Duration of reduction in enduring stress-induced hyperalgesia via FKBP51 inhibition depends on timing of administration relative to traumatic stress exposure

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Abstract

Chronic pain development is a frequent outcome of severe stressor exposure, with or without tissue injury. Enduring stress-induced hyperalgesia (ESIH) is believed to play a central role, but the precise mechanisms mediating the development of chronic posttraumatic pain, and the time-dependency of these mechanisms, remain poorly understood. Clinical and pre-clinical data suggest that the inhibition of FK506-binding protein 51 (FKBP51), a key stress system regulator, might prevent ESIH. We evaluated whether peritraumatic inhibition of FKBP51 in an animal model of traumatic stress exposure, the single prolonged stress (SPS) model, reversed ESIH evaluated via daily mechanical von Frey testing. FKBP51 inhibition was achieved using SAFit2, a potent and specific small molecule inhibitor of FKBP51, administered to male and female Sprague-Dawley rats via intraperitoneal injection. To assess timing effects, FKBP51 was administered at different times relative to stress (SPS) exposure. SAFit2 administration immediately after SPS produced a complete reversal in ESIH lasting >7d. In contrast, SAFit2 administration 72h following SPS produced only temporary hyperalgesia reversal, and administration 120h following SPS had no effect. Similarly, animals undergoing SPS together with tissue injury (plantar incision) receiving SAFit2 immediately post-surgery developed acute hyperalgesia but recovered by 4d and did not develop ESIH. These data suggest that (1) FKBP51 plays an important, time-dependent role in ESIH pathogenesis, (2) time windows of opportunity may exist to prevent ESIH via FKBP51 inhibition after traumatic stress, with or without tissue injury, and (3) the use of inhibitors of specific pathways may provide new insights into chronic post-traumatic pain development.

SUMMARY

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SAFit2-mediated inhibition of FKBP51 at variable times relative to traumatic stress exposure identified the importance of timing to the duration of reduction in persistent hyperalgesia.

Keywords

chronic pain; stress; FKBP51; SAFit2; rats; tissue injury; PTSD

INTRODUCTION

Traumatic stress exposure (TSE), with or without tissue injury, commonly results in chronic pain development.(e.g,[15; 18; 21; 42; 45; 57]) The chain of molecular events that generate the enduring stress-induced hyperalgesia (ESIH) after TSE that is the foundation of such chronic pain states remains poorly understood. In large part because of this lack of understanding, no effective interventions exist to prevent chronic pain development after common TSE. Interestingly, in the best-studied animal models of ESIH development after TSE, molecular mechanisms mediating the development of ESIH appear to be stress- and time-dependent (e.g.,[13; 28; 29; 49; 51]). An essential biological characteristic of ESIH development appears to be a switch in cellular phenotypes (e.g., such that mediators that were previously analgesic or innocuous become hyperalgesic[27; 50]).

One biological system implicated in development of ESIH after TSE is the hypothalamic–pituitary–adrenal (HPA) axis.[26; 40; 41] Within the HPA axis, evidence to date suggests that FK506-binding protein 51 (FKBP51), an intracellular protein that regulates glucocorticoid receptor (GR) sensitivity,[12; 53; 60] plays a particularly important role.[5; 34; 39; 58] Genetic variants in *FKBP5* that increase FKBP51 levels strongly predict chronic pain development across TSEs,[5; 34; 38; 39; 58; 65] and in animal models, knocking down/out FKBP51 reduces long-term pain behaviors after noxious inflammatory and spared nerve injury.[38; 39; 65] These data suggest that a better understanding of the role of FKBP51 in ESIH could improve understanding of mechanisms mediating chronic pain development after TSE, and that FKBP51 might even be a useful therapeutic target for chronic pain prevention.

In the current study, we assessed the hypothesis that FKBP51 inhibition would prevent ESIH development in a rat model of TSE, in both male and female animals. In addition, we hypothesized that the effect of FKBP51 inhibition on ESIH would be time-dependent relative to TSE, with the greatest duration of ESIH reduction observed with FKBP51 inhibition in the early aftermath of TSE. We also hypothesized that FKBP51 inhibition with SAFit2 would reduce ESIH after TSE with associated tissue injury. We assessed these hypotheses in a well-validated model of TSE, the single prolonged stress (SPS)[35] model, which produces a lasting hyperalgesic response in rats similar to the human experience of TSE. [20; 35; 55; 67] We modeled stress exposure with associated tissue injury by combining SPS with plantar incision.[6] To inhibit FKBP51, we used the highly potent and specific small molecule FKBP51 antagonist SAFit2.[17]

METHODS

Animals:

Experiments were performed on adult male and female CD Sprague Dawley rats 8–13 weeks old (males 290–450 g, females 170–280 g; Charles River, Raleigh, NC). Rats were pair-housed under a 12-hour light/dark cycle in the Division of Laboratory Animal Medicine at The University of North Carolina at Chapel Hill. Food and water were provided *ad libitum*. Animal care and use conformed to NIH guidelines. Experimental protocols were approved by the Institutional Animal Care and Use Committee.

