Differential Efficacy of Ketamine in the Acute *versus* Chronic Stages of Complex Regional Pain Syndrome in Mice

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ABSTRACT

Background: Complex regional pain syndrome (CRPS) is a painful, disabling, and often chronic condition, where many patients transition from an acute phase with prominent peripheral neurogenic inflammation to a chronic phase with evident central nervous system changes. Ketamine is a centrally acting agent believed to work through blockade of *N*-methyl-D-aspartate receptors and is being increasingly used for the treatment of refractory CRPS, although the basis for the drug's effects and efficacy at different stages of the syndrome remains unclear.

Methods: The authors used a mouse model of CRPS (n = 8 to 12/group) involving tibia fracture/cast immobilization to test the efficacy of ketamine (2 mg kg^{-1} day^{-1}; 7 days) or vehicle infusion during acute (3 weeks after fracture) and chronic (7 weeks after fracture) stages.

Results: Acute-phase fracture mice displayed increased limb temperature, edema, and nociceptive sensitization that were not reduced by ketamine. Fracture mice treated with ketamine during the chronic phase showed reduced nociceptive sensitization that persisted beyond completion of the infusion. During this chronic phase, ketamine also reduced latent nociceptive sensitization and improved motor function at 18 weeks after fracture. No side effects of the infusions were identified. These behavioral changes were associated with altered spinal astrocyte activation and expression of pain-related proteins including *N*-methyl-D-aspartate receptor 2b, Ca^{2+}/calmodulin-dependent protein kinase II, and brain-derived neurotrophic factor.

Conclusions: Collectively, these results demonstrate that ketamine is efficacious in the chronic, but not acute, stage of CRPS, suggesting that the centrally acting drug is relatively ineffective in early CRPS when peripheral mechanisms are more critical for supporting nociceptive sensitization. **(ANESTHESIOLOGY 2015; 123:1435-47)**

COMPLEX regional pain syndrome (CRPS) is a painful, disabling, and often chronic condition with an estimated 50,000 new cases in the United States each year.[1] The syndrome encompasses a disparate collection of signs and symptoms involving the sensory, motor, and autonomic nervous systems; bone demineralization; skin growth changes; and vascular dysfunction all limited to a single extremity in most cases. Although CRPS is described as one entity, it is often characterized by two distinct phases: a warm acute and a cold chronic phase.[2] In addition to changes in the clinical signs and symptoms, these two phases are accompanied by distinct biochemical changes in both human subjects[3] and rodent models of CRPS.[4] This transition from the acute to the chronic phase of CRPS suggests a shift in the underlying pain mechanisms, with peripheral mechanisms believed to

What We Already Know about This Topic

- In humans and animal models, complex regional pain syndrome exhibits an acute and a chronic phase with distinct clinical and mechanistic features
- Ketamine has efficacy in complex regional pain syndrome, but its mechanisms and specific effects in the acute and chronic phases of the syndrome are unclear

What This Article Tells Us That Is New

- Using a mouse model of complex regional pain syndrome, short-term systemic administration of ketamine reversed mechanical allodynia when administered in the chronic, not acute, phase
- The sustained behavioral effects of ketamine correlated with reduced changes in astrocyte activation and pain-related effectors in the spinal cord, identifying potential therapeutic targets

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Presented, in part, at the American Pain Society Annual Meeting, Tampa, Florida, April 30 to May 3, 2014.

Submitted for publication February 27, 2015. Accepted for publication August 5, 2015. From the Veterans Affairs Palo Alto Health Care System, Palo Alto, California (M.T., J.D.C.); Department of Anesthesiology, Stanford University School of Medicine, Stanford, California (M.T., J.D.C.); Palo Alto Veterans Institute of Research and Education, Palo Alto, California (M.T., D.L., P.Y., T.T.H., J.D.C.); Geriatrics Research Education and Clinical Center, Veterans Affairs Palo Alto Health Care System, Palo Alto, California (T.T.H.); Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, California (D.L., P.Y., T.T.H.); and Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California (W.S.K.).

Anesthesiology 2015; 123:1435-47

underlie at least some of the acute manifestations and central nervous system (CNS) changes evolving to support symptoms in the more chronic phases. Consequently, we might anticipate a shift in responses to therapeutic interventions depending on whether those interventions are peripherally or centrally targeted.

Ketamine is a centrally acting agent believed to work through the blockade of *N*-methyl-D-aspartate (NMDA) receptors used for postoperative, cancer-related, and neuropathic pain. This agent is being used increasingly for the treatment of refractory chronic CRPS, where several day infusions are a common method of administration[5–8]; however, neither the basis for the drug's effects in CRPS nor its efficacy at different stages of the syndrome is clearly defined at this point. Ketamine has been shown to have antidepressive effects in rodents[9] and is increasingly being used in patients with refractory depression and anxiety,[10,11] symptoms commonly co-occurring with CRPS. Furthermore, in a clinical setting, chronic ketamine treatment in patients with CRPS has been associated with improvement in motor function[12] and the reversal of the "pain brain" state, compared with that in control subjects, suggesting central sites of action.[13]

To study the therapeutic effects of chronic ketamine on CRPS, we have utilized a previously characterized mouse model of distal tibia fracture/cast immobilization displaying the nociceptive, functional, vascular, trophic, inflammatory, and immune aspects of this syndrome.[4,14–17] Our results are consistent with the hypothesis that short-term systemic administration of low-dose ketamine is efficacious in reversing mechanical allodynia displayed by fracture/cast mice when administered in the chronic, but not acute, stage of the syndrome. Furthermore, this study provides some of the biochemical correlates of this behavioral outcome, including changes in neuroinflammation and signs of plasticity at the level of the spinal cord (SC).

Materials and Methods

Animals

A total of five cohorts of mice were used. Male C57/B6J mice aged 12 to 14 weeks were purchased from a commercial supplier (Jackson Laboratory, USA) and were allowed to habituate to the animal facility for a minimum of 10 days before the experiments. Mice were housed in groups of four on a 12-h light–dark cycle and an ambient temperature of 22° ± 3°C, with food and water available *ad libitum*. All animal procedures and experimental designs were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, California) and followed the "animal subjects" guidelines of the International Association for the Study of Pain.

Limb Fracture and Cast Immobilization

After the random allocation to the control or the fracture/cast group, mice were anesthetized with 1.5% isoflurane and were subjected to a distal tibial fracture in the right leg. Briefly, a hemostat was used to make a closed fracture of the right tibia just distal to the middle of the tibia, and the hindlimb was wrapped in casting tape (cat. 82001, 3M Scotchcast Plus, United Kingdom), as previously described.[18] After the procedure, the mice were given subcutaneous buprenorphine (0.05 mg/kg) and enrofloxacin (5 mg/kg) for the next 2 days, as well as normal saline (1.5 ml once) for postoperative analgesia, prevention of infection, and prevention of dehydration. Mice were inspected daily to ensure the cast was positioned properly through the 3-week period of cast immobilization. Mice were provided with chow pellets postoperatively *ad libitum*; dietary gels were also made available on the cage floor for mice having undergone surgery. Casts were removed 3 weeks after surgery under brief isoflurane anesthesia.

Naive age- and sex-matched mice were used as control. We chose to use naive mice instead of cast immobilization (sham) animals because cast immobilization results in an intermediate phenotype that shows signs of transient mechanical allodynia.[14]

Pharmacology

Fracture and control mice were randomly assigned to one of two treatment groups: ketamine or saline infusion. Ketamine (2 mg kg^{-1} day^{-1}) or sterile saline was administered for 7 days using subcutaneous osmotic minipumps (total volume = 100 µl; rate of delivery = 0.5 µl/h; Alzet model 1007D, USA). Doses greater than 2 mg kg^{-1} day^{-1} were not used because of technical limitations pertaining to the commercially available concentration of the drug preparation, in addition to the volume of the pumps that can be used in mice.

