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Research Paper

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Evaluation of different drug classes on transient sciatic nerve injury–depressed marble burying in mice

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Abstract

A great need exists for the identification of new effective analgesics to treat sustained pain. However, most preclinical nociceptive assays measure behavioral responses evoked by noxious stimuli (ie, pain-stimulated behavior), which presents a challenge to distinguish between motor impairing and antinociceptive effects of drugs. Here, we demonstrate that chronic constriction injury (CCI) of the sciatic nerve elicits common pain-stimulated responses (ie, mechanical allodynia and thermal hyperalgesia) as well as reduces marble burying/digging behaviors that occur during the early stages of the neuropathy and resolve within 1 week. Although drugs representing distinct classes of analgesics (ie, morphine, valdecoxib, and gabapentin) reversed both CCI-induced and CCI-depressed nociceptive measures, diazepam lacked antinociceptive effects in all assays and the kappa-opioid receptor agonist U69593 reversed pain-stimulated, but not pain-depressed behaviors. In addition, we tested drugs targeting distinct components of the endocannabinoid system, including agonists at cannabinoid receptors type 1 (CB_1) and type 2 (CB_2), as well as inhibitors of the endocannabinoid-regulating enzymes fatty acid amide hydrolase and monoacylglycerol lipase. Each of these drugs reversed all CCI-induced nociceptive measures, with the exception of the fatty acid amide hydrolase inhibitor that reversed pain-stimulated behaviors, only. These findings support the use of the mouse marble-burying assay as a model of pain-depressed behavior within the first week of sciatic nerve injury to examine candidate analgesics. These data also support existing preclinical research that cannabinoid receptor agonists and inhibitors of endocannabinoid-regulating enzymes merit consideration for the treatment of pain.

Keywords: Cannabinoid, Pain depressed, CCI, SNI

1. Introduction

Pain is associated with numerous disease states and incurs a tremendous cost to society in terms of medical treatment and lost wages. Preclinical animal models of pain typically measure "pain-stimulated" behavior, such as withdrawal responses from mechanical or thermal stimuli. Although numerous drugs elicit antinociceptive effects in pain-stimulated animal models, drug-induced motor impairment often confounds interpretation of these studies. Clinically relevant pain is commonly associated with functional impairment, which is not measured in typical preclinical pain-stimulated assays.[43,51,53,64] A cadre of assays examine pain-depressed behavior using nest-building,[17,53] burrowing,[16] decreases in lever pressing for electrical intracranial self-stimulation,[43,47] locomotion,[7] and wheel running,[48] but the need persists for better preclinical assays to identify new analgesic drugs that have increased translational implications. In this study, we tested whether the mouse chronic constriction injury (CCI) of the sciatic nerve model of pain would alter marble-burying behavior. The marble-burying assay is sensitive to many environmental manipulations and different drug classes, including benzodiazepines, serotonin reuptake inhibitors, cannabinoids, and opioids. Although decreases in marble burying are often interpreted as evidence for anxiolytic-like activity,[37] an alternative view is that it is a consequence of natural digging behavior.[27]

First, we tested whether CCI alters marble-burying behavior and whether these changes correlate with time spent digging. In addition, we measured traditional pain-stimulated behavior, including hypersensitive withdrawal responses from mechanical stimuli (von Frey filaments) and thermal stimuli (hot plate).[5,22] As mice undergoing the spared nerve injury (SNI) procedure show increases in marble burying,[24,55] we also compared marble burying in mice subjected to the CCI procedure vs the SNI procedure. Second, we tested the effects of a variety of pharmacological agents representing different drug classes in this assay. We tested traditional analgesics, including morphine, gabapentin, and a cyclooxygenase-2 (COX2)-selective inhibitor. Two negative control drugs were also included. Kappa-opioid

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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receptor agonists commonly produce apparent antinociception in traditional assays of pain-stimulated behavior but fail to produce effective analgesia in clinical trials.[52,53] We also evaluated diazepam, well known to reduce marble-burying behavior, but lacks antinociceptive activity in animals and analgesic effects in humans.[37,60]

Our final objective was to test whether drugs targeting different components of the endogenous cannabinoid system would alter CCI-depressed and CCI-stimulated behavior. Although agonists of the cannabinoid 1 (CB_1) receptor and cannabinoid 2 (CB_2) receptor produce antinociceptive effects in numerous models of sustained pain,[21,73] the motor-depressive effects produced by CB_1 receptor stimulation not only reflect a side effect of concern but render tests of pain-stimulated behavior difficult to interpret.[14] CB_2 receptor agonists are devoid of locomotor effects.[59] Inhibitors of the respective chief hydrolytic enzymes of *N*-arachidonoylethanolamine (anandamide),[19] 2-arachidonylglycerol [2-AG],[46,65] fatty acid amide hydrolase [FAAH],[15] and monoacylglycerol lipase [MAGL],[20] produce antinociceptive effects in a variety of sustained pain models.[1,25,32,34,35] Thus, we tested a CB_1/CB_2 receptor agonist, a selective CB_2 receptor agonist, an FAAH, and an MAGL inhibitor for alterations in CCI-induced marble-burying behavior.

2. Methods

2.1. Animals

Adult male C57BL/6J mice (18-35 g; Jackson Laboratory, Bar Harbor, ME) served as subjects in these experiments. Mice were housed 4 per cage in a temperature (20-22°C), humidity (55 ± 10%), and light-controlled (12 hours light/dark; lights on at 0600) AAALAC-approved facility, with standard rodent chow and water available ad libitum. All procedures adhered to the ARRIVE guidelines[33] as well as those of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. The sample sizes selected for each treatment group in each experiment were based on previous studies from our laboratory. Specifically, we used a total of 8 to 12 mice per experimental group where the only, or main, behavioral measure examined was marble-burying behavior[37] and we used a total of 6 mice per experimental group where pain-evoked behaviors were examined in conjunction with marble-burying behaviors, as this number of animals is sufficient for the detection of significant effects for pain-evoked behaviors.[6,29,34,36,72] To ensure reproducibility of our data, we voluntarily triplicated the morphine dose–response study, including vehicle treatment, and finding no significant differences in the sets of data, collapsed them together, resulting in a study size of up to 18 mice per group for those experiments. A post hoc power analysis was performed (paired *t* test, PASS 15; NCSS LLC, Kaysville, UT) to verify that group sizes were adequate to detect significant effects.

