The stress regulator FKBP51 drives chronic pain by modulating spinal glucocorticoid signaling

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One Sentence Summary: The stress regulator FKBP51 is a modulator of long-term pain states,

ABSTRACT

Polymorphisms in *FKBP51* are associated with stress-related psychiatric disorders and influence the severity of pain symptoms experienced after trauma. Here, we report that FKBP51 (FK506 binding protein 51) is crucial for the full development and maintenance of long-term pain states and that this is independent from its effect on mood. Indeed, FKBP51 knock out mice but also mice with silencing of FKBP51 restricted to the spinal cord showed reduced hypersensitivity in a number of persistent pain models. FKBP51 deletion did not compromise the detection of acute painful stimuli, a critical protective mechanism. Moreover, the specific FKBP51 inhibitor SAFit2 intrathecally administered reduced the severity of an established pain state, confirming the crucial role of spinal FKBP51 in nociceptive processing. Finally, glucocorticoid signaling, which is known to modulate persistent pain states in rodents, was impaired in FKBP51 knock out mice. This suggested that FKBP51 regulates chronic pain by modulation of glucocorticoid signaling. In conclusion, FKBP51 is a central mediator of chronic pain, likely in humans as well as rodents, and is a new pharmacologically tractable target for the treatment of long term pain states.

INTRODUCTION

Genetic variants in *FKBP51* are associated with major depression and post-traumatic stress disorder in humans *(1, 2)*, and mice lacking the gene *FKBP51* show improved coping behavior under stress, without alterations to their attention or motor functions*(3–5)*. Variations in the gene *FKBP51* influence the severity of musculoskeletal pain symptoms experienced during the weeks after motor vehicle collision or after sexual assault*(6, 7)*. Furthermore, as we have previously reported in a microarray study, FKBP51 is up-regulated in the rat superficial dorsal horn after de-repression by the methyl CpG binding protein 2 (MeCP2) within 2h of ankle joint inflammation*(8–10)*. We therefore hypothesised that FKBP51 contributes to injury-induced pain hypersensitivity. We have tested this hypothesis here by using knock out (KO) mice, siRNA technology and the recently developed FKBP51 inhibitor SAFit2*(11)*.

FKBP51 is up-regulated after activation of the glucocorticoid receptor (GR) by steroids*(12)* and, in a negative feedback loop, modulates the stress response by antagonizing GR and regulating its sensitivity*(13)*. Moreover, it has been proposed that GR signaling regulates the hypersensitivity that develops in long term pain states *(14–16)*. Using behavioral, pharmacological and molecular tools, we here test the role of spinal FKBP51 on long term pain states and their regulation by glucocorticoid signaling.

RESULTS

FKBP51 increases in the spinal cord to after noxious stimulation

First, using immunohistochemistry with tyramide amplification, we found that FKBP51 was expressed exclusively in neurons of the superficial dorsal horn of both naïve and mice injected with CFA in the ankle joint (Fig.1). Using RT-qPCR, we then confirmed that FKBP51 was increased significantly in both rats and mice after ankle joint inflammation induced by injection of Complete Freunds' adjuvant (CFA) in the ankle joint: FKBP51 was up-regulated in the dorsal horn in the rat at

2h and 6h after CFA, and in the mouse 24h and 3 days after CFA (Fig.2A)and we had already reported that this increase in FKBP51 mRNA correlated with an increase in FKBP51 protein in the rat *(10)*. No changes were observed in L4 and L5 dorsal root ganglia (DRGs) or in the cervical cord, indicating a localized spinal response to the CFA injection. In people carrying specific variants of *FKBP51*, childhood trauma can induce a decrease in DNA methylation in the promoter of *FKBP51*. This demethylation enhances stress-dependent expression of FKBP51 and is associated with a higher risk of developing post-traumatic stress disorder (PTSD) in adult life*(2)*. We therefore examined whether noxious stimulation could also induce changes in *FKBP51* DNA methylation. For this, we performed bisulphite pyro-sequencing on rat superficial dorsal horn tissue after injection of CFA into the ankle joint and observed a fast (2 h post CFA) and long lasting (7 days post CFA) ipsilateral decrease in methylation at specific CpG sites previously identified in the promoter sequence of *FKBP51(17)* (Fig.2B, C). This suggested that changes in DNA methylation could contribute to the rapid increase in spinal FKBP51 levels after CFA and that FKBP51 may regulate both the development and maintenance of long-term pain states. To test this hypothesis, we next used FKBP51 KO mice and their WT littermates.