At 3 or 7 weeks after fracture, animals were anesthetized with 1.5% isoflurane, and the hair was clipped in the dorsal cervical region. After disinfecting the skin, a 0.5-cm incision was made through the dermis (between the two shoulder blades) using a small pair of scissors, and a skin "pocket" was created using sterile forceps. The osmotic pump was then inserted, and the wound was closed with staples. Antibiotic ointment was applied to prevent infection. All pumps were removed 8 days after insertion. For the mice that received ketamine 3 weeks after fracture, the pumps were inserted 2 days after cast removal. A summary of the experimental time course is shown in figure 1.

Physiologic Measures

Hind Paw Temperature. The temperature of the hind paw was measured using a fine wire thermocouple (Omega, USA) applied to the paw skin, as described previously.[19] Three sites were tested over the dorsal aspect of the hind paw: the space between the first and second metatarsals (medial), the second and third metatarsals (central), and the fourth and fifth metatarsals (lateral). The six measurements for each hind paw were averaged for the mean temperature.

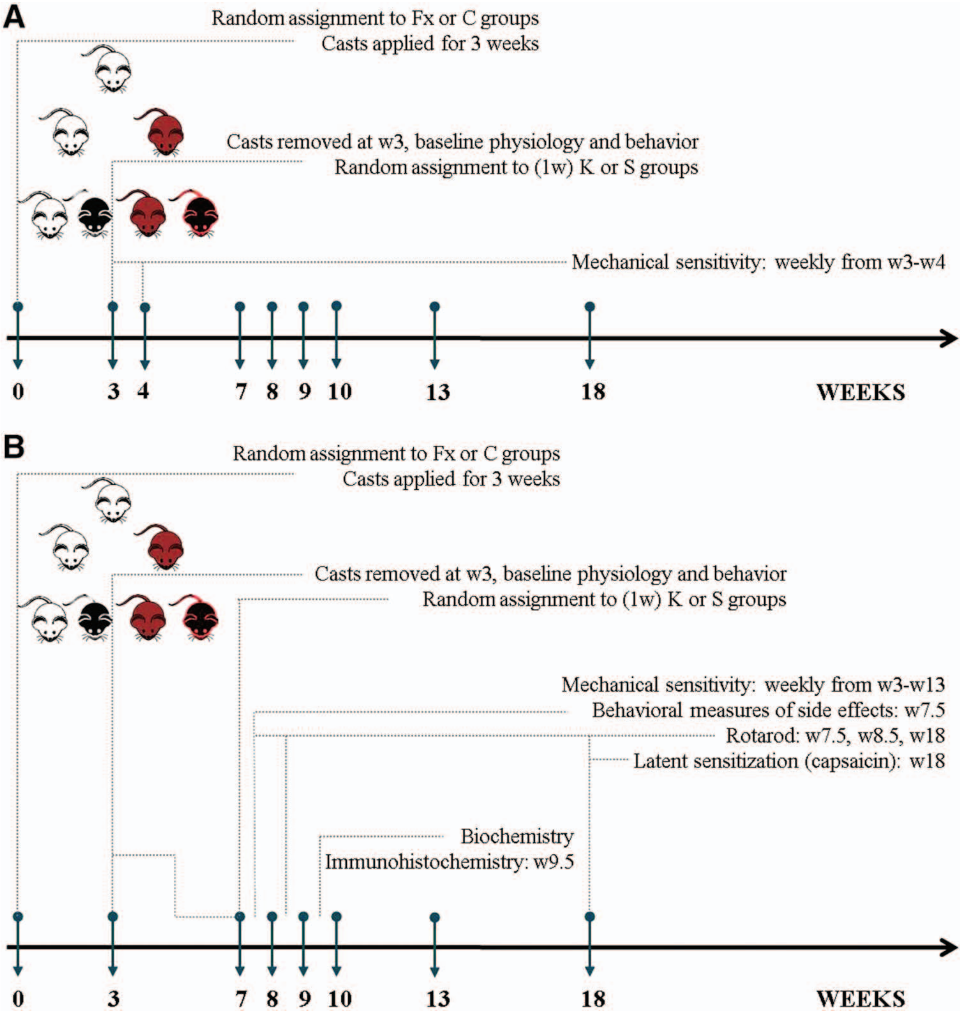

Fig. 1. Summary of experimental design. Timeline of surgical/pharmacologic interventions and behavioral/biochemical measurements when ketamine was administered at 3 weeks (one cohort of mice, *A*) or 7 weeks (four cohorts, *B*) after fracture. C = control; Fx = fracture; K = ketamine; S = saline; W = week.

Hind Paw Volume. A laser sensor device (measurement range of 200 mm, 0.01 mm resolution; cat. 4381-Precicura, Limab AB, Sweden) was used to determine the dorsal–ventral thickness of the hind paw, as described previously.[19]

Behavioral Testing

The experimenter was blind to the identity and experimental condition of the animals throughout the behavioral experiments and data analysis. Mice were habituated to handling by the experimenter for a few minutes each day for 7 days before initiation of the behavioral tests.

Mechanical Hypersensitivity. Calibrated monofilaments (Stoelting Co., USA) were applied to the plantar surface of the hind paw, and the 50% threshold to withdraw (grams) was calculated as described previously.[20] The stimulus intensity ranged from 0.004 to 1.7 g, corresponding to the filament numbers (1.65, 2.36, 2.44, 2.83, 3.22, 3.61, 3.84, 4.08, 4.17, and 4.31).

Rotarod. Locomotor capacity was measured by the use of an accelerating rotarod (cat. 47600, Ugo Basile, Italy). The task includes a speed ramp from 0 to 30 rotations per minute over 60 s, followed by an additional 240 s at the maximal speed. The latency to fall was reported for the initial exposure to the rotarod, in addition to 5 consecutive trials (30-min intertrial interval).

Capsaicin-evoked Behaviors. This assay was performed 18 weeks after fracture. This assay measures the duration of total behaviors (biting, scratching, licking, and shaking) during the 5 min after a local subcutaneous injection of capsaicin (2.5 µg in 5 µl, cat. MT028, Sigma-Aldrich, USA) or vehicle (0.25% dimethyl sulfoxide, 0.25% ethanol, 0.125% Tween-80 in saline) in the plantar surface of the right hind paw. Quantification was carried out in real time by a blind observer using a stopwatch.

Each animal was tested with both capsaicin and vehicle with a 5-day washout between treatments. To examine the long-term effects of capsaicin, mechanical hypersensitivity was measured on the plantar aspect of the right and left hind paws 1 h after injection.

Qualitative Measures of Sedation and Motor Impairment. Although this study uses a very low dose of ketamine that is administered systemically, we nonetheless elected to study the potential side effects of the drug in the home cage environment, particularly because they could present potential confounds in data collection and interpretation. Side effects were measured by scoring stereotypic behavior and activity levels, in addition to changes in body mass. Stereotypic behavior was scored on a 7-point scale as follows: −3, anesthesia; −2, sedation; −1, drowsiness; 0, normal; 1, moderately increased (increased explorative behavior); 2, increased (increased urge to move around the cage); and 3, greatly increased (inability to hold still with weaving, shaking, or twitching of the head and body). Activity level was defined as follows: −3, anesthesia; −2, sedation; −1, drowsiness; 0, normal; 1, moderately impaired (disturbances in paw support); 2, impaired (unable to maintain paw support with the ability to regain an upright position after falling over); and 3, greatly impaired (inability to regain an upright position after falling over). Mice were observed for 10 min each between 9:00 AM and 2:00 PM.