Single Prolonged Stress:

The details of the single prolonged stress (SPS) protocol have been reported previously.[30] Rats were exposed to serial stressors on one day as follows: restraint for 2 hours, forced swim for 20 minutes, a 15 minute recovery period, followed by exposure to diethyl ether until general anesthesia was induced (generally < 7 minutes). Rats were single housed following SPS.[30] Modified from the original SPS model, we chose to forgo the 7-day quiescent period in favor of daily monitoring of hyperalgesia. Animals not exposed to SPS were brought to a procedure room where they were left undisturbed for the same length of time as SPS exposed animals, then moved to single housing. Most experiments were performed in batches of n=8 animals with four experimental and four control/vehicle-treated animals per batch. A total of 146 animals were tested in 19 batches for 13 different experimental cohorts.

von Frey testing:

Paw withdrawal thresholds (PWT) were used to assess mechanical hyperalgesia-like behaviors. PWTs were determined by applying touch test sensory probes, von Frey monofilaments (Stoelting Co., Wood Dale, IL), at a right angle to the plantar surface of the left hind paw until the animal indicated sensation by pulling back its paw. The up-down method described by Chaplan et al was used, starting with a monofilament of 8g force.[9]

Prior to the start of experimental testing, animals were allowed to acclimate to the testing environment for 30 minutes each day over the course of four days. The acclimation period involved placing the animals in the testing chambers and applying a small filament to the paw such that no withdrawal response was elicited. Baseline PWTs were collected on two separate days following acclimation and the data averaged to determine a baseline sensitivity for each animal. For the determination of PWT on each day of testing, the up-down method was performed five times with five-minute intervals between each test. These five technical replicates were averaged to find the 50% pain threshold (i.e. the force required to elicit a response 50% of the time). Animals were assigned to groups such that average baseline PWT and average baseline weight were balanced across groups. Hyperalgesia-like behavior was assessed daily following SPS and/or PI or in naïve animals for 7–26 days, with select experiments extended due to available resources.

SAFit2 preparation and injections:

SAFit2 was synthesized by the Hausch lab (Technical University of Darmstadt, Germany). [1; 17] SAFit2 in powder form was combined with 100% EtOH at a concentration of 50mg/ml and stored at −20°C for the duration of the study. On the day of injection, SAFit2 was solubilized at a ratio of 4% ethanol-SAFit2 solution, 5% Tween80, and 5% PEG400 in 0.9% saline. SAFit2 was administered at a concentration of 20mg/kg via intraperitoneal (i.p.) injection. Vehicle animals received the solubilizing solution alone (4% ethanol, 5% Tween80, and 5% PEG400 in 0.9% saline). The dosage was chosen based on maximum solubility of SAFit2 in solution, injected in the maximum amount recommended for i.p. injection.

Timing of SAFit2 administration:

To test the effect of the timing of FKBP51 inhibition on ESIH following SPS, SPS+PI, PI, or in naïve animals, 20mg/kg injections of SAFit2 were administered via intraperitoneal injection at different time points following SPS. For animals receiving injections immediately following SPS or PI, both vehicle and SAFit2 were administered while the animals were still under the effects of ether anesthesia. For all experiments involving two injections, the timing between the first injection and the second injection remained constant. For the experiment in which SAFit2 was administered multiple times over the course of five days, twice-daily administration (i.e. two injections within a 24 hour period) was selected because of the known half-life of SAFit2 (n=9.7 hours).[17]

Plantar Incision:

In experiments where plantar incision (PI) was paired with SPS, PI was performed immediately following SPS. In experiments that assessed the effects of PI alone, PI was performed in animals at a similar post-natal date (within one week) to animals in the SPS+PI experiment. Modified from the Brennan model of postsurgical pain[6], animals were allowed to recover from ether exposure until normal ambulation, then were placed under a surgical plane of isoflurane anesthesia. A 1cm longitudinal incision was made on the left hind paw. The plantaris muscle was isolated and severed longitudinally without disruption of muscle insertions or surrounding connective tissue/muscles. The wound was sutured, and Bacitracin-Neomycin-Polymyxin B (BNP) ointment was applied to prevent infection. The animal was allowed to recover from anesthesia before being returned to single housing. von Frey testing was performed on the injured paw by applying monofilament pressure distal to the incision.

Statistical Analysis:

Force of PWT data is represented normalized by log transformation per Weber's law of nociception perception, which states that mechanical sensitivity is perceived on a logarithmic scale[46]. Comparison between groups, as defined in the manuscript, were performed using RStudio servers (v1.4.1103). The normality assumption was assessed using the Shapiro-Wilk test for each combination of factor levels. Significant differences between groups were primarily evaluated using two-way mixed analysis of variance (ANOVA) to assess group (SAfit2 vs vehicle or male vs female)*time interactions that incorporate data

from all time points assessed in the experiment, starting on the day of SPS until the last day that PWTs were collected. The results of two-way mixed ANOVA analyses of interaction terms are reported within the text of the Results section. Of note, in instances where the sample size in the groups differed at different time points, the smallest sample size across all data points was used in the two-way mixed ANOVA, and these are the sample sizes that are reported. Additionally, group effects at individual time points were compared using two-way mixed ANOVAs or pairwise t-tests followed by Bonferroni post-hoc adjustments to account for multiple comparisons. Statistical significance for individual time point comparisons are indicated in Figures using asterisks ($p<0.05$) or hashtags ($p<0.001$). All data are expressed as mean ± SEM.

RESULTS

Experimental protocol

Separate experiments evaluated the effect of administration of the FKBP51 inhibitor SAFit2 at five timepoints relative to SPS: prior to SPS, immediately following SPS, 24 hours following SPS, three days following SPS, and five days following SPS (Figure 1). In addition, the effect of SAFit2 administration immediately following SPS + plantar incision was also assessed (Figure 1). Weight and qualitative assessment for health anomalies (i.e. pale skin tint, coat cleanliness, etc.) were tracked daily over the course of the study.

Validation of Single Prolonged Stress as a model for enduring stress-induced hyperalgesia

The SPS model[36; 63] is a well-validated model that induces immediate hypersensitivity lasting 16 days in the absence[20; 55; 67] of tissue injury or 26 days in the presence[7; 62] of tissue injury. A previous study found no sex differences in post-SPS ESIH development. [68] Consistent with these studies, SPS produced ESIH (reduced PWT) in both male animals (two-way mixed ANOVA; n=3/condition (treatment*time), $F_{(7,28)}=8.78$, $p<0.001$) and female animals (two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(8,48)}=4.32$, $p<0.001$) (Figure 2A). No sex differences were observed in PWT in male and female animals that were exposed (two-way mixed ANOVA; n=6 male/8 female (sex*time), $F_{(7,63)}=0.73$, $p=0.645$) or were not exposed (two way mixed ANOVA; n=6 male/8 female (sex*time), $F_{(7,42)}=1.44$, $p=0.216$) to SPS (Figure 2A).