2.2. Drugs

Gabapentin, diazepam, and the kappa-opioid agonist (+)-(5α,7α,8β)-N-Methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4.5)dec-8-yl)-benzeneacetamide (U69593) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The COX2-selective inhibitor, valdecoxib, was gifted by Ironwood Pharmaceuticals. Morphine sulfate and the pan cannabinoid agonist (-)-*cis*-3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl)-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940)[44] were obtained from the National Institute on Drug Abuse Drug Supply Program (Bethesda, MD). The CB_2 receptor selective agonist 3-cyclopropyl-1-(4-(6-((1,1-dioxidothiomorpholino)methyl)-5-fluoropyridin-2-yl)benzyl)imidazolidine-2,4-dione hydrochloride (LEI101) was synthesized by the van der Stelt laboratory as described previously.[68] The MAGL inhibitor MJN110[11,54,72] and the FAAH inhibitor *N*-3-pyridinyl-4-((3-((5-(trifluoromethyl)-2-pyridinyl)oxy)phenyl)methyl)-1-piperidinecarboxamide (PF3845)[2] were synthesized by the Cravatt laboratory as described previously. All drugs except morphine were dissolved in a vehicle solution consisting of a mixture of ethanol, alkamuls-620 (Sanofi-Aventis, Bridgewater, NJ), and saline (0.9% NaCl) in a 1:1:18 ratio. Morphine was dissolved in sterile saline, (Hospira Inc, Lake Forest, IL). Each drug was given through the intraperitoneal (i.p.) route of administration. All drugs were administered in a volume of 10 μL/g body mass. The pretreatment times of drugs tested were based on previously reported time points in which maximal antiallodynic effects occurred, and dose ranges were based on previous work (**Table 1**).

2.3. Surgical procedures

2.3.1. Chronic constriction injury surgery

After baseline (BL) behavioral assessment, the surgical procedure for chronic constriction of the sciatic nerve was completed as previously described[5] but modified for mouse.[29] In brief, the mice were anesthetized with isoflurane (induction 5% vol. followed by

Table 1

Description of the drugs tested in mechanical allodynia, thermal hyperalgesia, and marble-burying assays.

Drug	Drug class	Doses (mg/kg)	Pretreatment time (min)	Reference(s)
Morphine	Opioid receptor agonist	0.3, 1, 3, 10	30	72
Gabapentin	Anticonvulsant	0.5, 20, 50	60	36
Valdecoxib	COX2 selective inhibitor	0.1, 2.5, 5	90	26
Diazepam	Benzodiazepine	0.3, 1, 3	60	37,60
U69593	K-opioid receptor agonist	0.01, 0.032, 0.1	15	8
CP55,940	CB_1/CB_2 receptor agonist	0.01, 0.1, 0.2	30	34
LEI101	CB_2 receptor agonist	5, 20, 40	120	68
MJN110	MAGL inhibitor	0.125, 0.5, 1.25	90	29,72
PF3845	FAAH inhibitor	3, 10, 30	60	23

FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase.

2.0% in oxygen), and the mid to lower back and the dorsal left thigh were shaved and cleaned with 75% ethanol. Using aseptic procedures, the sciatic nerve was carefully isolated, and loosely ligated with 3 segments of 5-0 chromic gut sutures (Ethicon, Somerville, NJ). Sham surgery was identical to CCI surgery, but without the loose nerve ligation. The overlying muscle was closed with (1) 4-0 sterile silk suture (Ethicon), and animals recovered from anesthesia within approximately 5 minutes. Use of chromic gut sutures in this model has been well characterized to produce bilateral allodynia, as well as robust upregulation of bilateral markers of inflammation in the dorsal horn of the spinal cord and the corresponding dorsal root ganglia.[49,57,58,71] Mice were randomly assigned to either CCI or sham surgical group, as BL measures were not incorporated into the assignment in any way. Mice in both groups were reassessed for allodynia and thermal hyperalgesia, as described above. Except for the characterization studies in **Figures 1 and 2**, a single acute administration of drugs or vehicle was used, with a cross-over design for administration on days 3 and 6 after surgery.

2.3.2. Spared nerve injury surgery

Spared nerve injury surgery was performed as described by Decosterd and Woolf.[18] Briefly, mice were anesthetized and prepared as described above for CCI surgery. After exposure of the sciatic nerve, the tibial and common peroneal nerve branches were ligated using silk sutures and transected while leaving the sural nerve intact. As with CCI surgery, the overlying musculature was closed using (1) 4-0 sterile silk suture and the animals recovered from anesthesia within 5 minutes.

2.4. Marble-burying assay

Mice were placed in Plexiglas cages (internal dimensions: 33 cm long × 21 cm wide × 19 cm high) with ~3 cm of wood chips (7090 Teklad Sani-Chips; Envigo, Somerset, NJ) on bottom and 20 marbles were spaced in grid-like manner (4 × 5). Marbles used in these experiments were clear, with exception to Digital Supplement Figure 2B (available online at http://links.lww.com/PAIN/A549) where black marbles were used, but this did not noticeably influence digging behavior, or the data obtained. After the 20-minute test period, subjects were carefully removed to minimize disturbance to the bedding. Marbles at least 75% covered with bedding were considered buried. In the model characterization studies, mice were recorded using Anymaze software (Stoelting Co, Wood Dale, IL) to determine time spent digging. Digging time was scored by an experimenter blinded with respect to group. Digging was operationally defined as use of the hind paws to kick, or forcefully move, the woodchip bedding. This scoring was highly reliable, as a correlation of $r = 0.98$ was observed between 2 independent scorers. The experimenters were blinded with respect to surgical and treatment condition throughout all experiments. For the pharmacological assessment studies that examined both pain-evoked behaviors and marble burying, the order of testing was von Frey, hot plate, and then marble burying was analyzed last. For these studies, time spent digging was not captured. To minimize the number of mice needed to complete these studies, mice were tested with drug or vehicle on both day 3 and day 6 after CCI, in a counterbalanced fashion.