Global deletion of FKBP51 has no effect on naïve thresholds and the short term inflammatory response

We found no difference in the cutaneous mechanical sensory threshold (Fig. 3A) or thermal heat (Fig. 3B) and cold (Fig. 3C) thresholds between KO and WT animals, suggesting that acute nociception was not impaired by FKBP51 deletion. This conclusion was supported by the normal expression of primary afferent markers IB4 and CGRP in the superficial dorsal horn in these mice (Fig. 3D and Fig. S1). Moreover, there was no difference between the two genotypes in the nociceptive sensitivity that follows the hindpaw injection of short-term inflammatory agents such as formalin (Fig. 2E) or IL6 (Fig.2F) These data suggested that deletion of FKBP51 does not impair acute pain responses.

Global deletion of FKBP51 prevents the full development of long term pain states

We next tested the effect of FKBP51 KO on long term pain states. Although both KO and WT mice showed a significant reduction in mechanical threshold after CFA injection in the ankle joint, KO mice were less sensitive than WT mice (Fig.4A). However, there was no difference in CFA-induced spinal phosphorylated extracellular signal-regulated kinase (pErk) (Fig.4B and Fig.S2A) and cFos (Fig.4B and Fig.S2B). Similarly, hindpaw injection of CFA, which induces long-term inflammation, caused significantly less mechanical hypersensitivity in KO than in WT mice (Fig.4C), despite causing the same degree of edema in the hindpaw (Fig.4D). Finally, to test whether the modulatory role of FKBP51 on persistent pain states extended beyond long-term inflammation, we also investigated a model of neuropathic pain. KO mice with spared nerve injury (SNI)[18] showed less mechanical hypersensitivity than WT mice, starting at 5 days after surgery (Fig.4E). They also performed better on the rotarod test (Fig.4F), likely a result of reduced mechanical hyperalgesia, as well as increased ability to cope with stress [3, 4]. However, glial activation, which contributes to the hypersensitivity seen in neuropathic pain states[19], was not different in KO mice 14 days after SNI surgery than in WT mice (Fig. S3, A and B). Together, these results showed that FKBP51 KO mice display reduced mechanical hypersensitivity in long-term pain states, suggesting that FKBP51 plays a key role in the full development of persistent pain states. Because our original findings [10] suggested a role for spinal FKBP51 in pain processing, we next used siRNA to ask whether FKBP51 specifically expressed in the spinal cord contributed to nociceptive signaling.

Spinal FKBP51 drives long term pain states

First, we specifically deleted FKBP51 in the spinal cords of WT mice using intrathecal injections of siRNA. Injections of siRNA reduced FKBP51 expression in the spinal cord by 24% but had no effect on FKBP51 expression in the DRGs (Fig.S4A). This decrease in FKBP51 expression is similar to that reported by others when injecting siRNA intrathecally that has a significant effect on behavior[20]. This deletion is likely to have occurred in neurons exclusively because in the superficial dorsal horn we found that only neurons expressed FKBP51 (by using immunohistochemistry with tyramide

amplification). Although siRNA injections did not affect mechanical threshold and motor performance in naïve animals (Fig.S4B and S4C, respectively), siRNA injections starting 48h before the CFA injection significantly reduced the mechanical hypersensitivity induced by CFA-triggered ankle joint inflammation when compared to scramble siRNA injections (Fig.5A). More important, because it mimics the clinical situation, siRNA silencing of FKBP51 starting 3 days after injury, when the hypersensitivity was maximal, significantly attenuated the pain state (Fig.5B).

