chamber with a neutral temperature of 30°C and varied temperature only for the cold plates. However, our TPPT apparatus has the flexibility to vary temperatures on both plates to evaluate nociception sensitivity under different pain conditions. It is also expected that temperature pairs other than those reported in this study may also serve well for TPPT. For example, in a slightly different apparatus setting, cold allodynia in CCI rats was previously demonstrated with a temperature pair of 10°C vs. 45°C.¹²

The TPPT reported here has also demonstrated its suitability for evaluating pharmacological intervention, which is a primary requirement for *in vivo* screening of new analgesics. Significant antinociceptive effects of pregabalin and morphine on the CCI rats as measured using the TPPT apparatus (Figure 5) establish the foundation for potential TPPT application in future analgesic drug screening. Because morphine and pregabalin have different mechanisms of action,²⁸ the observed positive responses from both drugs suggest that TPPT is a versatile test of candidate analgesics. Furthermore, the comparison of our diazepam results with results from a previous study²² suggests that our TPPT assay can better differentiate true and false-positive analgesics than the procedures of thermal and mechanical hypersensitivity assessment by Hargreaves and von Frey methods.

One may wonder why we chose cold instead of hot stimuli in our TPPT. A previous study has shown that CCI rats had a prolonged hypersensitivity to cold but not hot stimulations.¹² In addition, cold allodynia has been found to be more common than heat allodynia in patients with mechanical-nerve-injury-related neuropathic pain.²⁹ In a subpopulation of patients with neuropathy, their only clinical abnormality is cold allodynia.³⁰ Thus, cold instead of hot stimulation in TPPT is more reliable for experimental testing and more clinically relevant for the purpose of screening analgesics to treat neuropathic pain in humans.

In summary, we have demonstrated the utility of a new versatile, automated, and moderately high-throughput TPPT design for *in vivo* behavioral drug screening and development of the next generation of pain medications.

Materials & Methods

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were received at 5 weeks of age. The rats were housed in a temperature- and humidity-controlled facility under a 12-hour light-dark cycle with free access to food and water. Rats were randomly assigned to sham or injury groups. All animal protocols and procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Chronic constriction injury (CCI) and neuropathic pain model

Peripheral neuropathic pain was induced by a chronic constriction injury of the left sciatic nerve as reported previously.¹⁸ Anesthesia was induced by 2.5% isoflurane vapor in an oxygen and air mixture (50:50) within an induction chamber and maintained throughout the surgery by a nose cone. The fur on the left hind leg was removed by a thin application of a depilatory cream (Nair, Church & Dwight Co., Ewing, NJ, USA) and the exposed skin sterilized with a povidone-iodine solution. An incision was made at the thigh and the sciatic nerve was exposed by blunt dissection of the biceps femoris tissue. The connective tissue around the sciatic nerve was removed and four ligatures of chromic gut sutures (6-0 Chromic Gut, CP Medical, Norcross, GA, USA) were loosely tied at 1 mm spacing. The ligatures were tight enough such that they could not slide along the nerve but did not strangulate the sciatic nerve. Subsequently, the muscle and skin were closed with polyglycolic acid sutures (5-0 Polyglycolic acid, McKesson, Richmond, VA, USA). The wound was cleaned with povidone-iodine and treated with a layer of topical 2% lidocaine hydrochloride jelly to reduce interference of the sutures by the rats. To prevent the interference of surgical pain from measurements of cold allodynia, rats were given 10 days to recover after surgery and to develop peripheral neuropathic pain.³¹ Rats in the sham group received the same surgical procedure but without the ligatures around the sciatic nerve.