Immunohistochemistry

All analysis was blinded to the identity and experimental condition of the animal/tissue. Mice were anesthetized by 300 µl of ketamine/xylazine cocktail, followed by transcardiac perfusion with 0.9% saline solution at 7 weeks after injury. Ipsilateral lumbar SCs were carefully removed, postfixed in 4% paraformaldehyde for 2 days, and rinsed in phosphate-buffered saline for 3 days. Ipsilateral SCs were then embedded in agarose, and cross-sections were cut at room temperature at 40-µm thickness on a vibratome (Leica VT 1200S; Leica Biosystems, USA). Ten sections per mouse were randomly chosen for staining. Free-floating immunohistochemistry was done by using tris-buffered saline + 0.1% Tween-20 (TBST) as the wash buffer and 10% donkey normal serum (cat. #ab7475, Abcam, USA) in phosphate-buffered saline as the blocking buffer. A permeabilization step was added (before blocking and staining) using 0.1% triton and 0.6% hydrogen peroxide in 1X TBST. The following primary antibodies were used (diluted in blocking buffer): mouse monoclonal antiglial fibrillary acidic protein (GFAP; 1:5000, cat. MAB3402, Millipore, USA) and rabbit polyclonal antiionized calcium-binding adapter molecule 1 (Iba1, 1:800, cat. 019-19741, Wako, Japan). The following secondary antibodies were used (diluted in 1X TBST): donkey anti-mouse immunoglobulin G (IgG, H + L), Alexa Fluor 647 (AF647, 1:500, cat. ab150107, Abcam), and donkey anti-rabbit IgG (H + L) Alexa Fluor 488 (AF488, 1:500, cat. 711-545-152, Jackson Immunoresearch, USA).

SC sections were mounted on slides using fluoromount aqueous mounting medium (cat. F4680, Sigma,). Images were analyzed as a 3-µm step z-stack of 10 slices (20X objective magnification) using fluorescent imaging (Keyence BZ-X700, USA). Three sections per mouse were randomly chosen for analysis. GFAP$^+$ and Iba1$^+$ cells were manually quantified in each z-stack using the "Mark and Count" tool, and tissue area was measured in image J (National Institute of Health, USA). Data are presented as the number of cells per square millimeter.

Quantification of Target Proteins

All analysis was blinded to the identity and experimental condition of the animal/tissue. Mice were anesthetized (isoflurane) and killed by decapitation at 9 to 10 weeks after injury. The ipsilateral lumbar SC was carefully removed and stored at −80°C until use.

Extraction of Total Protein and Synaptosome Preparation. Tissues were homogenized using T-PER Protein Extraction Reagent (cat. 87793, Thermo Fisher Scientific, USA) in the presence of proteinase and phosphatase inhibitors (cat. 0490683700, Roche Applied Science, USA) and centrifuged at 12,000*g* for 5 min at 4°C. Supernatant fractions were then frozen at −80°C until use.

For the isolation of synaptosomes, tissue was homogenized in 1X Syn-PER reagent (cat. 87793, Thermo Fisher Scientific) containing "Halt" protease inhibitor cocktail (cat. 87785, Thermo Fisher Scientific), it was centrifuged at 1,000*g* for 10 min to remove cell debris, and the supernatant was transferred to a new tube where it was centrifuged at 17,500*g* for 20 min. The pellets, containing synaptosomes, were gently resuspended in 40 µl of the reagent (Syn-PER + Halt).

Two-color Fluorescent Western Blot Analysis. To assess the protein levels of the *N*-methyl-D-aspartate receptor 2b (NR2b) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK2), Western blot analysis was performed according to standard procedures. Briefly, after sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotting, proteins on the membranes were detected by overnight incubation at 4°C with the primary antibody (rabbit polyclonal anti-NR2b, 1:500, cat. ab65783; Abcam; and mouse monoclonal anti-CaMK2, 1:2000, cat. MA1-048; Thermo Fisher Scientific) followed by incubation with an infrared dye 800CW goat anti-rabbit IgG (H + L; 1:20,000; cat. 925–32211, LI-COR Biosciences, USA) or goat anti-mouse IgG (H + L; 1:20,000; cat: 926–32210, LI-COR Biosciences). β-Actin was used as an internal control and was detected with the mouse monoclonal anti–β-actin antibody (1:5000; cat. ab6276, Abcam) followed by incubation with an infrared dye 680CW goat anti-mouse IgG (H + L; 1:20,000; cat. 926–32220, LI-COR Biosciences). The signals were detected using Odyssey (LI-COR Biosciences) and quantified using the ImageJ software (USA).

Enzyme-linked Immunosorbent Assay. The mouse brain-derived neurotrophic factor (BDNF) enzyme-linked immunosorbent assay kit (total protein; cat. EK0309, Boster Immunoleader, USA) and the mouse synaptophysin (SYP)

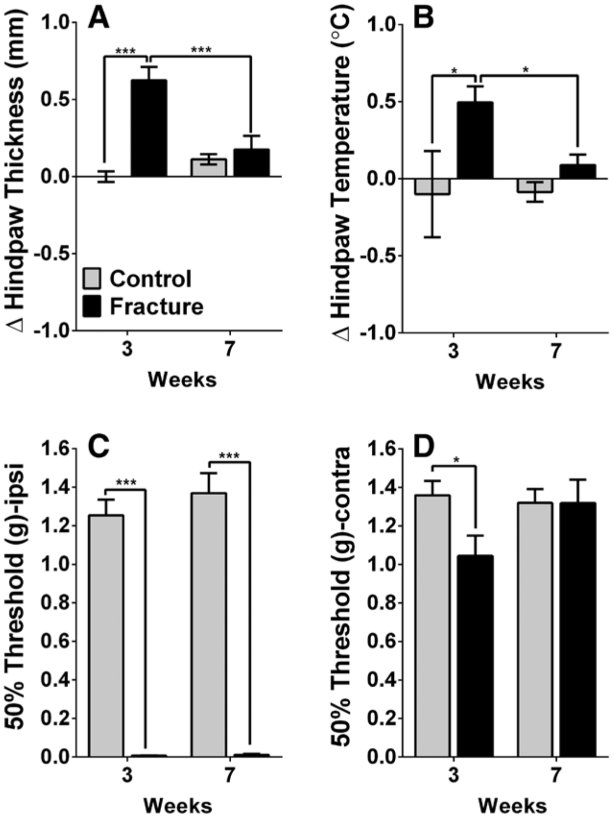

Fig. 2. Physiologic and behavioral changes in complex regional pain syndrome (CRPS) mice. CRPS mice display increased edema (*A*) and temperature (*B*) on the affected hind paw at 3, but not 7, weeks after fracture. In addition, they show signs of mechanical allodynia in the ipsilateral (ipsi, 3- and 7-week timepoints, *C*) and contralateral (contra, 3-week timepoint, *D*) hind paw. $^{*}P < 0.05$, $^{***}P < 0.001$. n = 16 mice for each of the four groups.

enzyme-linked immunosorbent assay kit (synaptosomal preparation; cat. SEA425Mu, Cloud-Clone Corp., USA) were used as per the manufacturers' instructions. Measures of the target proteins were normalized with respect to total protein as quantified by the Bradford assay (cat. 500-0001, Bio-Rad Laboratories, Inc., USA).