We then used the highly specific FKBP51 inhibitor SAFit2 *(11)* to block FKBP51 activity. Like anti-FKBP51 siRNA treatment, SAFit2 administered intrathecally improved the mechanical hypersensitivity induced by ankle joint inflammation when injected 3days after the CFA treatment (Fig.5C), suggesting that FKBP51 may be a promising therapeutic target for the treatment of chronic pain.

Together these results showed that the modulation of hypersensitivity by FKBP51 can be dissociated from its effect on mood: FKBP51 expressed in the spinal cord alone is sufficient to control the pain response. Moreover, spinal FKBP51 is not only sufficient for the full development of chronic pain but is also critical to the maintenance of this pain. We next explored the mechanism by which FKBP51 regulates nociceptive processing.

FKBP51 regulates injury induced hypersensitivity by modulating glucocorticoid signaling

FKBP51 is an antagonist of GR *(13)* and GR signaling regulates the hypersensitivity that develops after nerve injury *(15, 14)*. We therefore hypothesized that FKBP51 regulates the hypersensitivity that develops in long-term pain states by modulating glucocorticoid signaling. First, we studied the co-expression of FKBP51 and GR in WT superficial dorsal horn with immunohistochemistry. We found that colocalization of FKBP51 and GR was equivalent in tissue from both sham mice and mice injected with CFA in the ankle joint, with an average of 84 ± 1% of FKBP51 positive neurons expressing GR (85.5 ± 4%, CFA ipsilateral, 83.7 ± 1%, CFA contralateral and 82.1 ± 1%, sham) and 81 ± 1% of GR positive neurons expressing FKBP51 (78.7 ± 7%, CFA ipsilateral, 82.7 ± 2%, CFA

contralateral and 81.2 ± 4%, sham) (N=3 mice/group; 3 sections counted per mouse and 30 to 70 neurones counted per half section; Fig.6A).

GR has two main isoforms in both humans and rodents, GRα and GRβ, each associated with a distinct response to glucocorticoid hormones *(21)*. In the spinal cord, we found a small difference in total GR mRNA expression between FKBP51 KO and WT mice (Fig.6B). Nevertheless, CFA injection to the ankle joint did not induce any significant changes in GR mRNA levels in either genotype, and we found no difference in GR protein levels between KO and WT mice, in both naïve and CFA-injected animals (Fig. S5). There was also no difference in GRα mRNA levels between KO and WT and naïve and CFA-injected animals; however, both WT and KO mice showed increased GRβ levels on the side ipsilateral to the CFA injection compared to the contralateral side, and levels of GRβ were significantly lower in KO animals than in WT animals (Fig.6B). Increased GRβ levels have been associated with glucocorticoid resistance in inflammatory disorders *(22)*. Therefore our results suggest that in inflammatory pain states, KO mice are more sensitive to glucocorticoids than are WT mice. Supporting this interpretation, KO mice had lower corticosterone levels than WT mice, both in naïve and in persistent pain states (Fig.6C), indicating a stronger suppression of corticosterone secretion in *FKBP51*-deficient mice, as reported previously *(3)*.

Next, to test whether FKBP51 drives chronic pain by modifying GR signaling in the spinal cord, we used the GR antagonist mifepristone (RU38486). First, we injected mifepristone intrathecally in naïve animals. Both naïve WT and naïve KO mice showed increased mechanical hypersensitivity after mifepristone administration (Fig.6D), indicating that GR was anti-nociceptive under basal conditions, regardless of FKBP51 expression. However, when mifepristone was administered 3 days after CFA injection in the ankle joint, the GR antagonist eliminated the CFA-induced hypersensitivity in WT animals (Fig.6E and Fig.S6A for full time course of behavior), as seen in neuropathic pain models *(15, 16)*, but aggravated CFA-induced mechanical hypersensitivity in KO mice (Fig.6F and Fig.S6B for full time course of behavior). Mifepristone also increased the CFA-induced hypersensitivity in mice with reduced levels of FKBP51 only in the spinal cord (Fig.6G and Fig.S6C for full time course of behavior). This result suggested that after CFA injection, spinal cord GR signaling had switched to a pro-

nociceptive function in WT animals but that the deletion of spinal cord FKBP51 had prevented this switch and restricted GR to an anti-nociceptive function.