2.5. Assessment of pain-stimulated behavior

Mechanical allodynia and thermal hyperalgesia were used to assess nociceptive behavior after sham or CCI surgery (see above). Before surgery, mice were habituated to the testing environment. Next, von Frey monofilaments (North Coast Medical, Morgan Hills, CA) were used to establish BL responses to light mechanical touch and to assess the development and presence of allodynia after surgery.[29] Specifically, the mice were placed on top of a wire mesh screen, with spaces 0.5 mm apart

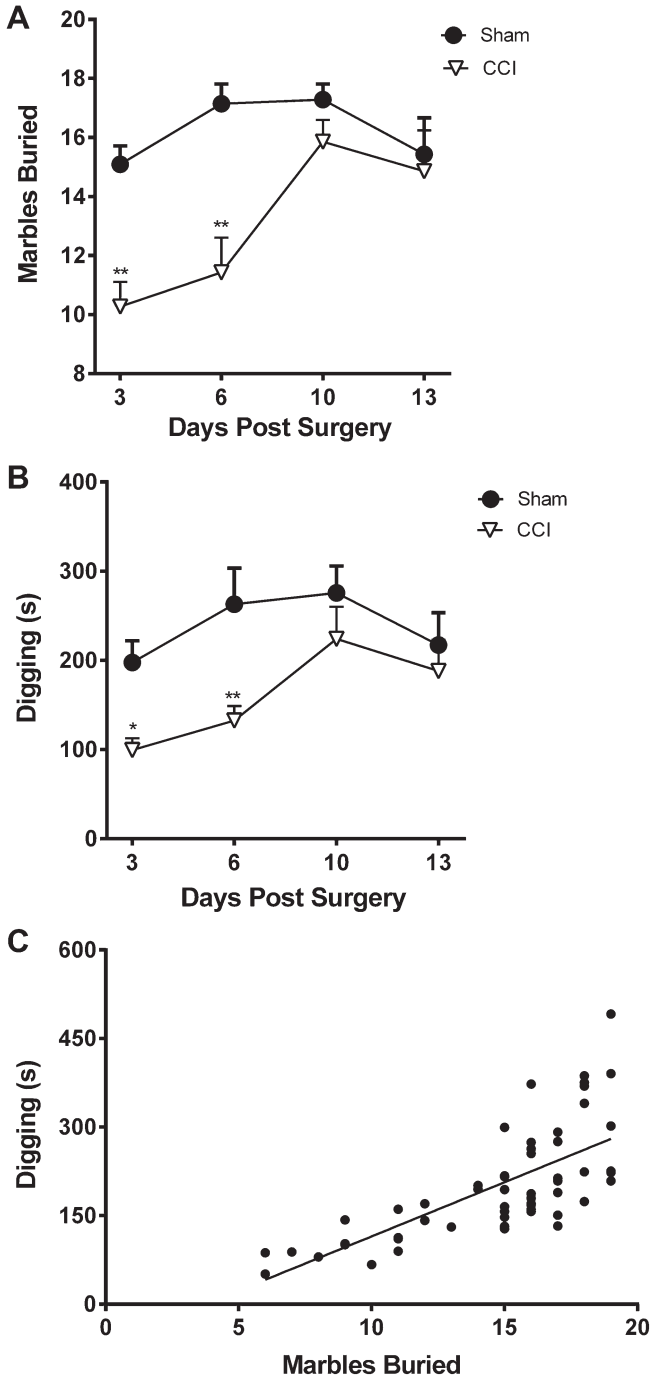

Figure 1. Mice subjected to CCI surgery bury fewer marbles and spend less time digging than sham mice. (A) On postsurgical days 3 and 6, mice in the CCI condition bury fewer marbles than mice in sham surgery group, but marble burying is normalized by day 10. (B) Chronic constriction injury surgery leads to reductions in time spent digging on postsurgical days 3 and 6, and digging behavior is again normalized by day 10. (C) The time spent digging and number of marbles buried from panels A and B were highly correlated ($r = 0.84$). ***$P < 0.0001$, **$P < 0.005$, and *$P < 0.05$ vs sham. Data reflect mean ± SEM, n = 8 mice per group. CCI, chronic constriction injury.