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis between experimental groups was carried out using a two-way ANOVA followed by the Holm–Sidak method to correct for multiple comparisons (figs. 2–8). Repeated measures two-way ANOVA was used for figures 3, 4, D–F, and 5. Significance was set at $P < 0.05$ (Prism 5; GraphPad Software, USA). Grubb outlier test was used for the detection and subsequent exclusion of outlier data points. Analyses conducted with n less than or equal to 4 are presented as scatter plots (instead of bar graphs) to place the reader closer to the actual data (figs. 7 and 8). Sample size determination was guided by previous experience with the reported assays and is indicated in the figures.

Results

Fracture Mice Displayed a Transient Increase in Edema and Temperature in Addition to Persistent Mechanical Sensitivity in the Affected Hind Paw

Ipsilateral and contralateral measurements of hind paw edema and temperature, measurements related to commonly observed signs in CRPS patients, were performed at 3 and 7 weeks after fracture. Both temperature and edema demonstrated similar profiles, exhibiting transient increases identified at the 3-week timepoint (temperature: 3-week fracture *vs.* control mean difference = 0.59, 95% CI = 0.16 to 1.03, $P < 0.001$; edema: 3-week fracture *vs.* control mean difference = 0.63, 95% CI = 0.41 to 0.84, $P < 0.001$), but not at the 7-week timepoint (fig. 2, A and B; reported values reflect the difference between the values acquired from ipsilateral and contralateral hind paw measurements). In contrast, mechanical sensitivity in the ipsilateral hind paw, assessed using von Frey filaments, was evident in the 3-week (fracture *vs.* control mean difference = −1.25, 95% CI = −1.45 to −1.04, $P < 0.001$) and 7-week timepoints (fracture *vs.* control mean difference = −1.36, 95% CI = −1.56 to −1.15, $P < 0.001$; fig. 2C). Furthermore, a transient decrease in mechanical thresholds was observed in the contralateral hind paw of the fracture group at the 3-week timepoint only (fig. 2D).

Chronic Systemic Ketamine Infusion Was Antiallodynic When Administered at the Late (7 Weeks), but Not Early (3 Weeks), Timepoint after Fracture

The infusion of ketamine for 1 week through subcutaneous osmotic pumps did not result in increased mechanical thresholds when administered 3 weeks after fracture (fig. 3, A and B). In contrast, when administered 7 weeks after fracture, ketamine had a significant antiallodynic effect in fracture mice, starting at day 7 posttreatment. Furthermore, this antiallodynic effect was maintained for 4 weeks after the termination of the ketamine infusion (two-way ANOVA surgery factor *P* value < 0.0001 and time factor *P* value < 0.0001), until the resolution of mechanical hypersensitivity was observed in the saline-treated fracture mice (at 12 weeks after fracture). No significant ketamine effects were observed in control mice or in the ipsilateral hind paw (fig. 3, C and D). Because of the lack of efficacy at the acute phase (3 weeks), all subsequent experiments were carried out in cohorts of mice treated at the chronic (7 weeks) phase.

Chronic Systemic Ketamine Infusion Had Long-term Effects on Motor Performance

Our findings regarding the antiallodynic effects of ketamine prompted us to examine the short- and long-term functional outcomes of ketamine treatment administered at 7 weeks after fracture. We used three different cohorts because our aim was to examine rotarod performance after first-time exposure to the apparatus. At the 7-weeks timepoint, when ketamine was chronically administered through the osmotic pumps

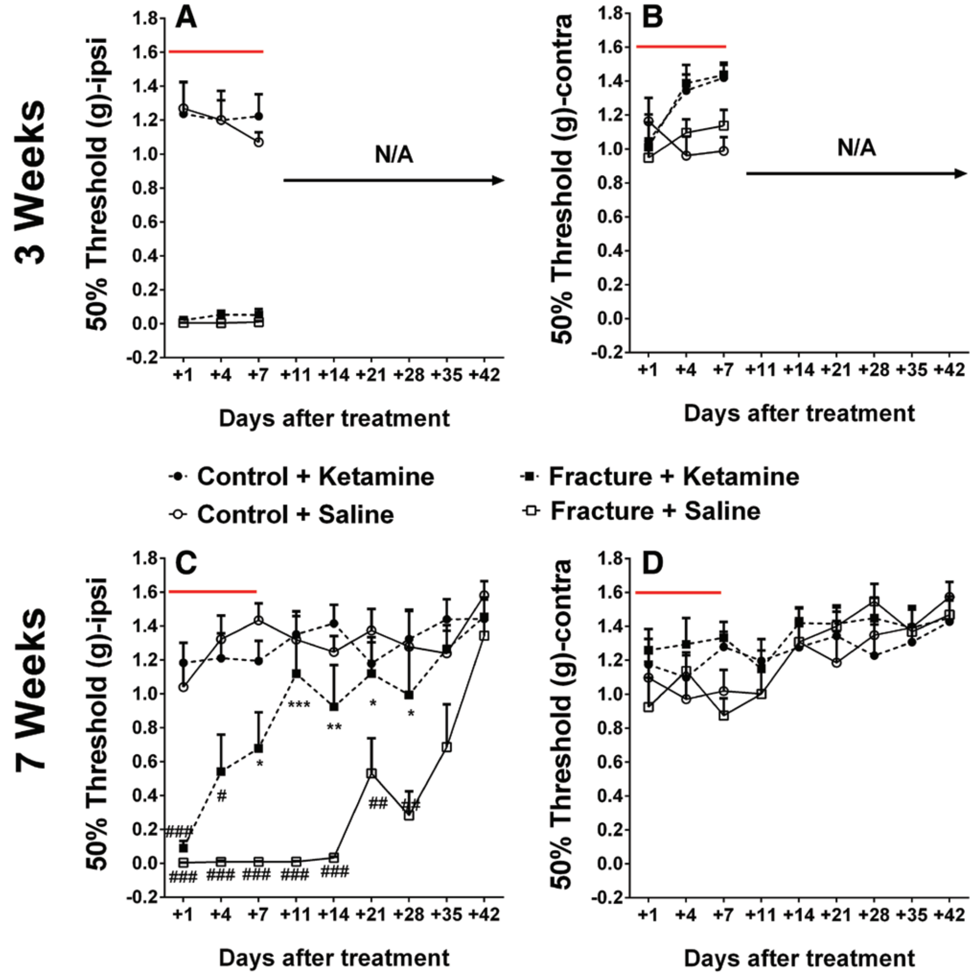

Fig. 3. Ketamine is efficacious when administered at the late, but not early, phase of complex regional pain syndrome. Ketamine shows no efficacy in reversing mechanical allodynia when administered at 3 weeks after fracture (*A, B*). In contrast, ketamine reverses mechanical allodynia on the ipsilateral (ipsi) hind paw when administered at the 7-week timepoint (*C, D*). No changes were observed in the contralateral (contra) hind paw. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ compared with the Fracture + Saline group. #$P < 0.05$, ##$P < 0.005$, ###$P < 0.001$ compared to the Control + Saline group. (*A*, *B*) n = 6 mice for Control + Saline, n = 6 mice for Control + Ketamine, n = 8 mice for Fracture + Saline, and n = 11 mice for Fracture + Ketamine. (*C*, *D*) n = 8 mice for Control + Saline, n = 8 mice for Control + Ketamine, n = 6 mice for Fracture + Saline, and n = 9 mice for Fracture + Ketamine. The *red lines* indicate the duration of ketamine administration.

for 7 days, we observed no significant changes between the saline-treated control and fracture groups and no ketamine efficacy in the fracture group (fig. 4A, rotarod testing was performed on the third day of chronic infusion, and one animal was excluded from the fracture + saline group based on the Grubb outlier test). Similarly, at the 8-week timepoint, which marks the removal of the osmotic pumps, there were no differences among the four experimental groups (fig. 4B). These data suggest that fracture-induced functional deficits are not easily observed at these timepoints (although trends of diminished motor performance are seen) and that ketamine does not impair performance at the dose administered. It is noteworthy that the lack of significant fracture effect at this timepoint could be because of reduced motor performance in the control mice because of the surgical procedures used for implanting and removing the pumps. In contrast, when the mice were first introduced to the rotarod apparatus 18 weeks after fracture (long after mechanical thresholds were normalized), fracture mice showed significant impairment in the rotarod assay (ketamine group: fracture *vs.* control mean difference = −61.03, 95% CI = −94.76 to −27.29, $P < 0.001$; saline group: fracture *vs.* control mean difference = −112.0, 95% CI = −155.1 to −68.97, $P < 0.001$), which was reduced in the group of mice that was treated with chronic ketamine many weeks before (fig. 4C; fracture group: saline *vs.* ketamine mean difference = −34.93, 95% CI = −66.73 to −3.140, P = 0.033). These data suggest that sustained ketamine infusion can positively affect the performance of a novel motor task many weeks after exposure to the drug.