Finally, IL6 is a cytokine upregulated with neuroinflammation *(23, 24)*, and GR can directly induce or inhibit the transcription of IL6 by complex binding to the IL6 promoter regulatory elements *(25)*. We measured IL6 levels in the spinal cord of WT and KO mice and found that IL6 was upregulated in the ipsilateral superficial dorsal horn in WT mice after CFA injection (Fig.6H), supporting the idea that GR could be promoting pro-inflammatory cytokine expression in WT animals after CFA treatment. However, IL6 levels were significantly lower in the spinal cord of the KO mice on the side ipsilateral to the CFA injection when compared both to WT animals and to the KO contralateral side, suggesting that GR could be suppressing IL6 transcription on the ipsilateral side in KO mice after CFA injection (Fig.6H).

Altogether, our data indicate that spinal FKBP51 regulates the full development and maintenance of chronic pain by modulating glucocorticoid signaling. We propose that inhibition of FKBP51 function by deletion or antagonism allows spinal cord GR signaling to perform an anti-nociceptive function in long-term pain states. As a result, CFA-induced hypersensitivity never develops to its full extent in FKBP51 KO animals and resolves after deletion or inhibition of spinal cord FKBP51 in WT mice.

DISCUSSION

This study identifies the stress modulator FKBP51 as a regulator of injury-induced hypersensitivity. Our experiments show that in mice deletion of FKBP51 did not compromise the detection of acute noxious stimuli but did attenuate the full development and the maintenance of mechanical hypersensitivity, in our animal models of chronic pain states. This was true whether FKBP51 was deleted before the pain-causing injury or silenced after the long-term pain state had been established. Moreover, FKBP51 regulation of pain states could be dissociated from its regulation of mood since deletion of FKBP51 or inhibition with the state of the art inhibitor SAFit2*(11)* that was restricted to the spinal cord improved the CFA-induced hypersensitivity. As a result, we propose that

FKBP51 is a potential therapeutic target for the control of chronic pain with promising clinical applications. Chronic pain is an area of immense unmet medical need that requires new analgesic drugs.

FKBP51 regulates GR signaling, and GR mediates the action of steroid hormones required for physiological homeostasis. Prior to hormone binding, GR is part of a large protein complex localised to the cytoplasm where it is bound to heat-shock protein 90 (Hsp90), which itself recruits to its COOH terminus a co-chaperone such as FKBP51 or FKBP52. Although FKBP51 likely does not directly bind GR, its presence in the protein complex induces a conformational change of the ligand-binding pocket that reduces GR hormone binding affinity *(26, 27)*. FKBP51 also prevents the nuclear translocation of the GR complex that occurs when Hsp90 binds FKBP52 *(28)*. After steroid hormone binding to GR, GR translocates to the nucleus where it serves as a regulator of differential gene expression. Among other targets, GR can upregulate FKBP51, thereby providing an inhibitory feedback mechanism *(29, 30)*. However, other factors contribute to the upregulation of FKBP51 in the spinal cord, such as neuronal activity--induced relief of MeCP2 repression [observed both in rats *(10)* and mice *(31, 32)*] and the decrease in FKBP51 DNA methylation reported here and also seen in mice after increased cortisol concentrations *(17, 33)*.

Intact GR signaling is known to be critical to the full development of pain states. Indeed, both adrenalectomy and GR knock down in the spinal cord by intrathecal administration of siRNA prevent the full development of mechanical hypersensitivity *(15)*. Moreover, the GR agonist dexamethasone can unmask the hypersensitive state in injured adrenalectomized animals *(15)*. Here, we show that deletion of FKBP51 modulates GR signaling. Indeed, KO mice had lower blood glucocorticoid concentrations than did WT animals, as well as lower levels of GRβ, the GR isoform upregulated with glucocorticoid resistance *(22)*, suggesting that FKBP51 KO mice are more sensitive to glucocorticoids that are WT mice. We propose that the up-regulation of FKBP51 after CFA-treatment, together with the subsequent binding of FKBP51 to the GR complex, could be a trigger that switches GR from an