SAFit2 inhibition of FKBP51 initiated <24 hours following SPS and continuing twice a day for 5 days prevents ESIH

Intraperitoneal SAFit2 administration 4 hours following SPS, followed by continued twice-daily injections × 5 days, prevented the development of post-TSE hyperalgesia (Figure 2B). SAFit2 prevented SPS-induced decrease in PWT, continuing for at least seven days (Figure 2B, two-way mixed ANOVA; n=3/condition (treatment*time), $F_{(10,40)}=10.61$, $p<0.001$. Significance for individual timepoints indicated in graph). Of note, repeated administration of SAFit2 did not influence animal weight (Figure 1s) or other qualitative measures of health (e.g. scruffy coat, dull eyes or nose, lumps at the injection site).

Two doses of SAFit2 administered < 24 hours after SPS produces enduring reduction in hyperalgesia

We next assessed whether continued inhibition of FKBP51 following SPS (via multiple injections of SAFit2) as performed above was necessary for persistent reduction in post-SPS hyperalgesia or whether a shorter duration of SAFit2 administration could lead to similar results when administered early following TSE. Therefore, we assessed whether two doses of SAFit2, administered during the 24-hour period after SPS, could prevent the development of long-lasting mechanical hyperalgesia. In this experiment, we found that the magnitude and duration of reduction in hyperalgesia observed with two doses of SAFit2 within 24 hours of SPS was at least as long as that observed with continued twice daily administrations of SAFit2 for an additional four days This effect was observed in both male rats (Figure 3A; two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(7,42)}$=6.74, p<0.001) and female rats (Figure 3B; two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(8,48)}$=3.24, p=0.005). No sex differences in the effect of SAFit2 were observed at any individual timepoints (two-way ANOVA with Bonferroni post-hoc comparison; p>0.05 at each time point). However, we did detect an overall difference in pain-like trajectories when comparing SAFit2 treated animals across sex (two-way mixed ANOVA; n=4/condition (sex*time), $F_{(6,36)}$=2.58, p=0.035). In addition, we also assessed whether SAFit2 influences basal nociception by administering the same two dose regimen of vehicle or SAFit2 in SPS-naïve rats. In this experiment, we found that there was no statistically significant difference between vehicle and SAFit2 treated animals (Figure 2s; two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(9,54)}$=0.22, p=0.99), and no difference between post-injection PWTs and baseline measures of hyperalgesia in either the vehicle (within subjects ANOVA; $F_{(9,30)}$=0.95, p=0.49) or SAFit2 treated animals (within subjects ANOVA; $F_{(9,30)}$=1.74, p=0.12).

Two doses of SAFit2 administered 72 hours after SPS produces only transient reduction in hyperalgesia

When the same dosing strategy (two doses within 24 hours) was administered on day 3–4 (72–96 hours after SPS) rather than day 1, the reduction in hyperalgesia observed was transient rather than persistent (Figure 4). In male animals, this transient reduction in ESIH lasted for four days (Figure 4A; two-way mixed ANOVA; n=8/condition (treatment*time), $F_{(12,72)}$=9.1, p<0.001). In female animals, this transient reduction in ESIH lasted for two days (Figure 4B; two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(11,66)}$=5.51, p<0.001).

Two doses of SAFit2 administered 24 hours after, five days after, and the day before SPS produce differing effects

As with two doses of SAFit2 administered < 24 hours after SPS, two doses of SAFit2 administered 24–48 hours after SPS resulted in persistent reversal of stress-induced hyperalgesia. (Figure 5A; two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(9,54)}$=8.59, p<0.001). In contrast, two doses of SAFit2 administered 120 hours after SPS had no effect on stress-induced hyperalgesia at any individual timepoints (Figure 5B). However, a significant interaction between treatment and time was detected (two-way mixed

ANOVA; n=4/condition (treatment*time), $F_{(11,66)}$=2.73, p=0.006). Two doses of SAFit2 administered during the 24 hour period prior to SPS (24 hours and one hour prior) resulted in a statistically significantly reduction in hyperalgesia on each of the first two days following SPS. This reduction continued over time, for the full 16-day experiment (Figure 5C; two-way mixed ANOVA; n=4/condition (treatment*time), $F_{(10,60)}$=2.71, p=0.008). However, only two individual timepoints (day 1 and day 2) showed statistically significant differences in PWT when comparing SAFit2 treated animals to vehicle treated animals (pairwise t-test with Bonferroni correction, p<0.05).

Two doses of SAFit2 administered within 24 hours of SPS+plantar incision prevents enduring but not acute hyperalgesia

SPS with plantar incision (PI) [6] was used to model stress exposure plus tissue injury. Two doses of SAFit2 administered < 24 hours following SPS+PI, as performed above after SPS alone, prevented enduring but not acute hyperalgesia. Animals administered SAFit2 after SPS+PI still developed acute hyperalgesia, but this hyperalgesia returned to baseline levels by day 4. In contrast, vehicle-treated animals demonstrated persistent hyperalgesia for twenty-six days (Figure 6; two-way mixed ANOVA; n= 4/condition (treatment*time), $F_{(13,78)}$=4.36, p<0.001). We also assessed the influence of SAFit2 administration in animals exposed to PI alone (i.e. without SPS). Consistent with previous studies[6; 62], we detected a robust acute hyperalgesia in vehicle treated animals (Figure 3s). Interestingly, and in contrast to SPS+PI-exposed animals, SAFit2 administration completely abolished this acute hyperalgesia (Figure 3s; two-way mixed ANOVA; n= 4/condition (treatment*time), $F_{(13,78)}$=21.54, p<0.001).