Finally, training the fracture mice on the rotarod for five consecutive trials (intertrial interval = 30 min) showed no difference between the treatment groups on the third day of

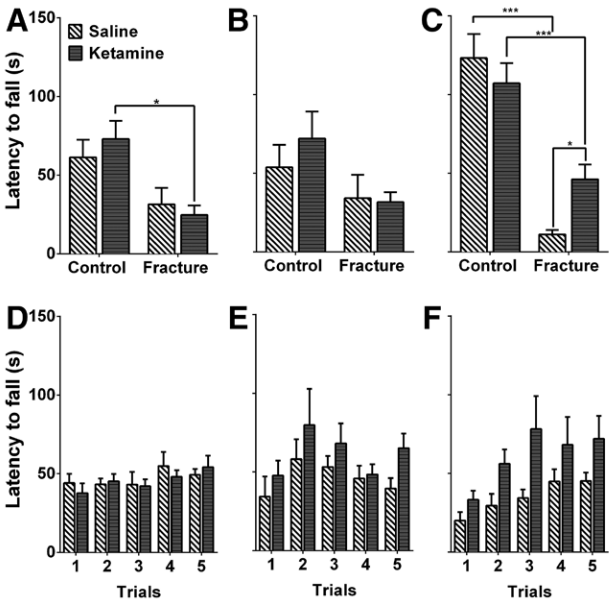

Fig. 4. Ketamine exposure improves rotarod performance 18 weeks after fracture. Three different cohorts were used to measure initial rotarod performance at three different timepoints. Fracture animals show no obvious impairment in the rotarod assay at the (*A*) 7- and (*B*) 8-week timepoints; however, they do show impairment at the 18-week timepoint, which can be ameliorated by ketamine administration (*C*). Subsequent training in fracture/cast mice showed no statistically significant differences between the ketamine- and saline-treated groups (*D*–*F*). *$P < 0.05$, ***$P < 0.001$. (*A*, *D*) n = 10 mice for Control + Saline, n = 8 mice for Control + Ketamine, n = 8 mice for Fracture + Saline, and n = 8 mice for Fracture + Ketamine. (*B*, *E*) n = 6 mice for each of the four groups. (*C*, *F*) n = 8 mice for Control + Saline, n = 8 mice for Control + Ketamine, n = 6 mice for Fracture + Saline, and n = 12 mice for Fracture + Ketamine.

infusion (7 weeks), at the completion of infusion (8 weeks), and at 18 weeks after fracture (at this timepoint, there was a significant effect of training, but *post hoc* comparisons show no significant differences between the two groups at any of the trials; fig. 4, D–F), suggesting that the observed results are unique to the initial exposure to a new motor task (one animal was excluded from the fracture + saline group at the 8-week timepoint based on the Grubb outlier test).

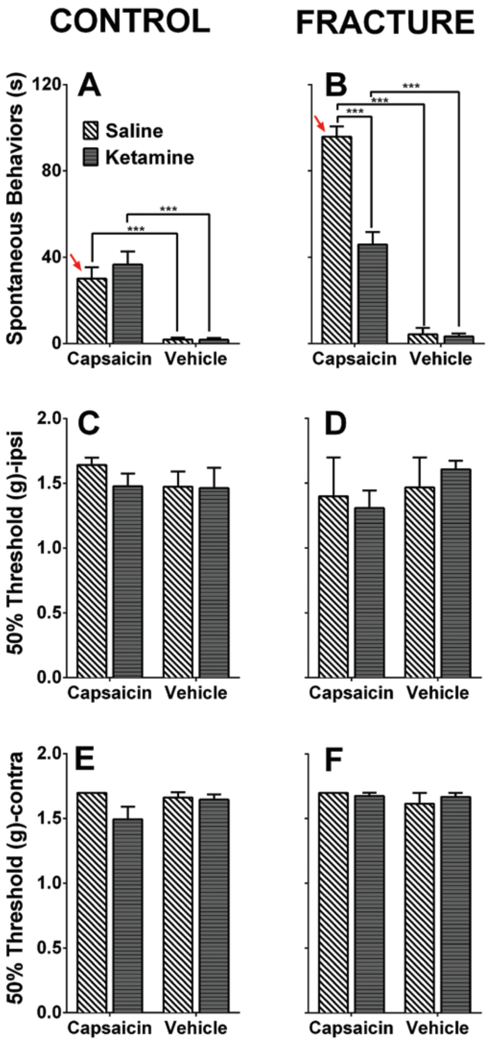

Fig. 5. Ketamine exposure improves capsaicin-induced hypersensitivity 18 weeks after fracture. Eighteen weeks after injury, fracture mice spend more time engaging in capsaicin-evoked behaviors (licking, biting, scratching, and shaking) compared with control mice (*A*, *B*, *red arrows*, $P < 0.001$). This is indicative of persistent hind paw sensitization in the absence of notable differences in mechanical thresholds. Furthermore, the ketamine-treated group displayed less capsaicin-evoked behaviors when compared with the saline-treated group (*B*). No changes were observed in mechanical thresholds 1 h after capsaicin or vehicle treatments in the ipsilateral (ipsi, *C, D*) and contralateral (contra, *E, F*) hind paws. ***$P < 0.001$. n= 8 mice for Control + Saline, n = 8 mice for Control + Ketamine, n = 6 mice for Fracture + Saline, and n = 8 mice for Fracture + Ketamine.

Fracture Mice Displayed Hypersensitivity to Subcutaneous Capsaicin that Was Ameliorated by Previous Ketamine Exposure

The rotarod data suggested that low-dose chronic ketamine administration (a 7-day infusion at 7 weeks after fracture) had functional effects at 18 weeks after fracture, long after the ketamine treatment ended. We then further explored the chronic effects of ketamine infusion on overall nociception. Because mechanical thresholds had already normalized by 18 weeks after fracture, we challenged the four groups of mice with a subcutaneous injection of capsaicin. Capsaicin-evoked behaviors were interpreted as an indication of latent sensitization not detectable by measuring hind paw mechanical sensitivity thresholds.