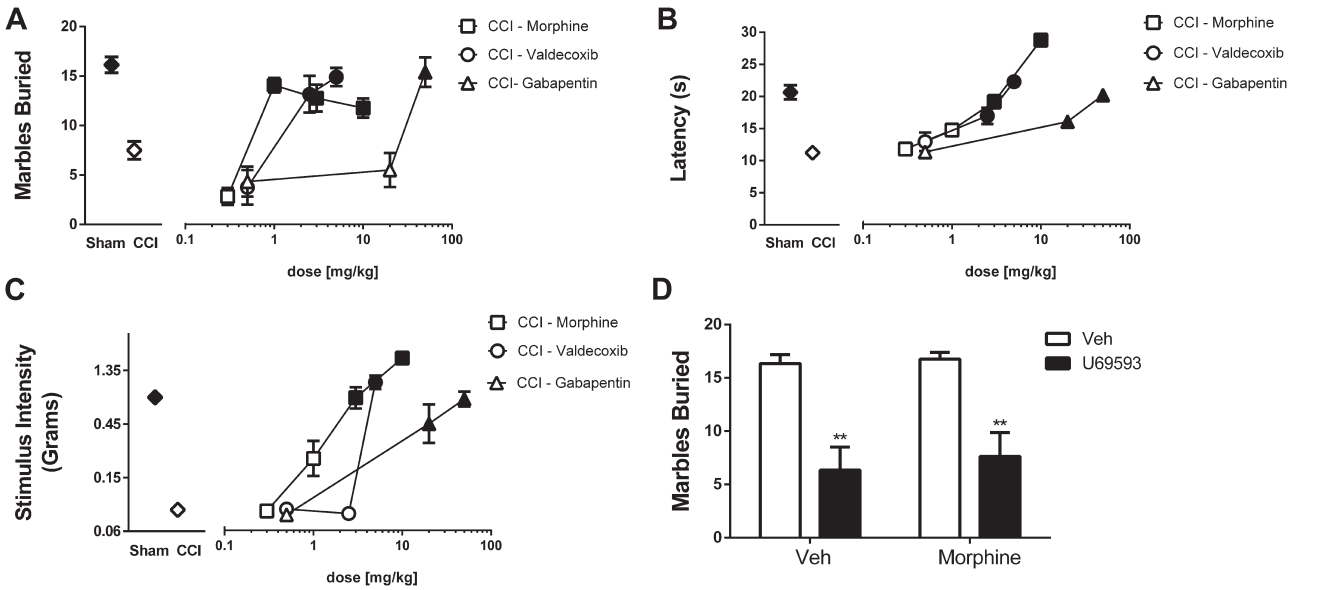

Figure 2. Standard analgesics reverse pain-depressed marble burying and pain-stimulated allodynia and thermal hyperalgesia. Morphine, valdecoxib, and gabapentin dose dependently reverse CCI-induced (A) decreases in marble burying, (B) thermal hyperalgesia, and (C) allodynia. Filled symbols denote significance from CCI controls, ($P < 0.05$). (D) Morphine (10 mg/kg) does not affect marble-burying behavior in vehicle-treated mice and does not alter U69593 (0.1 mg/kg)-induced decreases in marble burying. $^{**}P < 0.001$, vs vehicle–vehicle. Data reflect mean ± SEM, n = 6 to 18 mice/group. CCI, chronic constriction injury.

and habituated for approximately 30 minutes on 4 consecutive days before surgical procedures commenced. Mice were unrestrained, and singly placed under an inverted Plexiglas basket (8 cm diameter and 15 cm height), with a wire mesh top to allow for unrestricted air flow. The von Frey test uses a series of calibrated monofilaments, (2.83-4.31 log stimulus intensity) applied randomly to the left and right plantar surface of the hind paw for 3 seconds, using a modified "up-down" method.[10] Each paw was stimulated 5 times with each filament, starting with the 3.61 log stimulus intensity filament and increasing until the mouse responded 5 out of 5 times.[29] Lifting, licking, or shaking the paw was considered a response. Three or more responses out of 5 stimulations at the 3.61 log stimulus intensity filament were coded as a positive response. If a positive response was detected, at the 3.61 log stimulus intensity filament, lower weight filaments, starting at 2.84 and then sequentially increasing were used to assess the sensory threshold for each paw until the paw responded 5 out of 5 times. After completion of allodynia testing, the mice were placed on a heated (52°C) enclosed Hot Plate Analgesia Meter (Columbus Instruments, Columbus, OH). The latency to jump or lick/shake a hind paw was assessed. A 30-second cutoff time was used to avoid potential tissue damage.[22] For each assay, testing was performed in a blinded fashion. For dose–response analysis of each drug, data from the same sham-vehicle and CCI-vehicle groups are included in each appropriate graph depicting marble burying, mechanical allodynia, and thermal hyperalgesia.

2.6. Data analysis

All data are presented as mean ± standard error (SEM). For allodynia testing, psychometric behavioral analysis was performed to compute the log stiffness that would have resulted in the 50% paw withdrawal rate, as previously described.[50, 67] Briefly, thresholds were estimated by fitting a Gaussian integral psychometric function to the observed withdrawal rates for each of the tested von Frey hairs, using a maximum-likelihood fitting method.[49,71] Pearson correlations were conducted to examine the relationship between marble burying and digging time, and these data were transformed in a best-fitting curve analysis. Data were analyzed using appropriate inferential statistical analysis of *t* tests, 1-way analysis of variance (ANOVA), or 2-way ANOVA.[13] Except where indicated, the Tukey test was used for post hoc analyses of significant 1-way ANOVAs. Multiple comparisons after 2-way ANOVA were conducted with Bonferroni post hoc comparison.

3. Results

3.1. Chronic constriction injury surgery reduces marble-burying and digging behaviors

The first experiment tested whether CCI surgery would alter marble burying and time spent digging compared with mice that had undergone sham surgery. As shown in **Figure 1**, mice in the CCI group buried fewer marbles than mice in the sham control ($P < 0.01$, **Fig. 1A**), and displayed a reduction in time spent digging (main effect of surgery, $P < 0.01$, main effect of time, $P < 0.05$, **Fig. 1B**) compared with controls. The reduction in these behaviors occurred on postsurgical days 3 and 6 and increased to levels of the control mice by days 10 and 13. Further analysis revealed a positive correlation of time spent digging and the number of marbles buried, ($r = 0.84$, $P < 0.0001$, **Fig. 1C**). Locomotor speed ($P = 0.4$, Supplemental Digital Content Figure 1A, available online at http://links.lww.com/PAIN/A549) and distance traveled ($P = 0.5$, Supplemental Digital Content Figure 1B, available online at http://links.lww.com/PAIN/A549) did not differ between sham and CCI groups. To determine whether repeated exposure to the marble-burying assay accounted for the complete resolution of the observed effects, new groups of mice undergoing sham or CCI surgery were prepared and tested on postsurgical days 3 and 13, only. On day 3, mice in the CCI group buried significantly fewer marbles than the sham mice ($P < 0.01$, Supplemental Digital Content