DISCUSSION

Previous studies have demonstrated temporary analgesia with FKBP51 inhibition via SAFit2 in animal models of established neuropathic and inflammatory pain.[38; 39; 65] Consistent with and extending these data, FKBP51 inhibition via SAFit2 72–96 hours after SPS temporarily reversed stress-induced hyperalgesia. Further, we show that FKBP51 inhibition within 24 hours of stress exposure, or between 24–48 hours after stress exposure, produced long-lasting reversal of hyperalgesia without influencing basal nociception. Similarly, FKBP51 inhibition via SAFit2 administration within 24 hours of stress exposure with concomitant tissue injury (plantar incision) also prevented persistent (but not acute, unless in the absence of stress) hyperalgesia. Across all experiments, FKBP51 inhibition after TSE reduced hyperalgesia in both male and female animals.

Preventing and treating chronic pain after TSE is an important clinical question, as TSEs such as motor vehicle collision, sexual assault, surgery, and/or military service are experienced by most individuals during life and commonly result in chronic pain.[15; 21; 24; 37; 57] Our findings have several potential implications for understanding and preventing the development of chronic post-TSE pain. First, these data suggest that FKBP51 may play an essential role in the series of molecular events that lead to ESIH development, as FKBP51 inhibition in the early aftermath of TSE prevented ESIH. Second, the fact that FKBP51 inhibition in the early aftermath of TSE prevents but later inhibition only

transiently reverses hyperalgesia suggests that FKBP51 contributes to a phenotypic switch. Using inhibitors of FKBP51, and perhaps inhibitors of other components of the stress response, to probe the timing and molecular biology underpinning this/these phenotypic switch(s), and defining similarities and differences with physiologic mechanisms identified in other animal models of ESIH (e.g.,[13; 28; 29; 49; 51]), is a promising area of study.

Our findings that tissue injury produces short-term hyperalgesia, but stress exposure is necessary for persistent hyperalgesia, is consistent with data in humans that tissue injury in general plays a relatively modest role in chronic pain development after TSE,[15; 21; 23; 45] and that tissue injury is not necessary for stress-induced hyperalgesia[8; 44; 57]. These data are also consistent with evidence from other animal models (e.g.,[28; 32]). Over the arc of the 20th century, the realization that TSE without tissue trauma could produce enduring changes in neurobiology manifest as posttraumatic stress was first appreciated by the scientific community, and then spread to popular culture. Despite the continued accrual of evidence such as in the experiments presented above, a similar dissemination of the concept that ESIH can cause chronic pain and somatic symptoms after TSE has not yet occurred.

In addition to the many biological insights that have been/can be gained from this work, the above findings suggest that FKBP51 is also a promising therapeutic target to prevent chronic pain development after TSE. The fact that FKBP51 influences long-term nociceptive processing,[34; 39] but not always acute nociceptive processing (SPS+PI vs PI alone) makes it attractive, because acute nociception is important for protection against potentially harmful behaviors, but long-term pain behaviors lose their protective effects and become pathogenic[59; 61]. In addition to potential preventive effects on chronic post-TSE pain, evidence suggests that FKBP51 inhibition may prevent adverse outcomes frequently comorbid with chronic post-TSE pain nociception/pain,[43] including depression,[4; 19] posttraumatic stress,[3; 64] anxiety,[2] suicidality,[14; 16] and dissociation[31].

These study findings indicate that the early posttraumatic period following TSE may provide a unique therapeutic window to initiate preventive interventions. Consistent with these data, a number of studies have now shown that administration of small molecules that modulate aspects of the stress response[33] [10; 22; 69] are efficacious in diminishing the long-term adverse effects of TSE. A great many preventive intervention studies for adverse posttraumatic neuropsychiatric sequelae have been performed, yet only handful have attempted to exploit the potential window of opportunity represented by the acute post-traumatic period, and the majority of these studies (such as those referenced above) have shown promise. Clinical translational studies to test the ability of interventions administered in the early aftermath of TSE to prevent chronic pain development represent a promising area of study.

While the mechanisms via which FKBP51 influences ESIH in a time dependent manner are not understood, previous studies have demonstrated that the early post-TSE period is marked by profound molecular changes in the nervous, stress, and immune systems.[25; 54] These molecular-level changes commence immediately following TSE and persist for varying, often unknown, periods of time following TSE. FKBP51 expression is known to

increase following TSE[19; 39; 52], but the exact timing and tissue specificity of these changes, and potential additional molecular changes that induce the phenotypic changes underlying ESIH, are unknown. Preliminary evidence from the SPS and other pain models suggest that molecular effectors such as immune cells/trafficking in the CNS,[11; 49; 50] NFkB pathways,[65] IL-6,[39] and specific microRNA[47; 56] may be important mediators in the process. Further experiments are needed to test the timing and nature of the underlying molecular events (and corresponding physiological changes to e.g. brain morphology and brain signaling networks) that are driving time dependent effects of FKBP51 on ESIH.