anti- to a pro- nociceptive role. Our data obtained with the GR antagonist mifepristone indicate that GR signaling was anti-nociceptive in naïve WT mice but became pro-nociceptive after CFA injection and pain state development (Fig.6D and E). We were confident that mifepristone acted as an inhibitor of GR since others had reported that injury induced hypersensitivity could be reduced by both mifepristone and another more specific GR antagonist, dexamethasone 21-mesylate, in mice(16). Our result indicating a change in GR activity is consistent with recent data suggesting that GR can switch from an anti-inflammatory state under naïve conditions to a pro-inflammatory state after exposure to stressors (34). In contrast, after FKBP51 deletion, GR signaling induced a constant anti-nociceptive state (Fig. 5D, F and G). The rapid effects of the GR antagonist mifepristone reported here are most likely to reflect non-genomic activity of GR, such as modulation of the MAPK and PI3K signal transduction pathways. However, GR can act both genomically (up-regulation and inhibition of gene expression) and non-genomicallyafter activation by glucocorticoids and/or post translational modification such as phosphorylation (35). We hypothesised that for optimal efficiency, non-genomic and genomic actions of GR worked in concert towards pro- or anti-nociceptive signaling at a given time point. That this is the case is supported by our finding that the increase in expression of the pro-inflammatory cytokine IL6 (24), observed in the ipsilateral dorsal horn of WT animals after CFA treatment, did not occur in KO mice. GR is able to bind to the IL6 promoter sequence in rodent neurones and the GR agonist dexamethasone can induce IL6 transcription in these neurones (36). However, GRis known to both promote and inhibit the expression of IL6 (34, 25). Gene regulation by GR is indeed highly complex and depends on a number of factors including the tissue, the concentration of circulating glucocorticoids and GR post-translational modifications.

We also report here that levels of DNA methylation within the *FKBP51* DNA promoter sequence in the rat spinal cord are significantly lower 7 days after CFA injection. However, we have previously shown that there were no significant changes in FKBP51 mRNA expression at this time point (9). Thus, changes in DNA methylation are not sufficient to change FKBP51 expression in spinal cord but need to be combined with other events, such as relief of MeCP2 repression, as we had previously

reported *(10)*. . Others have shown that stress in early life can induce long term changes in DNA methylation in the FKBP51 sequence. These modifications lead to a much greater induction of FKBP51 when further stress is encountered later on in life) *(2)*. Together with our report of injury induced long lasting changes in DNA methylation in the FKBP51 promoter, this offers the interesting perspective that FKBP51 might contribute to the excessive hypersensitivity that develops after repetitive injuries, both in rodents and humans *(37–39)*.

In conclusion, this study identifies the stress regulator FKBP51 as a key modulator of long-term pain states. Our finding that the deletion or inhibition of spinal cord FKBP51 does not compromise acute pain detection but reduces hypersensitivity in mice with established inflammatory pain points to FKBP51 as a possible new target for the treatment of long term pain states. FKBP51 inhibition is known to successfully treat mood disorders, but the development of FKBP51 antagonists has been challenging *(40)*. New and highly specific inhibitors for FKBP51 developed very recently, such as SAFit2 *(11)*, can reduce signs of depression in mice in vivo *(11)* and, as we have shown here, inhibit established injury-associated mechanical hypersensitivity in mice. The lack of FKBP51 does not seem to induce any deleterious side effects; *(5)* therefore, FKBP51 may be a suitable target for the long term treatment that would be required for chronic pain.

MATERIAL AND METHODS

Study design

This study represents a series of experiments using multiple molecular and behavioural techniques in rodents. In pharmacological studies, mice were randomly assigned to experimental groups. The experimenter was always blind to genotype and treatment. While the effect size of genotype and drug effects were not predictable, sample sizes in this study are consistent with norm in the field*(41)*.

Animals

Subjects in all experiments, except DNA methylation studies, were adult male mice (6-12 weeks old). WT mice in all siRNA experiments were C57Bl/6J from Harlan Laboratories. In the remaining experiments, mice were FKBP51 KO and their WT littermates obtained from FKBP51 heterozygous from C.A. Dickey's group (University of South Florida, USA). These mice were from mixed genetic background, C57Bl/6J and Swiss Webster. In the DNA methylation studies, adult rats weighing 200-250g and obtained from UCL animal house were used. All animals were kept in their home cages in a temperature-controlled (20 ±1 ºC) environment, with a light-dark cycle of 12h (light on at 7:30 a.m.) food and water were provided *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animal used (UK Animal Act, 1986).