When capsaicin was injected into the hind paw at the 18 weeks postfracture timepoint, fracture mice spent more time engaging in capsaicin-evoked behaviors (licking, biting, scratching, and shaking the hind paw) than control nonfractured mice (fig 5, A and B, red arrows). This observation could represent persistent hind paw sensitization (because of

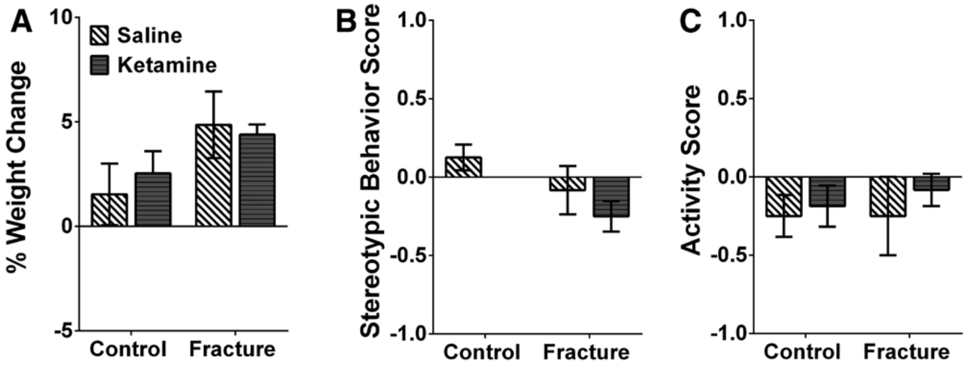

Fig. 6. No side effects were observed during chronic ketamine administration. No changes in body weight (*A*), stereotypic behaviors (*B*), and activity levels in the home cage (*C*) were observed between ketamine- and saline-treated groups 5 days after ketamine exposure (at 7 weeks after fracture). n = 7 mice for Control + Saline, n = 8 mice for Control + Ketamine, n = 6 mice for Fracture + Saline, and n = 12 mice for Fracture + Ketamine.

peripheral or central mechanisms) in the absence of notable differences in mechanical thresholds[19] or alternatively could be an indication of ongoing mechanical sensitivity that is not detected by reflexive measures. Furthermore, the group of mice that had been treated with a 7-day infusion of ketamine at 7 weeks after fracture displayed less capsaicin-evoked behaviors when compared with the saline-treated fracture group (fig 5B; fracture group receiving capsaicin: saline *vs.* ketamine mean difference = −49.92, 95% CI = −66.52 to −33.33, $P < 0.001$). No changes were observed in mechanical thresholds 1 h after capsaicin or vehicle treatment in the ipsilateral (fig 5, C and D) and contralateral hind paws (fig 5, E and F).

It is important to note that these data do not imply that the capsaicin hypersensitivity is necessarily caused by central mechanisms, particularly because we limited our studies to the hind paw corresponding to the fractured limb.

Low-dose Systemic Ketamine Administration Was Not Accompanied by Observable Side Effects

Preclinical studies in animal models can be heavily confounded by motor ability and/or changes in overall activity levels. To test whether ketamine administration results in any visible side effects, changes in body weight (% change in body mass immediately before and after 1 week of ketamine treatment) in addition to stereotypic behaviors and activity levels in the home cage were scored. Although these measures might be better suited for a more global assessment of side effects and may not be able to detect side effects that are mild, our results show no differences in any of observed measures between ketamine- and saline-treated groups (fig. 6, A–C). These observations are in agreement with our data from the rotarod (fig. 4A) assay, where ketamine treatment was not accompanied by motor dysfunction.

Fracture Mice–induced Activation of Spinal Astrocytes Was Inhibited in Mice Previously Treated with Ketamine

To study the immunohistochemical correlates of the behavioral signs of antiallodynia, we examined the spinal levels of neuroinflammation markers at 10 weeks after fracture. This timepoint was chosen for the following reasons: first, the thresholds of mechanical sensitivity were different among the treatment groups at this timepoint, and second, any observed results would not be due to the acute effects of ketamine because there would be a washout period of approximately 10 days before the biochemical measurements.

Immunohistochemical staining for GFAP showed that, in comparison with control animals, fracture mice exhibit increased numbers of immunostained astrocytes in the dorsal horn of the ipsilateral lumbar SC at 10 weeks after fracture (saline group: fracture *vs.* control mean difference = −141.4, 95% CI = −250.3 to −32.44, $P = 0.013$). This increase was absent in the ketamine-treated group (fig. 7A, fracture group: saline *vs.* ketamine mean difference = −135.6, 95% CI = −252.1 to −19.18, $P = 0.024$). In contrast, no changes were seen in the number of Iba1$^+$-immunostained spinal microglia at this timepoint (fig. 7B).

Ketamine Exposure Was Accompanied by Biochemical Changes in the Lumbar Spinal Cord

In addition to our immunohistochemical studies, we studied biochemical changes in the lumbar SC 10 weeks after fracture. NR2b was chosen based on both our preliminary studies and a recent study showing NR2b-containing receptor/ion channels to play a prominent role in synaptic transmission in the superficial lamina of the rodent SC.[21] CaMK2 was included based on our ingenuity pathway analysis of our previously published SC microarray data[4] and its known binding to, and activation by, stimulated NR2bs; SYP was chosen as a proxy indicator of synaptic abundance; and BDNF was chosen because of its known involvement in pain and central sensitization.

NR2b. There was a decrease in the protein levels of the glutamate receptor subunit NMDAR2b in the SC tissue from the fracture group at 10 weeks after fracture (saline group: fracture *vs.* control mean difference = 0.35, 95% CI = 0.12 to 0.57, $P = 0.004$). Ketamine exposure was associated with increased NR2b in both the fracture (ketamine *vs.* saline

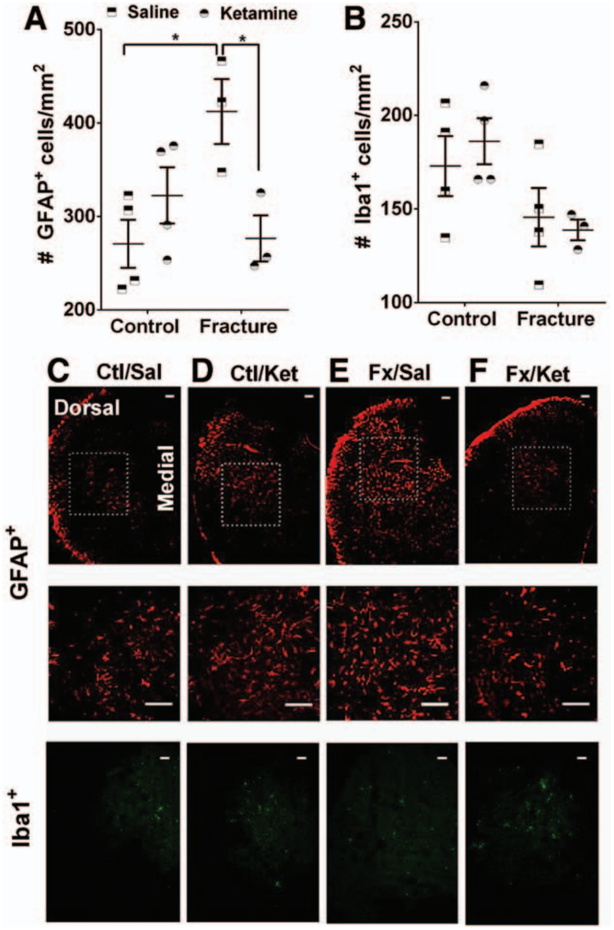

Fig. 7. Ketamine exposure ameliorates complex regional pain syndrome-related up-regulation of spinal astrocytes. Immunohistochemical staining for GFAP shows that, in comparison with control animals, fracture mice exhibit increased numbers of astrocytes in the dorsal horn of the ipsilateral spinal cord 10 weeks after fracture. This increase is absent in the ketamine treated group (*A*). In contrast, no changes were seen in the number of Iba1⁺ cells at this timepoint (*B*). Examples of GFAP- and Iba1-stained sections are included (*C–F*). The areas enclosed by the *dotted squares* are further enlarged in the central panels. Scale bar = 100 µm, $*P < 0.05$. Sample sizes are shown in the scatter graph. Ctl/Ket = Control + Ketamine; Ctl/Sal = Control + Saline; Fx/Ket = Fracture + Ketamine; Fx/Sal = Fracture + Saline; GFAP = glial fibrillary acidic protein; Iba1 = ionized calcium-binding adapter molecule 1.

mean difference = 0.70, 95% CI = 0.43 to 0.96, $P < 0.001$) and the control (ketamine *vs.* saline mean difference = 0.35, 95% CI = 0.10 to 0.60, $P = 0.007$) groups (fig. 8A).