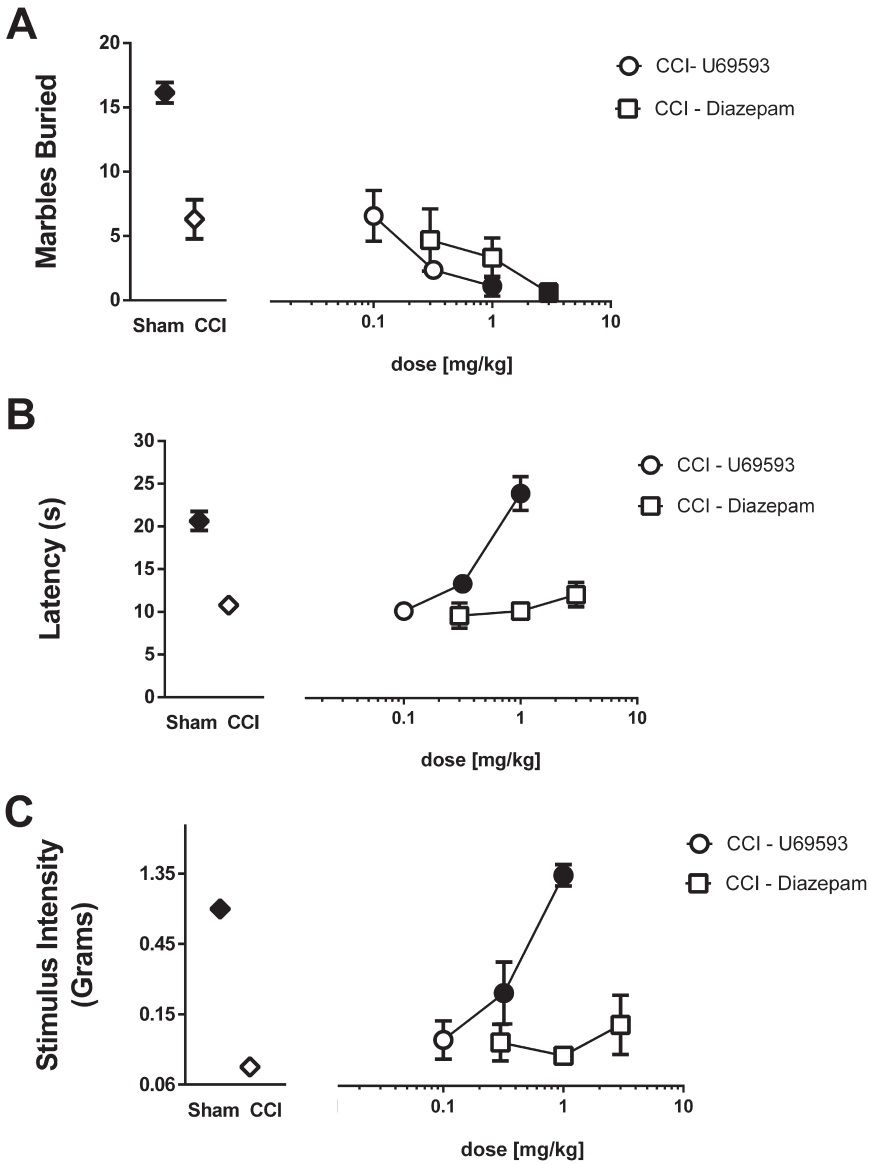

Figure 3. Diazepam and U69593 decrease marble-burying behavior but show differential effects in pain-stimulated behavioral assays. (A) Neither diazepam nor U69593 reverses decreases in marble burying, but further decreased marble burying at the highest doses tested. (B) U69593, but not diazepam reversed thermal hyperalgesia (C) and allodynia. Filled symbols denote significance from CCI controls, ($P < 0.05$). Data reflect mean ± SEM, n = 6 to 8 mice/group. CCI, chronic constriction injury.

Figure 1C, available online at http://links.lww.com/PAIN/A549) and spent less time digging than mice in the sham group ($P < 0.05$). However, on postsurgical day 13, both groups buried a similar number of marbles ($P = 0.40$) and spent a comparable duration of time digging, ($P = 0.39$, Supplemental Digital Content Figure 1D, available online at http://links.lww.com/PAIN/A549). Thus, CCI surgery depressed marble burying and digging time for the first postsurgical week.

Because previous research using the SNI neuropathic pain model reported increases in marble burying,[24,55] we next compared marble burying behavior among SNI, CCI, and sham-operated mice on days 3 and 14 postsurgery. Both the CCI and SNI groups exhibited significant decreases in marble burying compared with sham-operated controls on day 3 ($P < 0.05$, Supplemental Digital Content Figure 2A, available online at http://links.lww.com/PAIN/A549), no group differences were observed on day 14 ($P = 0.7$, Supplemental Digital Content Figure 2B, available online at http://links.lww.com/PAIN/A549). However, mice undergoing SNI or CCI surgery exhibited significant ipsilateral mechanical allodynia ($P < 0.0001$, Supplemental Digital Content Figure 2C, available online at http://links.lww.com/PAIN/A549) and thermal hyperalgesia on day 13 compared with sham mice ($P < 0.0001$, Supplemental Digital Content Figure 2D, available online at http://links.lww.com/PAIN/A549).

3.2. Evaluation of standard analgesics and diazepam in chronic constriction injury–depressed marble burying and chronic constriction injury–evoked behaviors

We next examined if a panel of drugs (ie, morphine, gabapentin, and valdecoxib) known to reverse CCI-induced mechanical allodynia and thermal hyperalgesia would also ameliorate CCI-depressed marble burying. Morphine significantly reversed CCI-induced decreases in marble burying ($P < 0.05$, **Fig. 2A**),

thermal hyperalgesia ($P < 0.0001$, **Fig. 2B**), and mechanical allodynia ($P < 0.0001$, **Fig. 2C**). Similarly, gabapentin significantly reversed CCI-induced decreases in the marble-burying assay ($P < 0.001$, **Fig. 2A**), thermal hyperalgesia ($P < 0.0001$, **Fig. 2B**), and mechanical allodynia ($P < 0.0001$, **Fig. 2C**). Similarly, valdecoxib reversed CCI-depressed marble burying ($P < 0.001$, **Fig. 2A**), thermal hyperalgesia ($P < 0.001$, **Fig. 2B**), and mechanical allodynia ($P < 0.0001$, **Fig. 2C**).

None of the drugs altered marble burying in sham mice (Supplemental Digital Content Figure 3A; morphine: $P = 0.7$, valdecoxib: $P = 0.1$, gabapentin: $P = 0.7$, available online at http://links.lww.com/PAIN/A549). Morphine elicited antinociception in the hot-plate test ($P < 0.0001$), but neither valdecoxib ($P = 0.94$) nor gabapentin ($P = 0.06$) significantly altered basal hot-plate responses (Supplemental Digital Content Figure 3B, available online at http://links.lww.com/PAIN/A549). In sham mice, morphine elevated mechanical stimulus thresholds ($P < 0.01$), but gabapentin ($P = 0.9$) and valdecoxib ($P = 0.06$) did not significantly affect this measure (Supplemental Digital Content Figure 3C, available online at http://links.lww.com/PAIN/A549).

Next, we examined whether the standard analgesic morphine would reverse the depressive effects of the kappa-opioid receptor agonist U69593 on marble burying. Naive mice given U69593 (0.1 mg/kg) buried fewer marbles compared with the vehicle control mice (**Fig. 2D**). Unlike its effects in reversing CCI-induced depression of marble burying, morphine (10 mg/kg) did not alter marble-burying behavior in either vehicle or U69593-injected mice (main effect of U69593, $P < 0.0001$).