High-throughput TPPT apparatus

The TPPT apparatus consists of two rows of six adjacent chambers with each chamber having the dimensions of 24 × 45 × 30 cm (W x L x H) with black glass surface. The chamber walls were constructed from a 6 mm thick black expanded PVC sheet (Curbell Plastics, Orchard Park, NY) and assembled by evenly spaced interlocking slots at the junctions. The floor of each chamber was divided equally into warm and cold zones. The warm zone was formed by a commercial glass warming countertop (45.7 × 152.4 cm, W x L; Hatco HBG-6018, Milwaukee, WI) and controlled by a variable autotransformer (Staco Energy Products, Dayton, OH) for fine-tuning temperatures. The central warming countertop was flanked by two cooled glass surfaces of an equal size (24 × 152 cm) to form the cold zone in each chamber. The cold zone was constructed by placing a 0.6-cm thick black glass plate (One Day Glass, Vancouver, WA) on the top of a 1.3-cm aluminum plate (McMaster-Carr, Princeton, NJ). A 144W Peltier cooler assembly (Asixx B07DJC9V4Y) was bolted to the aluminum underneath the center of the cold zone in each chamber. Graphite film (Mineral Seal Corporation, 2010B-02039100, Tuscon, AZ) was sandwiched between the glass and aluminum plate to ensure good thermal contact. Each Peltier cooler assembly was wired to a 160W DC Buck converter (DROK B078Q1624B, www.droking.com) to provide

individual control of voltage and thereby temperature. Every six Peltier assemblies were wired in parallel to a power supply (60A 16VDC switching mode power supply, BK Precision 1900B, Yorba Linda, California). Extruded polystyrene insulation board (2" thick, Kingspan, Atlanta GA) was stacked between the Peltier assemblies for thermal insulation. The target temperatures of the warm and cold plates in each chamber were well maintained with a margin of $\pm 0.5^{\circ}\text{C}$ error and measured using an IR thermometer (Fluke-59 Max+; Fluke Corp., Everett, WA, USA) before and after each experiment. The experiments were recorded using two low light cameras (SVPRO 2MP 1080P Sony IMX322 2.8–12mm varifocal SV-USBFD06H-SFV) mounted above the TPPT apparatus.

Experimental procedure

Rats were typically moved into the testing room at least 30 minutes before the experiments. All rats had gone through habituation with both plates at room temperature before being used for TPPT. They were initially placed in the center of each chamber and roamed freely for 10 minutes. The location data of each rodent during habituation were collected as a baseline test using the Any-Maze behavioral tracking software (Stoelting Co., Wood Dale, IL, USA), which was also used for TPPT. All experiments were conducted in a low-light environment using red light lamps (Universal Red, Bright Lab, Dallas, TX, USA). For TPPT, a 10-minute measurement session was typically used except for the experiment to optimize time windows, for which an extended session up to 2 hours was used. Each rat can be used multiple times over the course of 10 to ~21 days after surgery, but no more than twice (temperature test) in a single day. For drug testing, rats were injected subcutaneously with phosphate buffered saline (PBS, pH = 7.4) as vehicle control or a dose of a given drug. Rats received a lower dose before a higher dose on a subsequent day. Animals were returned to their home cages after injection and waited for 30 minutes before TPPT to allow the drugs to take effect. To prevent a biased outcome due to the rats' potential learning of the apparatus, rats were placed into different chambers between experiments to ensure that they encountered the warm and cold plates on opposite sides of the apparatus for counterbalancing. The floor of each chamber was cleaned with 70% ethanol between tests.

Drugs

Morphine (morphine sulfate; McKesson, Richmond, VA, USA) was purchased as a solution (10 mg/mL) and used directly for experiments. Pregabalin ((S)-pregabalin; Toronto Research Chemicals, North York, ON, CN) was dissolved in PBS at 30 mg/mL and sterilized with a 0.2 μm syringe filter. Diazepam (Sigma-Aldrich Chemicals, St. Louis, MO, USA) powder was suspended in sterile PBS at 2 mg/mL.