CaMK2. Protein levels of the serine/threonine-specific protein kinase CaMK2 were increased in the fracture group at 10 weeks after fracture when compared with that in the nonfracture controls (saline group: fracture *vs.* control mean difference = 0.41, 95% CI = 0.04 to 0.78, $P = 0.029$), consistent with our previously published mRNA results from 3- and 7-week postfracture mice.[4] Furthermore, ketamine exposure was associated with further increases in CaMK2 levels in both the fracture (ketamine *vs.* saline mean difference = −0.35, 95% CI = −0.65 to −0.05, $P = 0.021$) and the control (ketamine *vs.* saline mean difference = −0.54, 95% CI = −0.98 to −0.10, $P = 0.015$) groups (fig. 8B).

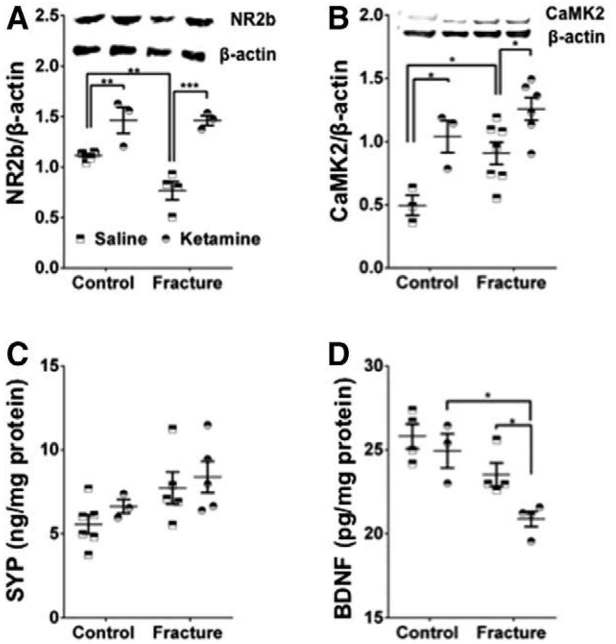

Fig. 8. Ketamine exposure is accompanied by biochemical changes in the lumbar spinal cord. Ten weeks after fracture, our data show a decrease in the protein levels of NR2b in the fracture group, with ketamine exposure being associated with increased NR2b in both the fracture and the control groups (*A*). In addition, we show an increase in CAMK2 in the fracture group, with ketamine exposure linked to increased CAMK2 levels in both the fracture and the control groups (*B*). No significant differences in synaptophysin levels were observed among the four groups (*C*). Finally, ketamine administration was accompanied by a decrease in BDNF levels in the fracture group only (*D*). $*P < 0.05$, $**P < 0.005$, $***P < 0.001$. Sample sizes are shown in the scatter graph. BDNF = brain-derived neurotrophic factor; CaMK2 = Ca^{2+}/calmodulin-dependent protein kinase II; NR2b = *N*-methyl-D-aspartate receptor 2b; SYP = synaptophysin.

Synaptophysin. No significant differences were observed in the protein levels of SYP, a proxy marker for synaptic abundance caused by either fracture or ketamine treatment (fig. 8C).

BDNF. There were no significant changes in the spinal levels of BDNF 10 weeks after fracture in comparison to control animals. However, ketamine exposure was associated with a decrease in BDNF in the fracture group only (fig. 8D, ketamine *vs.* saline mean difference = −2.66, 95% CI = −5.178 to −0.1352, $P = 0.039$).

Discussion

Many options for the treatment of CRPS have been described, but none are universally effective. Given its changing nature over time, there may be value in knowing when, over the course of the condition, specific therapies become most efficacious. One promising but controversial approach is the use of low-dose ketamine infusions,[5,22] where periodic infusions are used repeatedly, with antihyperalgesic effects lasting for 6 months or more in some patients.[23] By using the tibia fracture/cast model of CRPS in mice, we have shown that 1-week systemic infusions, at an allo-scaled dose (comparable with what is used in human subjects), are efficacious when administered in the late (7 weeks), but not early (3 weeks), stages of the syndrome. We believe that our preclinical data have significant clinical relevance because, when administered in the late phase, ketamine could ameliorate three key features relevant to the clinical management of this condition: (1) persistent allodynia: mechanical thresholds were reduced for up to 12 weeks after fracture and this allodynia completely resolved after ketamine treatment. (2) Latent sensitization: exaggerated capsaicin-induced nociceptive responses were observed in our model after reflexive measures of mechanical allodynia had subsided and were reversed or prevented by earlier ketamine treatment. (3) Motor dysfunction: similar to clinical observations,[12] ketamine ameliorated motor dysfunction at a chronic timepoint long after the normalization of the mechanical sensitivity thresholds; this is an important finding because pain relief and recovery of motor function go hand in hand when considering rehabilitation programs for CRPS patients.

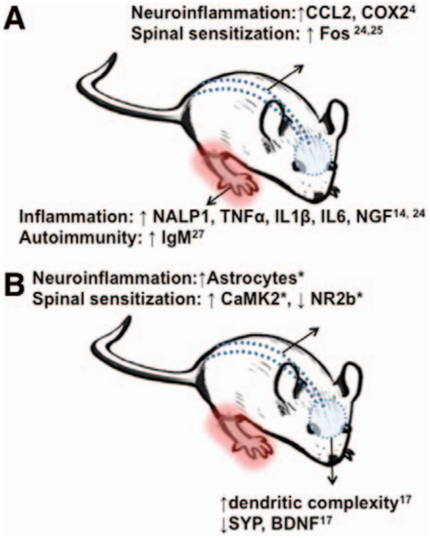

Fig. 9. Summary. Illustration of potential nociceptive mechanisms observed in the acute (*A*) *versus* chronic (*B*) stages of complex regional pain syndrome in the fracture/cast rodent model. *Data shown in this study. BDNF = brain-derived neurotrophic factor; CaMK2 = Ca^{2+}/calmodulin-dependent protein kinase II; CCL2 = chemokine (C-C motif) ligand 2; COX2 = cytochrome *c* oxidase subunit 2; IgM = immunoglobulin M; IL1-β = interleukin 1β; IL6 = interleukin 6; NALP1 = NAcht Leucine-rich repeat protein 1; NGF = nerve growth factor; NR2b = *N*-methyl-D-aspartate receptor 2b; SYP = synaptophysin; TNF-α = tumor necrosis factor α.

Acute versus *Chronic Stages of CRPS*

Similar to the two distinct phases (acute warm *vs.* chronic cold[2]) observed in patients, fracture/cast mice exhibit distinct physiologic, behavioral, biochemical, and transcriptional changes in the early and late phases.[4] Therefore, we postulate that it is likely that mechanism-based treatment outcomes would vary when administered at these different stages.