We next sought to investigate if either the anxiolytic diazepam or U69593 would alter CCI depression of marble burying or CCI-induced allodynia or thermal hyperalgesia. Both diazepam and U69593 produced further decreases in marble burying within the CCI group ($P < 0.05$, **Fig. 3A**), but these drugs had differential effects on CCI-stimulated behavior. Although diazepam failed to alter CCI-induced thermal hyperalgesia ($P = 0.4$, **Fig. 3B**), or mechanical allodynia ($P = 0.6$, **Fig. 3C**), U69593 produced a dose-responsive reversal of CCI-induced thermal hyperalgesia ($P < 0.0001$, **Fig. 3B**), and mechanical allodynia ($P < 0.001$, **Fig. 3C**).

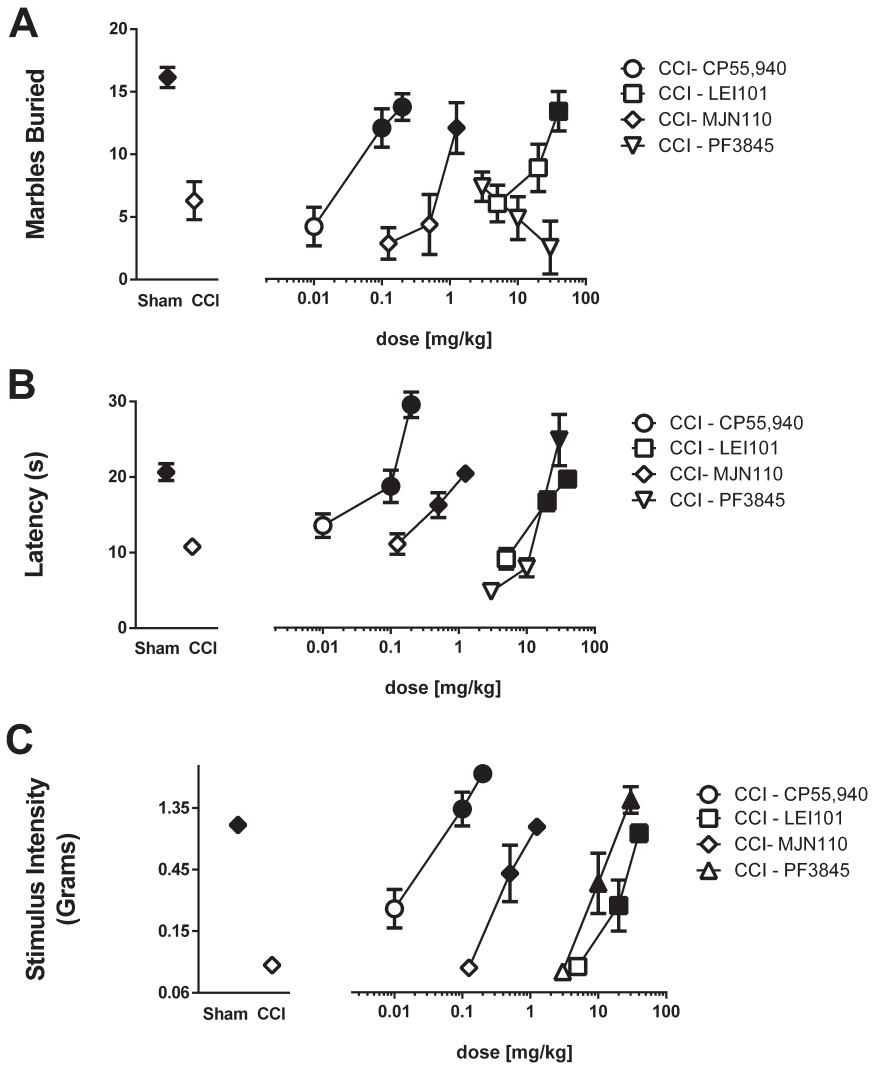

Figure 4. Drugs targeting different components of the endogenous cannabinoid system differentially reverse CCI-induced nociceptive behavior. The mixed CB_1/CB_2 receptor agonist CP55,940, the selective CB_2 receptor agonist LEI101, and the MAGL inhibitor MJN110 dose dependently reverse CCI-induced decreases in (A) marble burying, (B) thermal hyperalgesia, and (C) mechanical allodynia. The FAAH inhibitor PF3845 does not reverse CCI-induced depression of (A) marble burying but reverses CCI-induced (B) thermal hyperalgesia, and (C) mechanical allodynia. Filled symbols denote significance from CCI controls, ($P < 0.05$). Data reflect mean ± SEM, n = 6 mice/group. CCI, chronic constriction injury; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase.

Both diazepam and U69593 decreased marble burying in sham mice (diazepam: $P < 0.0001$, U69593: $P < 0.01$; Supplemental Digital Content Figure 4A, available online at http://links.lww.com/PAIN/A549), but neither drug altered hot-plate latencies (diazepam: $P = 0.1$; U69593: $P = 0.6$; Supplemental Digital Content Figure 4B, available online at http://links.lww.com/PAIN/A549) or mechanical stimulus thresholds (U69593: $P = 0.4$; diazepam: $P = 0.9$; Supplemental Digital Content Figure 4C, available online at http://links.lww.com/PAIN/A549) in sham control mice.

3.3. Modulation of the endocannabinoid system reverses both pain-evoked and pain-depressed behavior

Finally, because the endogenous cannabinoid system contains multiple targets that show promise for the treatment of sustained pain, we tested cannabinoid receptor agonists as well as inhibitors of FAAH and MAGL in CCI-induced depression of marble burying. In addition, the same mice were tested for mechanical allodynia and thermal hyperalgesia.

The mixed CB_1/CB_2 receptor agonist CP55,940 dose dependently reversed marble burying ($P < 0.001$, **Fig. 4A**), thermal hyperalgesia ($P < 0.0001$, **Fig. 4B**), and mechanical allodynia ($P < 0.0001$, **Fig. 4C**) in the CCI group. Likewise, the CB_2 receptor agonist LEI101 dose dependently reversed CCI-induced decreases in marble burying ($P < 0.05$, **Fig. 4A**), thermal hyperalgesia ($P < 0.01$, **Fig. 4B**), and mechanical allodynia ($P < 0.05$, **Fig. 4C**).