The strengths of this body of work include the use of a highly potent and specific small molecule inhibitor of FKBP51, SAFit2, inclusion of both male and female animals, and utilization of a well-validated animal model of TSE. A number of limitations should also be considered when interpreting the results of this work. First, while male and female animals were included in many of the timing experiments, they were not utilized in every timing permutation experiment performed. Therefore, the generalizability of these results to both sexes is not fully understood. However, our results showing mostly sex independent effects (where tested) is consistent with human cohort studies indicating a lack of sex differences in the influence of *FKBP5* on chronic pain[34], and with previous studies of other pain models showing sex-independent effects[38]. Second, pain-like behaviors were assessed exclusively via mechanical von Frey testing. Recent studies indicate that more novel approaches to assessing hypersensitivity in animals, including testing of operant-conditioned[48] or voluntary behaviors[66], can provide a more comprehensive and informative assessment of pain-like behaviors that capture more of the complexity of pain inherent in humans who suffer from posttraumatic chronic pain. Future studies should perform a battery of outcomes that capture more than just pain-like sensitivity (e.g. psychological aspects of pain, interference with normal behavior). Third, the overall length of each experiment varied (e.g. Figure 3 vs Figure 2B), limiting the extent that conclusions can be made on the longevity of protection from FKBP51 inhibition in certain scenarios. Fourth, the underlying molecular mechanisms driving the timing effects we observe have not yet been elucidated. Such work has the potential to elucidate the exact timing of the "switch" from effectiveness to non-effective administration of SAFit2. It could also lead to important discoveries indicating how one might augment later administration of the inhibitor to increase its effectiveness at later time points.

In conclusion, we showed that inhibition of FKBP51 following TSE dramatically reduces the development of ESIH in male and female animals. We also show the importance of administration of SAFit2 to the early posttraumatic period. If extended, this work has the potential to increase our understanding of the potential to prevent highly morbid and costly posttraumatic chronic pain outcomes in the millions of individuals each year who experience life-threatening TSEs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Disclosures

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HIGHLIGHTS

- *FKBP51* is a critical regulator of the stress response

- Inhibition of FKBP51 following stress exposure reduces hyperalgesia in male and female animals

- The duration of reduction in hyperalgesia is dependent on the timing of FKBP51 inhibition

- FKBP51 signaling might contribute to the underlying mechanisms mediating post-stress chronic pain

PERSPECTIVE

The current work adds to a growing body of literature indicating that FKBP51 inhibition is a highly promising potential treatment strategy for reducing hyperalgesia. In the case of posttraumatic chronic pain, we show that such a treatment strategy would be particularly impactful if administered early after traumatic stress exposure.