The differential efficacy of ketamine at the acute *versus* chronic timepoints could be due to many factors. One possibility is that ketamine's CNS-mediated analgesic effects are obscured by peripheral pronociceptive mechanisms at the early stages of the syndrome. This hypothesis is supported by previous evidence where, during the acute phase, we have shown prominent effects of neuroinflammation and increased levels of inflammatory mediators in the skin of the affected tissue including interleukin-1β, tumor necrosis factor-α, interleukin-6, and others, in both our rodent model[15,19,24–26] and human biopsy samples.[27] Another potentially ketamine-resistant mechanism of nociceptive sensitization is the peripheral autoimmune response observed during the acute stage of the syndrome.[28] A centrally acting NMDA receptor antagonist may be ineffective in suppressing sensitization supported by these mechanisms. Consequently, once these peripheral inflammatory responses subside at 7 weeks after fracture, as evidenced by the resolution of increase of temperature and edema, ketamine CNS analgesic action may be more effective. Alternatively, it is possible that ketamine acts on signaling pathways mediating central sensitization that are not present or fully developed at the acute timepoint. This hypothesis is supported by our behavioral evidence showing ketamine efficacy in providing persistent reversal of sensitization only during infusion in the chronic phase. In addition, ketamine reduced latent sensitization to capsaicin injections performed after the resolution of allodynia (our experiments do not discern whether this latent sensitization is established entirely during the acute phase, the late chronic phase, or both) and normalized motor performance on the rotarod, measures that are ostensibly, although not exclusively, sensitive to CNS changes. A summary of the peripheral and central changes at the acute and chronic stages of CRPS in the fracture/cast rodent model is shown in figure 9.

Hypothesizing that ketamine's effects might be most clearly demonstrated in the CNS, we performed immunohistochemical and molecular experiments to determine whether CNS cells and proteins associated with persistent nociceptive sensitization were chronically inhibited after 7 days of ketamine infusion.

Spinal Mechanisms of Ketamine Activity

A 7-day infusion of ketamine chronically inhibited the postfracture activation of SC dorsal horn astrocytes for several weeks after the pumps were removed. Although initially considered as passive bystanders, it is now clear that glia constitute a dynamic part of the chronic pain equation. This is particularly true of the more abundant glial cells, astrocytes.[29] At the chronic time point, we studied that astrocytic, but not microglial, activation was evident. This is consistent with the findings from preclinical models of pain where microglial up-regulation has been shown during the acute phase,[30] whereas astrocytic activation is often prominently observed in the more persistent states.[31] Data from pharmacologic studies in preclinical pain models have shown that ketamine administration inhibits spinal nerve ligation-induced activation of astrocytes and the associated hyperalgesia.[32] In addition, our data are consistent with *in vitro* studies showing the inhibition of lipopolysaccharide-induced production of prostaglandin-E2 and tumor necrosis factor-α in primary astrocytic cultures.[33] Our data provide further evidence that glial inhibitory changes can be persistent after ketamine treatment.

The various immune mediators released in the SC after peripheral injury could result in central sensitization by direct modulation of excitatory synaptic transmission. Central sensitization is a mechanism common to many chronic pain syndromes including CRPS, where increased glutamate levels in the cerebrospinal fluid of patients are observed.[34] Despite the fact that the exact role of NMDA receptors in this mechanism is unknown, it is generally believed that an important pathway through which peripheral noxious stimulation leads to persistent pain states is through central sensitization at the level of the SC, where, in concert with other systems, the corelease of peptides and glutamate from peripheral nerves induces the activation of NMDA receptors.[35] For our biochemical studies, the NR2b subunit was chosen based on both our preliminary data and a recent study showing NR2b-containing receptors to play a predominant role in synaptic transmission in the superficial lamina of the rodent SC[21]; CaMK2 was included based on the ingenuity pathway analysis of our previously published SC microarray data[4] and its known binding to, and activation by, stimulated NR2bs; SYP was chosen as a proxy indicator of synaptic abundance; and BDNF was chosen because of its established involvement in pain and central sensitization. We show that persistent sensitization in our model is associated with decreased NR2b and increased CaMK2, with no changes in the spinal levels of SYP and BDNF. Furthermore, data from the ketamine-treated groups did not provide us with a clear biochemical mechanism that could account for the antiallodynic effects of the drug. Therefore, it is possible that, at least at the 10-week timepoint, ketamine mechanisms are mainly glial and not neuronal and might even be independent of NMDA receptors entirely. As such, future studies that fully evaluate the role of glial cells, particularly astrocytes, in this pain model are needed. We have obser-ved in a rat model of CRPS that the intrathecal administration of the astroglial inhibitor L-α-aminoadipate reduces mechanical allodynia (data not shown). This concept is consistent with the lack of therapeutic efficacy of drugs that target aberrant neuronal activity alone.[36] However, it is also possible that examining only a few biochemical targets did not provide an adequately comprehensive account of central sensitization in our model.

Nonspinal Mechanisms of Ketamine Activity

We examined the spinal effects of a 7-day systemic infusion of ketamine in a mouse model of CRPS. However, ketamine may have effects on both supraspinal structures and peripheral tissues. For example, human imaging shows that subanesthetic doses of ketamine reduce connectivity between the somatosensory network and the areas involved in the affective processing of pain.[37] In our data, a 7-day systemic ketamine infusion had no immediate effects of measures of anxiety in the fracture model (data not shown); however, it is possible that some of the antihyperalgesic/antiallodynic effects could be attributed to supraspinal mechanisms. Furthermore, subanesthetic doses of ketamine have been shown to have antidepressant activity. Ketamine used in mouse models of depression and anxiety has been demonstrated to reverse deleterious neuroplastic changes, *e.g.*, dendritic, synaptic protein, and BDNF changes, in the prefrontal cortex and hippocampus.[38] Similarly, improvements in neuropsychologic testing, along with normalization of resting-state networks, have been documented clinically in CRPS patients treated with ketamine.[13]

In addition to their role in the CNS, NMDA receptors are expressed at the peripheral processes of unmyelinated fibers, and their activation is linked to the development of hyperalgesia in neuropathic rats.[39] In addition, topical ketamine treatment has shown mixed effects in reducing mechanical allodynia in patients with CRPS,[7,40] where analgesic effects seem to be superior during the acute phase of the syndrome. These local mechanisms were not fully evaluated in our studies, and hence, we cannot exclude peripheral sites in addition to central sites of action for ketamine in our model. Furthermore, ketamine has well-known antiinflammatory effects through interactions with toll-like receptors on immune cells, which could account for some of its analgesic actions.[41] This is an important mechanism to consider in CRPS because in addition to peripheral inflammation that is associated with the syndrome, peripheral vascular changes could also be sensitive to ketamine treatment which is believed to be involved in improved blood flow and even direct relaxation of blood vessels.[42]

Limitations and Future Directions

Animal models have inherent limitations regarding the validity and translational value of the findings. Using a nonspecific NMDA antagonist, while being clinically relevant, does leave the need to establish that the effects observed are, in fact, mediated by NMDA receptors, particularly because ketamine could act through NMDA-independent mechanisms of action, by binding to opioid receptors, monoamine transporters, muscarinic and nicotinic cholinergic receptors, serotonin receptors, *etc.*[41] In this study, ketamine was administered systemically. Despite the fact that ketamine is believed to cross the blood–SC and blood–brain barriers, a more thorough approach would have been to study, in parallel, the administration of ketamine to the periphery, SC, and various brain regions. In addition, only a single dose of ketamine was used; it is possible that higher doses could have resulted in some analgesic action, although our methods were not suitable to address this question. Finally, this study focused on the long-term effects of ketamine administration; more granular time course studies might better elucidate the effects of ketamine infusion on CRPS-related behaviors and cellular and biochemical changes.

Acknowledgments

The authors thank Tian-Zhi Guo, Ph.D., Wenwu Li, Ph.D., and Hamda Khan (Palo Alto Veterans Institute of Research and Education, Palo Alto, California) for their technical assistance.

This study was supported by National Institutes of Health grant NS072168 (to Drs. Kingery and Clark).

Competing Interests

The authors declare no competing interests.

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