The MAGL inhibitor MJN110 reversed CCI-induced reduction in marble burying ($P < 0.01$, **Fig. 4A**), thermal hyperalgesia ($P < 0.001$, **Fig. 4B**), and mechanical allodynia ($P < 0.0001$, **Fig. 4C**). By contrast, the FAAH inhibitor PF3845 did not reverse marble-burying behavior ($P = 0.1591$, **Fig. 4A**), but reversed thermal hyperalgesia ($P = 0.001$, **Fig. 4B**) and mechanical allodynia ($P = 0.0004$, **Fig. 4C**) in the CCI group. A summary of the drug effects on CCI-induced decreases in marble-burying and pain-evoked behaviors can be found in **Table 2**.

In sham mice, 2 mg/kg CP55,940 significantly decreased marble burying ($P < 0.05$, Supplemental Figure 5A, available online at http://links.lww.com/PAIN/A549), as well as elevated hot-plate latencies ($P < 0.05$, Supplemental Digital Content Figure 5B, available online at http://links.lww.com/PAIN/A549) and withdrawal thresholds in the von Frey assay ($P < 0.01$, Supplemental Digital Content Figure 5C, available online at http://links.lww.com/PAIN/A549). Meanwhile, 40 mg/kg LEI101 and 1.25 mg/kg MJN110 did not alter the number of marbles buried (LEI101: $P = 0.4$, MJN110: $P = 0.4$, Supplemental Digital Content Figure 5A, available online at http://links.lww.com/PAIN/A549), hot-plate latencies (LEI101: $P = 0.3$, MJN110: $P = 0.3$, Supplemental Digital Content Figure 5B, available online at http://links.lww.com/PAIN/A549), or paw withdrawal thresholds (LEI101: $P = 0.6$, MJN110: $P = 0.1$, Supplemental Digital Content Figure 5C, available online at http://links.lww.com/PAIN/A549) in sham mice. PF3845 produced a small, but significant decrease in the number of marble buried ($P < 0.001$, Supplemental Digital Content Figure 5A, available online at http://links.lww.com/PAIN/A549) and reduced hot-plate latencies ($P < 0.05$, Supplemental Digital Content Figure 5B, available online at http://links.lww.com/PAIN/A549), but did not affect von Frey thresholds ($P = 0.6$, Supplemental Digital Content Figure 5C, available online at http://links.lww.com/PAIN/A549).

4. Discussion

Pain disrupts performance of otherwise routine behaviors such as housekeeping, social function, grooming, and can severely impact job performance as well as overall quality of life.[41,45,62] Despite pain-depressed normative life-oriented endpoints, most preclinical studies screening new analgesics use a cadre of assays using pain-evoked behaviors, such as lifting or licking hind paws in response to light mechanical touch, cold, or heat.[50, 51] Conversely, evaluation of pain-depressed behaviors offers parallel preclinical lines of evidence for screening potential therapeutics and has been hypothesized to predict clinical efficacy better than pain-stimulated pain assays.[43,51,53,64] Examples of pain-depressed assays include reductions in voluntary wheel running[70] and burrowing behavior[3] in rats after CCI or SNI surgery, respectively. We report that CCI or SNI surgery transiently decreases marble-burying behavior and overall time spent digging. Moreover, established antinociceptive agents from distinct drug classes (ie, morphine, gabapentin, and valdecoxib) and a variety of drugs targeting different components of the endocannabinoid system (ie, cannabinoid receptors and MAGL) reversed CCI-induced depression of marble burying, as well as CCI-evoked behaviors of thermal hyperalgesia and mechanical allodynia.

Although CCI and SNI surgery led to mechanical allodynia and thermal hyperalgesia that persisted beyond the second post-surgical week, the decreased marble-burying effect occurred

Table 2

Description of whether test drugs reverse, have no change, or further diminish CCI-induced alterations in marble burying, thermal hyperalgesia, and mechanical allodynia.

Drug	Marble burying	Thermal hyperalgesia	Mechanical allodynia	Clinically used?
Morphine	Reverse	Reverse	Reverse	Yes
Gabapentin	Reverse	Reverse	Reverse	Yes
Valdecoxib	Reverse	Reverse	Reverse	Yes
Diazepam	Further diminish	No change	No change	No
U69593	Further diminish	Reverse	Reverse	No
CP55,940	Reverse	Reverse	Reverse	No
LEI101	Reverse	Reverse	Reverse	No
MJN110	Reverse	Reverse	Reverse	No
PF3845	No change	Reverse	Reverse	No

In addition, it is noted if these compounds currently have a clinical indication for the treatment of pain.
CCI, chronic constriction injury.

during the first postsurgical week, only. However, these decreases are likely not due to the surgical procedure itself, as sham mice display similar rates of marble burying to surgically naive vehicle–vehicle treated mice (**Fig. 2D**). Our findings agree with other evidence for robust and sustained expression of many pain-stimulated behaviors in comparison with expression of pain-depressed behaviors.[12,43,61] The high correlation between the number of marbles buried and the time spent digging is consistent with work from Gyertyan,[27] who concluded that this assay reflects overall digging behavior. Other data suggest that marble burying reflects a repetitive and perseverative behavior,[66] rather than a model of anxiety or depression. Given that CCI surgery led to a transient decrease in marble-burying behavior compared with the long duration of hypersensitive withdrawal responses from mechanical and thermal stimuli,[36] this may be related to inflammatory and pronociceptive mediators associated with the early-phase after the nerve ligation. Alternatively, the relatively quick resolution of this pain-depressed behavior compared with the pain-stimulated behaviors may reflect an adaptive response in which mice rely on such behaviors such as digging to survive.[16] Mechanical allodynia and thermal hyperalgesia are regulated largely through the spinothalamic tract,[56, 63] whereas numerous regions within the central nervous system (eg, inputs from the hippocampus, prefrontal cortex, hypothalamus, thalamus, and spinal cord[16,40]) regulate digging behavior. Thus, clear anatomical distinctions, specifically regarding higher order brain region inputs, may be responsible for the facilitated resolution of CCI-induced depression of marble burying, compared with CCI-induced thermal hyperalgesia or mechanical allodynia.