Genotyping

For genotyping, DNA was extracted from a small portion of ear tissue and the following primers were used for the PCR: forward primer (for WT and 51KO): AAAGGACAATGACTACTGATGAGG; reverse WT primer: AAGGAGGGGTTCTTTTGAGG; reverse 51KO primer: GTTGCACCACAGATGAAACG. Samples from WT animals showed a single PCR product of 363bp; samples from KO animals showed a single PCR product of 510bp and samples from HET animals would present both bands.

Pain models

*Inflammatory models (*Sham treatment consisted of anesthetizing the animals)

CFA induced ankle joint inflammation

Inflammation was induced by injection of Complete Freud's Adjuvant (CFA; Sigma, UK) at the volume of 5 µl (mice) or 10 µl (rats), in the left ankle joint, under isoflurane anesthesia as described *(10)*

Hindpaw inflammation

Formalin (20 µl, 5%), IL6 (20 µl) and CFA (20 µl) were injected with a micro syringe with a 27-gauge needle subcutaneously into the plantar surface of the left hind paw of mice. Mice were maintained under isoflurane anaesthesia during the injection except for formalin injections.

Neuropathic model: spared nerve injury

The spared nerve injury (SNI) was performed as described *(18)*.

Behavioral testing

The experimenter was always blind to genotype and treatment.

Von Frey's hairs

Animals were placed in Plexiglas chambers, located on an elevated wire grid, and allowed to habituate for at least 1 hour. After this time, the plantar surface of the paw was stimulated with a series of ascending forces Von Frey's monofilaments. The threshold was determined by using the up-down method as described by Chaplan and colleagues *(42)*. The data were expressed as log of the mean of the 50% pain threshold ± SEM.

Hargreaves test

The latency of foot withdrawal to noxious heat stimuli was measured as described *(43)*.

Formalin behavior

Mice were allowed to habituate to a Plexiglas chamber for at least 30 minutes. Behavioral testing was initiated immediately after formalin injection and lasted 60 min. The incidences of licking and biting were counted at intervals of 5 min.

Rotarod test

Motor performance was evaluated by an accelerating rotarod apparatus with a 3 cm diameter rod starting at an initial rotation of 4 RPM and slowly accelerating to 40 RPM over 100 seconds. Mice were expected to walk at the speed of rod rotation to keep from falling. The time spent on the rod during each of three trials per day was measured and expressed in seconds. Testing was completed when the mouse fell off the rod (*i.e.* from a height of 12 cm).

Intrathecal injections and drugs

The intrathecal injections were performed as described *(44)*. All intrathecally delivered drugs were injected in a 2 µl volume. Mifepristone (1 nmol in 2 µl) was purchased from Sigma and was dissolved in 10% ethanol in saline. Control animals received 2 µl of 10% ethanol in saline. SAFit2 was obtained

from Felix Hausch (MPI) and used at a concentration of 2mg/ml (4 µg in 2 µl), in 4% Ethanol, 5% PEG400, 5% TWEEN 80 in 0.9% saline. Control animals received 2 µl of 4% Ethanol, 5% PEG400, 5% TWEEN 80 in 0.9% saline.

siRNA

In vivo silencing of the protein FKBP51 was achieved with a mouse-specific small interference RNA (siRNA; Thermo Scientific; SMARTpool E-040262-00-0020). The siRNA was reconstituted in RNase-free H_2O to a final concentration of 0.5 or 2 µg/µl. Intrathecal injection of the siRNA solution (2 µl/mouse) was performed, as described above, for three consecutive days according to the experimental protocol. Control treated animals received 2 µl of Accell Non-targeting Pool (D-001910-10-20) prepared in the same concentrations as the targeting siRNA. Intrathecally delivered siRNA reached the spinal cord (Fig. S7) and the siRNA treatment was optimized to 1 daily injection on 3 consecutive days of 4 µg siRNA.