Data Analysis

All statistical analyses were performed using Prism 8.3 (GraphPad Software, La Jolla, CA, USA). Data are reported as mean $\pm$ standard error of the mean (SEM). $P < 0.05$ was considered statistically significant for all analyses. One-way ANOVA with Dunnett's post-hoc test was used to analyze drug effects on percent time spent on the cold plate and the average traveling speed of rats (locomotor activity). Two-way ANOVA with Fisher's LSD post-hoc test was used to identify temperature pairs that lead to a statistical difference between sham and CCI groups in TPPT. Repeated measures mixed-effects analysis was

performed to determine the time windows showing significantly different percent time spent on the cold plate by sham and CCI rats. For habituation data, one sample t-test against a theoretical value of 50% was performed to determine if the sham or CCI rats had any inherent bias for one plate over the other. The same data were further analyzed using the two-tailed t-test to determine if there was significant difference between two groups of rats.

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Figure 1:

(A) A schematic top view of the TPPT apparatus designed for high-throughput *in vivo* nociceptive testing. The TPPT apparatus includes 12 chambers. Each chamber has a dimension of 45 cm x 22 cm x 30 cm with an equal area for cold (light gray) and warm (dark gray) plates. The cold temperatures are controlled by independent Peltier assemblies fastened below individual chambers and the warm temperatures are controlled by a commercial surface warming device. Two representative traces over 10 minutes of movement are shown for sham (left) and CCI (right) rats. (B) A photograph of the TPPT apparatus. The six Peltier assemblies for controlling “cold” temperatures for the individual front chambers are visible.

Figure 2: Baseline testing of apparatus.

Sham (n=12) and CCI rats (n=24) were introduced to the apparatus for habituation and a baseline test at room temperature (~25°C) with both cold and warm temperature control devices turned off. Neither animal group had an inherent bias for one plate over another. Data are expressed as means $\pm$ SEM.

Figure 3: Temperature optimization for testing neuropathic-pain-induced cold allodynia. Sham (open bars) and CCI (hashed bars) rats were tested in the TPPT apparatus at a constant warm temperature of 30 °C but varied cold temperatures. The significant differences of occupancy on the cold plate between sham (11 n 20) and CCI (18 n 26) rats were observed at 12, 13, and 14 °C ($\pm 0.5^{\circ}\text{C}$). Data are expressed as means $\pm$ SEM. Statistical analyses were performed using two-way ANOVA with Fisher's LSD post-hoc test (** $p < 0.01$).

Figure 4: Optimization of time window to effectively capture significant difference in the cold plate reaction between the CCI and sham rats.

CCI and sham rats were placed into the TPPT apparatus with temperatures of $12.5 \pm 0.5^\circ\text{C}$ and $30^\circ\text{C} \pm 0.5^\circ\text{C}$ for the cold and warm plates, respectively. The animals were allowed to explore the chamber freely for up to 2 hours. Each data set was fit with a double exponential decay function. The CCI rats ($n=21$, 20 min; $n=7$, >20 min) spent significantly less time on the cold plate than the sham group ($n=15$, 20 min; $n=7$, >20 min) for up to 90 minutes. Data are expressed as means $\pm$ SEM. Statistical analysis was performed using mixed-effects analysis with Fisher's LSD post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 5: Pharmacologic intervention of cold allodynia with common analgesics for neuropathic pain.

Sham (n=16) and CCI (n=30) rats treated with vehicle (PBS) and CCI rats treated with the indicated doses (s.c.) of pregabalin (n=8–10), morphine (n=8–10), or diazepam (n=10) were placed for TPPTs 30 minutes after injection. (A) % time on the cold plate and (B) total distance traveled during 10 min experiment recorded and compared among different groups. The cold and warm plate temperatures were held at $12.5 \pm 0.5^\circ\text{C}$ and $30 \pm 0.5^\circ\text{C}$, respectively. Data are expressed as means $\pm$ SEM. Statistical analysis with one-way ANOVA and Dunnett's post-hoc test to compare CCI without and with drug treatment. Unpaired t-test was performed for sham vs CCI injected with vehicle. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).