SIGNIFICANCE

FKBP51 is a critical regulator of the stress response. Previous studies have shown that dysregulation of the expression of this gene plays a role in the pathogenesis of chronic pain development as well as a number of comorbid neuropsychiatric disorders. Using the FKBP51 inhibitor SAFit2, the current study explored the effect of FKBP51 inhibition on the development of enduring stress-induced hyperalgesia in an animal model of traumatic stress exposure. The influence of FKBP51 inhibition was time-dependent, such that early post-stress administration prevented hyperalgesia, whereas the effect of later administration was temporary. These data suggest that FKBP51 inhibition may prevent/ treat hyperalgesia, and that the study of underlying mechanisms mediating observed timing effects may provide new insights into chronic post-traumatic pain pathogenesis.
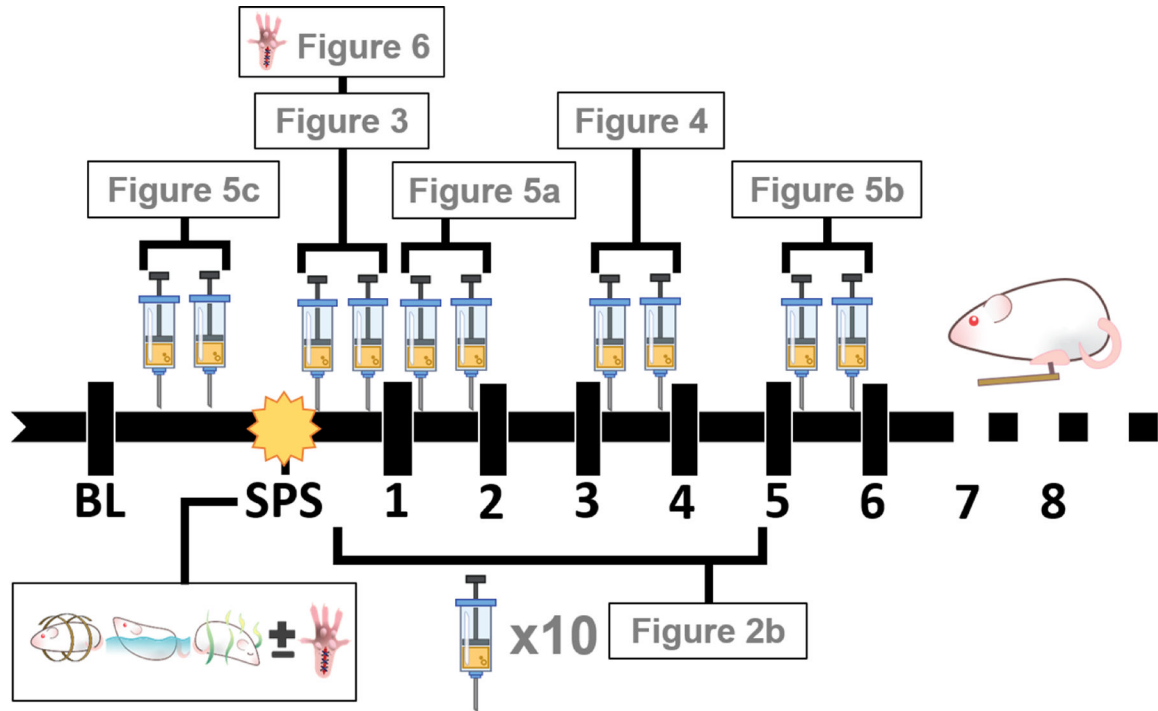
Figure 1.
Schematic showing experimental design with timing of administration of the FKBP51 inhibitor SAFit2 and indication of which figures contain data corresponding to the different permutations of the timing of SAFit2 administration. Experiments were performed in male and female animals. *Timeline left to right*: Baseline (BL) measurements were assessed prior to single prolonged stress (SPS) and included two separate days of mechanical Von Frey. Following baseline measurements, the SPS protocol was performed. Von Frey testing was performed daily following SPS for up to 26 days. *Bottom left box*: The SPS protocol is a multimodal stress protocol that exposes animals to restraint stress, swim stress, and ether exposure in a single day. Here, it was performed in the absence of tissue injury or in the presence of hind paw incision. *Syringes*: Subcutaneous injections of 20mg/kg SAFit2 or vehicle were administered at various time points throughout the study, as indicated by syringes. *"Figure" boxes*: Figure boxes indicate where data resulting from different time/ duration of SAFit2 administration can be found throughout the manuscript.
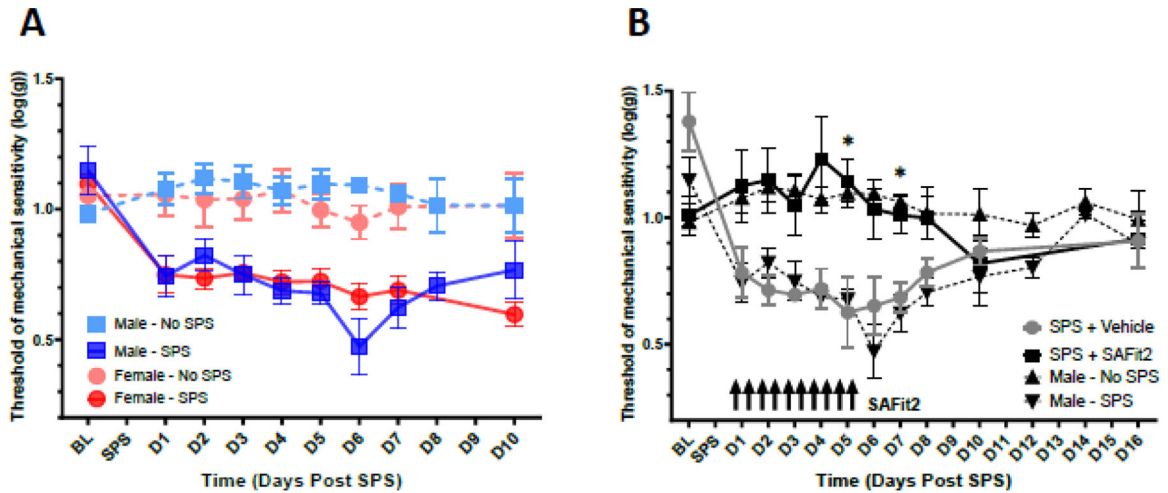
Figure 2.