Blood sampling and corticosterone assay

Blood was collected in the morning between 9:00 to 11:00 from mice tail vein. Before sampling, mice were placed in a warming cabinet (39°C for 10 to 15 min) in order to dilate the blood vessel. The levels of corticosterone were measured using an ELISA kit (ab108821, Abcam) following the manufacturer protocol.

RNA preparation and RT-qPCR

For fresh tissue collection, animals were terminally anesthetized with CO_2 3 d after CFA or sham surgery. Total RNA was extracted using an acid phenol extraction method (TRIzol reagent, RNeasy mini-colums; Quiagen) and cDNA obtained as described *(10)*. Reactions were performed at least in triplicate and the specificity of the products was determined by melting curve analysis. The ratio of the relative expression of target genes to β-actin was calculated by using the $2^{\Delta Ct}$ formula. Efficiencies of qPCRs were calculated for each gene using serial dilution.

DNA methylation analysis

DNA extraction and bisulphite treatment: DNA was isolated from dorsal horn quadrants of rat spinal cord (see Fresh tissue collection) using DNeasy Blood and Tissue kit (Qiagen). Bisulphite conversion of 100ng of genomic DNA was achieved through use of the Epitect bisulfite kit (Qiagen), according to the manufacturer's instructions.

PCR and Pyrosequencing: Details of PCR and pyrosequencing can be found in Supplementary Material.

Immunohistochemistry and western blot.

Immunohistochemistry and western blot were performed as described *(10)*. For details see Supplementary Material.

Data analysis

All statistical tests were performed in IBM SPSS statistic 20. For the behavioral experiments, statistical analysis was performed on the data normalized by log transformation (Von Frey data; as suggested by Mills et al. *(45)*) or raw data, as presented in the figures. Behavioural data were also presented and analysed without log transformation and statistically significant effects for all groups were also observed with data presented as Von Frey force in grams (Fig.S8). The significance of any differences in sensitivity was assessed using repeated-measured two-way or one-way ANOVA, as appropriate. In all cases, a significant effect of the main factor(s), or interactions between them, was taken as the criterion for progressing to post hoc analysis. Bonferroni analysis was the preferred post-hoc analysis; however, if the general ANOVA was significant but no Bonferroni significance observed, we also reported the results of the LSD post-hoc analysis. In all cases 'time' was treated as a within subjects factor and 'genotype' and 'treatment' were treated as between subject factors. For the RTqPCR experiments, data were analysed by univariate analysis for individual genes followed by the appropriate post-hoc analysis (as above). Biological samples for the DNA methylation

experiments (n=6 in each group) and the measures of glucocorticoid levels (n=4 in each group) were analysed twice and data of the technical replicates were examined by nested ANOVA.

LIST OF SUPPLEMENTARY MATERIALS

Table 1: All data values presented in the manuscript in tabular format.

Figure S1: Global deletion of FKBP51 does not affect the expression of CGRP and IB4 in the superficial dorsal horn.

Figure S2: Global deletion of FKBP51 does not affect CFA-induced cFos or pERK protein levels in the superficial dorsal horn.

Figure S3: Global deletion of FKBP51 does not affect SNI-induced Iba1 expression in the superficial dorsal horn.

Figure S4: Reduction of spinal FKBP51 expression with siRNA has no effect on basal mechanical threshold and locomotor activity.

Figure S5: Global deletion of FKBP51 does not affect GR protein expression in naïve and injured WT and KO mice.

Figure S6: FKBP51 regulates long term pain states by modulating glucocorticoid signaling (full time course).

Figure S7: GFP labelled anti-cyclophilin siRNA spinal delivery.

Figure S8: Behavioural data presented as Von Frey force in grams.

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the mice, Paul Guilhamon for his help with the DNA methylation experiment and the laboratories of Chad Dickey and Mathias Schmidt for their help with the breeding of the FKBP51 KO colony. FKBP51 KO mice were obtained from FKBP51 heterozygous from Chad Dickey's colony.