Morphine, gabapentin, and valdecoxib are reported to elicit antinociceptive effects in preclinical models of pain.[26,34,63] In this study, each drug fully reversed both CCI-stimulated nociceptive behaviors and the depressive effects of CCI surgery on marble-burying behavior, despite producing pharmacological effects through distinct mechanisms. Specifically, morphine dampens transmission of the sensory and affective components of nociception through activation of the mu-opioid receptor, gabapentin dampens neuronal excitability, and valdecoxib elicits anti-inflammatory effects through the inhibition of COX-2. Similarly, the selective COX-2 inhibitor celecoxib decreases upregulation of $P2X_3$ receptors in dorsal root ganglia and decreases CCI-stimulated nociceptive behaviors when administered early after CCI.[69] Although U69593 reversed CCI-stimulated behaviors, it depressed marble burying irrespective of CCI surgery. Similarly, U69593 reverses lactic acid–induced stretching behavior, but not lactic acid-depressed nest-building behaviors.[53] Although morphine reversed CCI-depressed marble burying, which at higher doses might be due to enhanced locomotion rather than digging, it did not reverse drug-induced depression of marble burying by U69593. This pattern of findings suggests a degree of selectivity for morphine in reversing depression of marble burying by a pain stimulus, but not by a nonpain stimulus. It also highlights the sensitivity of the marble-burying assay to drugs that increase locomotion. Diazepam was tested as another negative control, and it did not alter mechanical allodynia or thermal hyperalgesia, and only exacerbated CCI-induced suppression of marble-burying behavior. The inhibitory effects of diazepam on marble-burying behavior are well described.[37,60]

This study also demonstrates that drugs targeting multiple components of the endocannabinoid system reverse both CCI-stimulated and CCI-depressed behaviors at approximately comparable doses for a given drug. Specifically, the CB_1/CB_2 receptor agonist CP55,940 or the selective CB_2 receptor agonist LEI101, reversed CCI-stimulated and CCI-depressed behaviors. This effect is consistent with a recent report that Δ^9-tetrahydrocannabinol, another mixed CB_1/CB_2 receptor agonist, alleviated migraine pain-related depression of wheel running in rats.[30] However, Δ^9-tetrahydrocannabinol and CP55,940 lacked efficacy in other pain-depressed assays, including i.p. acid-induced depression of feeding or wheel running in mice,[48] i.p. acid-induced depression of feeding or positively reinforced operant behavior in rats, intraplantar formalin-induced depression of operant responding in rats, or noxious heat-induced depression of operant responding squirrel monkeys.[31,39,42,48] The effects of LEI101 are consistent with previous reports showing that CB_2 receptor agonists reverse CCI-induced behaviors.[36,71] Thus, the effectiveness of cannabinoid receptor agonists in reversing pain-depressed behavior may depend on multiple procedural factors, such as the type of noxious stimulus/injury, the behavioral endpoint, and species.

Indirect modulation of cannabinoid receptors through inhibitors of endocannabinoid-regulating enzymes also holds promise as a potential strategy to treat pain. Specifically, MAGL inhibitors block 2-AG degradation leading to increased levels of this endocannabinoid, and consequently increased signaling at cannabinoid CB_1 and CB_2 receptors.[29,34,36,66] In this study, the MAGL inhibitor MJN110 reversed CCI-induced behaviors in the von Frey, hot-plate, and marble-burying assays. Notably, although MJN110 did not affect marble burying in sham mice, the MAGL inhibitor JZL184 reduced marble burying in naive mice.[37] As shown previously, MJN110 and JZL184 differentially alter rates of operant responding for food administration and locomotor behavior, which may be time and dose dependent.[29]

The FAAH inhibitor PF3845 led to a different pattern of results than the other drugs acting on the endocannabinoid system. Although PF3845 reversed thermal hyperalgesia and allodynia, as reported elsewhere,[6,23] it failed to reverse CCI-induced decreases of marble burying. Likewise, the FAAH inhibitor PF-0445784 did not reverse pain-related decreases of burrowing behavior in a rat model of osteoarthritis,[9] which is consistent with its failure in a clinical trial for osteoarthritis pain.[28] By contrast, another FAAH inhibitor, URB597, reversed acetic acid–depressed feeding and wheel running behaviors[48] and partially reversed lactic acid–depressed rates of intracranial self-stimulation.[38] Translation of preclinical studies to the clinic may be affected by multiple factors, including the type of noxious stimulus used, the dependent measures of pain-depressed and pain-stimulated behavior, and differential pharmacokinetics and pharmacodynamics of drugs categorized in the same class of drugs.

In contrast to the observation that CCI decreased the number of marbles buried and time spent digging, others reported that SNI increased marble burying in mice beginning at 2-week postsurgery.[55,74] To ascertain whether the type of nerve injury model accounted for these disparate findings on marble burying, we compared the consequences of CCI and SNI surgery in the marble burying on days 3 and 14. Both surgeries elicited a similar pattern of effects in which marble burying was reduced on day 3 compared with the sham controls, and this pain-depressed behavior resolved by day 14. Although previous studies concluded that increased marble burying equated to "pain-induced anxiety," this study revealed a high correlation between marble-burying and digging behavior.[4] Similarly, other research suggests that marble burying reflects a nongoal directed digging behavior,[66] which can be affected by numerous environmental and pharmacological manipulations. However, this study did not

assess digging behavior in experiments testing the various pharmacological agents and did not distinguish between goal-directed and incidental behavioral responses that resulted in the wood chips covering the marbles.

The marble-burying assay offers a straightforward procedure with sensitivity to pain-depressed behavior during the early stages of SNI- and CCI-induced neuropathy, and is readily reversed by known analgesics (ie, morphine, gabapentin, and valdecoxib). In addition, a variety of pharmacological agents targeting distinct components of the endocannabinoid system (ie, cannabinoid receptors and MAGL) reverse both CCI-induced depression of marble-burying behavior and CCI-stimulated nociceptive behavior, which adds credence for potential clinical efficacy. One important caveat of our findings is that this assay is only useful for 1 week after surgery, which may limit its preclinical drug discovery utility. Thus, this assay is not useful for measuring changes in chronic pain. Nonetheless, incorporation of pain-depressed behaviors, such as marble burying, in conjunction with pain-stimulated behaviors is relatively straightforward behavioral assays and may serve to identify new analgesic drugs with increased translational implications for treating patients suffering from pain.

Conflict of interest statement

Z.A. Curry declares that he received personal fees and other from National Institutes of Health, during the conduct of the study. The remaining authors have no conflict of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A549.

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