Paw withdrawal threshold (PWT) following single prolonged stress (SPS) in SAFit2-treated and untreated animals. (***A***) Male (male symbol) and female (female symbol) Sprague Dawley rats were exposed to SPS (solid lines, n=6 males, n=8 females) or left undisturbed (dashed lines, n=6 males, n=8 females). PWTs were assessed via von Frey monofilaments at the indicated days post-SPS. (***B***) SAFit2 (20mg/kg, black squares and solid lines) or vehicle (gray circles and solid lines) was administered to male rats (n=3) twice-daily via intraperitoneal injection. The first dose was administered within four hours of the completion of SPS. Subsequent injections were performed each day in the morning and in the evening for a total of ten injections. For comparison, dotted lines represent untreated animals either exposed to SPS or left undisturbed (n=6). Asterisks signify statistical significance when comparing SAFit2 vs vehicle treated animals at individual time points, after adjusting for multiple comparisons using Bonferroni adjustment (* p ≤ 0.05). Error bars signify SEM.
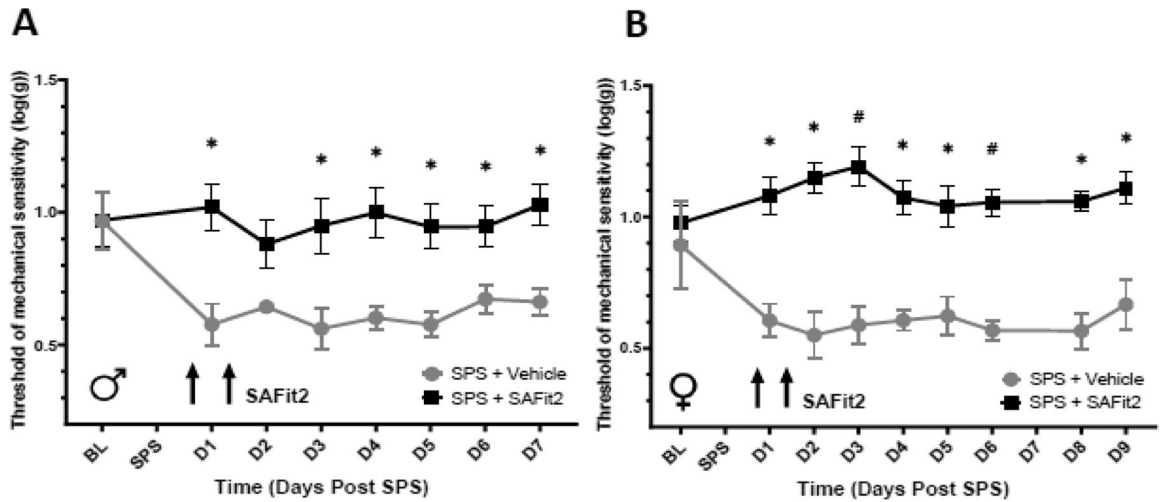
Figure 3.
Mechanical sensitivity in male and female animals following single prolonged stress (SPS) in experiments testing whether two intraperitoneal injections of the FKBP51 inhibitor SAFit2 administered starting immediately following SPS are efficacious in reducing long-term hyperalgesia. (*A*) Male Sprague Dawley rats (n=8) and (*B*) Female Sprague Dawley rats (n=8) were exposed to SPS and administered either Vehicle (gray circles and lines) or SAFit2 (20mg/kg; black squares and lines) via two injections (black arrows) within 24 hours of SPS. Hyperalgesia was assessed using von Frey monofilaments to the left hind paw. Asterisks and hashtags signify statistical significance at each timepoint after adjusting for multiple comparisons using Bonferroni adjustment (* p   0.05; # p   0.001). Error bars signify SEM.
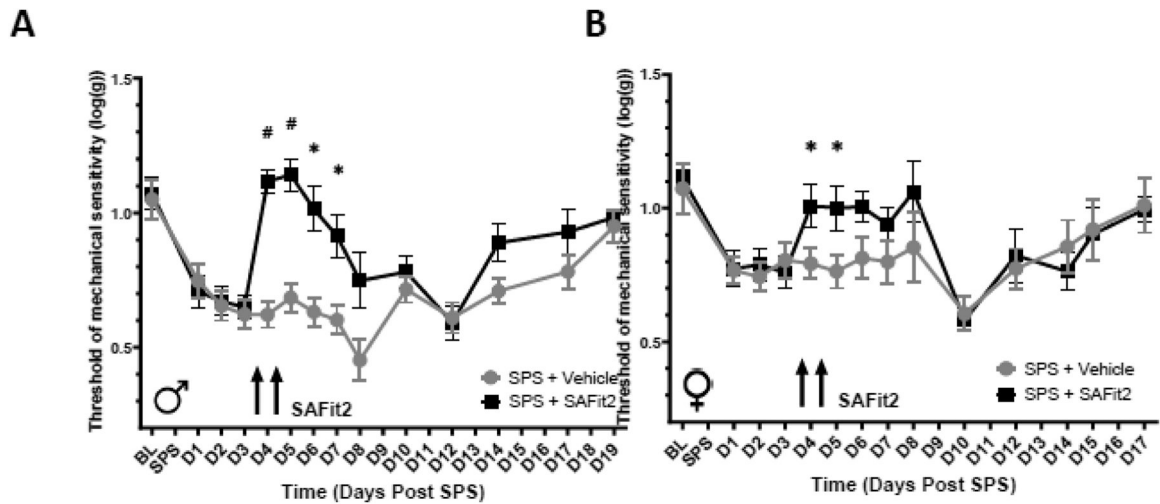
Figure 4.
Mechanical sensitivity following two intraperitoneal injections of the FKBP51 inhibitor SAFit2 administered 72 hours following SPS in male and female Sprague Dawley rats. (*A*) Male rats (n=8) and (*B*) female rats (n=8) were exposed to SPS and administered either Vehicle (gray circles and lines) or SAFit2 (20mg/kg; black squares and lines) via two injections (black arrows) commencing 72 hours following SPS. Hyperalgesia was assessed using mechanical Von Frey applications to the left hind paw. Asterisks and hashtags signify statistical significance of individual time point comparisons after adjusting for multiple comparisons based on Bonferroni adjustment (* p ≤ 0.05; # p ≤ 0.001). Error bars signify SEM.
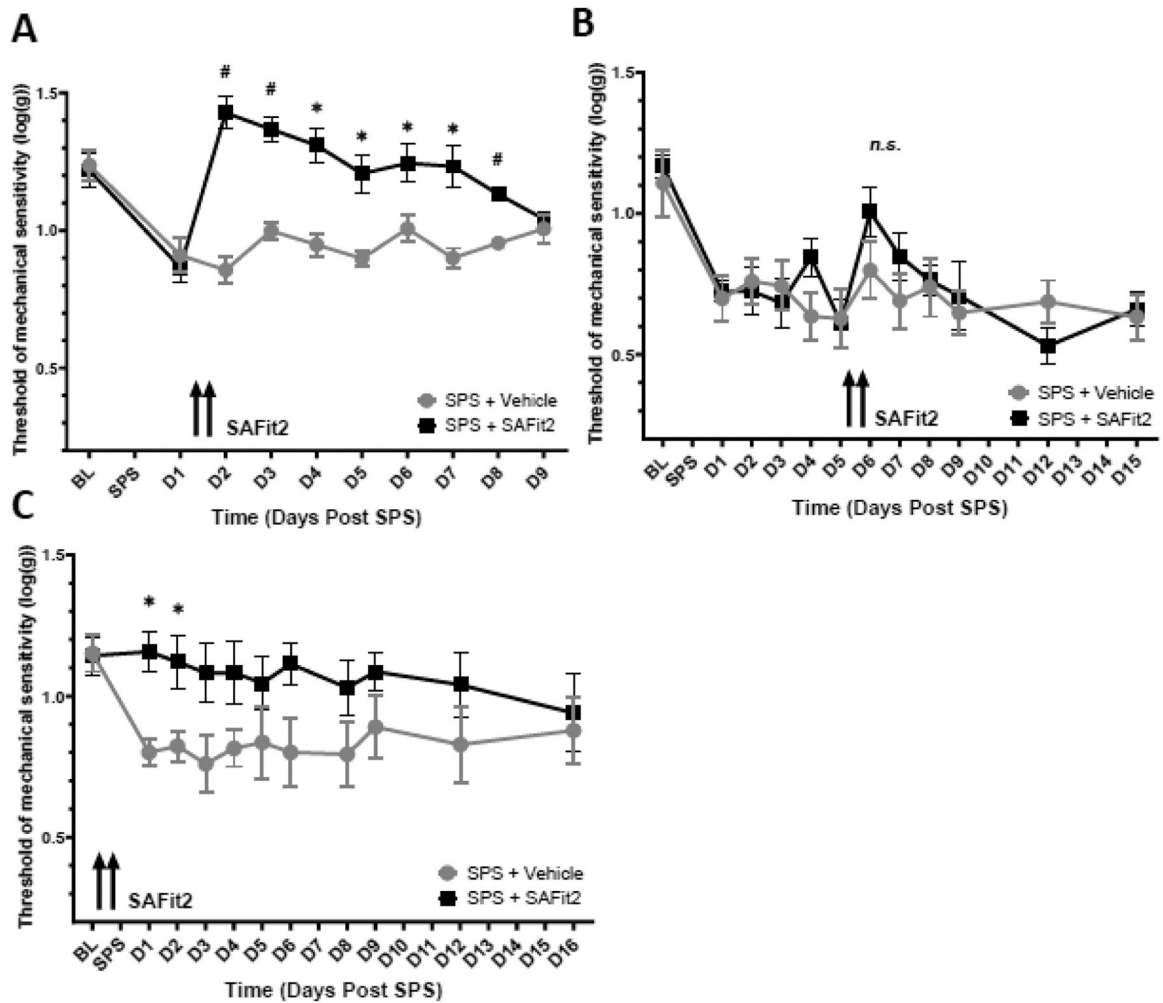
Figure 5.
Mechanical sensitivity following two intraperitoneal injections of the FKBP51 inhibitor SAFit2 administered at different time points following SPS, as indicated by black arrows. SAFit2 was administered starting (*A*) 24 hours following SPS, (*B*) 120 hours following SPS, and (*C*) 24 hours prior to SPS in male Sprague Dawley rats (n=4). Hyperalgesia was assessed using mechanical von Frey applications to the left hind paw. Vehicle (gray circles and lines) or SAFit2 (black squares and lines) was administered via two injections (black arrows). Asterisks and hashtags signify statistical significance of individual time point comparisons after adjusting for multiple comparisons based on Bonferroni adjustment (* p 0.05; # p 0.001). Error bars signify SEM. n.s. = not significant.
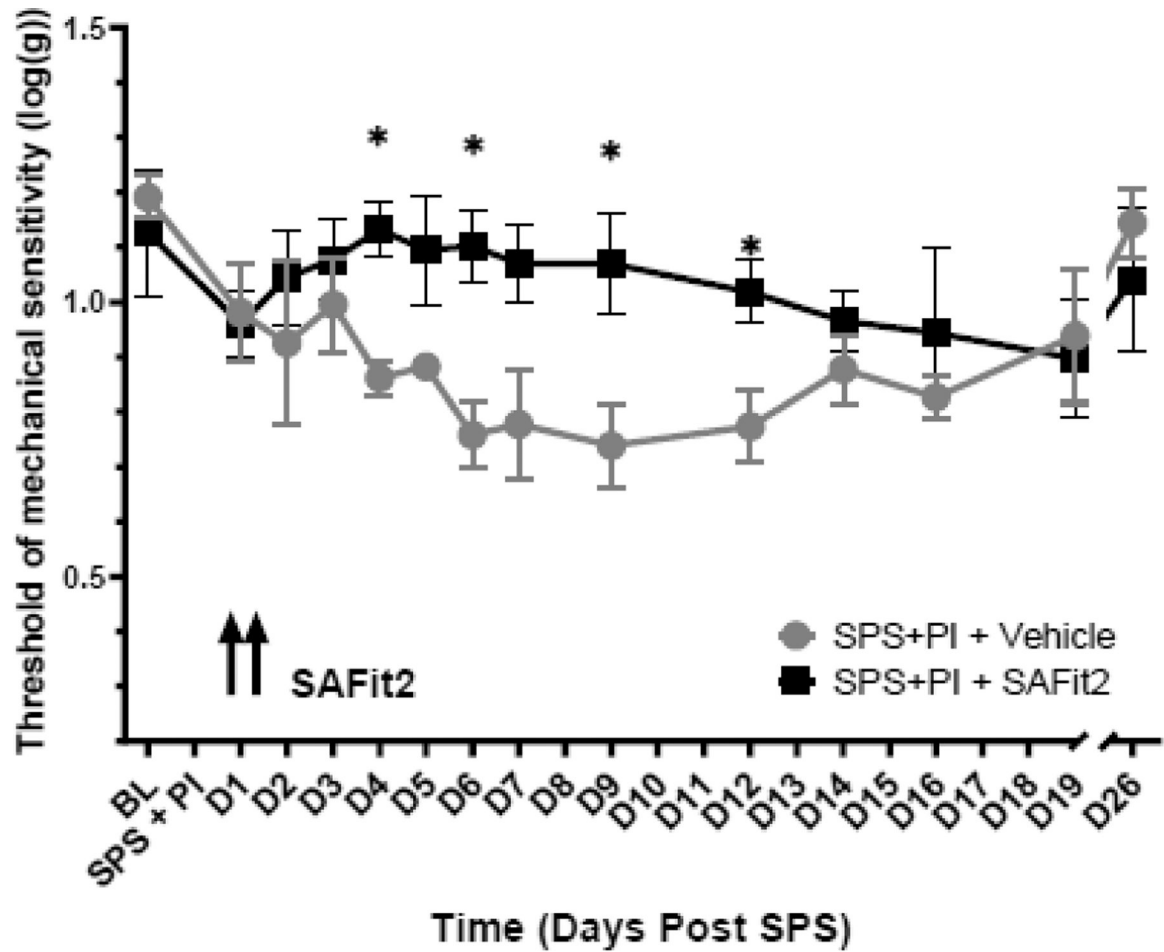
Figure 6.
Mechanical sensitivity following the inhibition of FKBP51 via two intraperitoneal injections of SAFit2 immediately following SPS + plantar incision ("SPS+PI). SAFit2 was administered (black arrows) to male Sprague Dawley rats immediately following SPS+PI and the following morning. Sensitivity was assessed using mechanical von Frey applications to the left hind paw, distal to the plantar incision. Vehicle treated animals (n=4; gray circles and lines) or SAFit2 treated animals (n=4; black squares and lines) were assessed over the course of twenty-six days. Asterisks signify statistical significance after adjusting for multiple comparisons via Bonferroni adjustment (* p   0.05). Error bars signify SEM.