Author contributions: SMG and MM designed the experiments, analyzed the data and wrote the manuscript. MM conducted all the behavioral experiments. MM and SMG conducted the RTqPCR and the glucocorticoid experiments. SG, MM and KKT conducted the immunohistochemical experiments. SMG and LVA conducted the DNA methylation studies. CGB designed the primers for the DNA methylation study. MBC provided the FKBP5 KO mice. FX and FH provided SAFit2.

Competing interests: The authors declare that they have no competing interests.
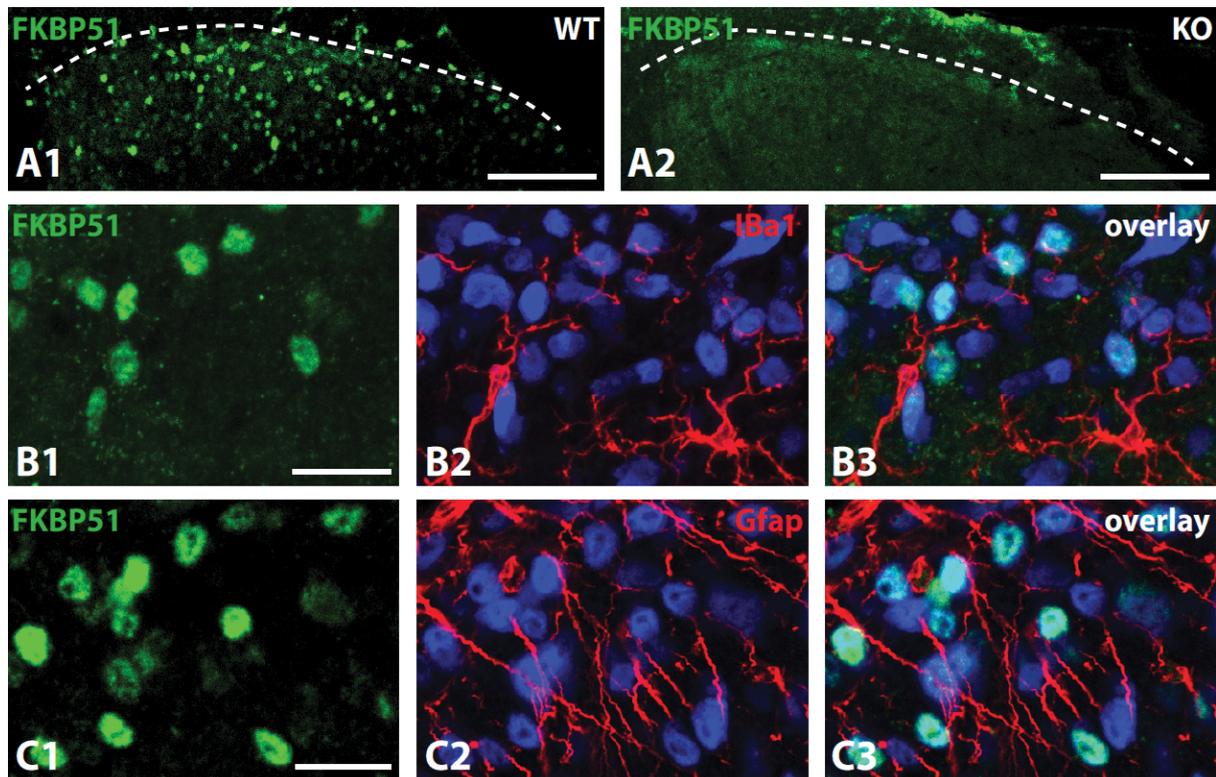
Fig. 1: FKBP51 is expressed exclusively in neurons in mouse dorsal horn. (A) Typical images of FKBP51 immunoreactivity in the superficial dorsal horn of WT and KO animals. Dotted line indicates the boundary of the superficial dorsal horn. Scale bar, 100μm. **(B)** Ipsilateral dorsal horn obtained 3d after CFA injection in the ankle joint. FKBP51 expression was never seen in microglia. Green, FKBP51; red: Iba1, a marker of microglia; blue, NeuN, a marker of neurons. Scale bar, 20μm. **(C)** Ipsilateral dorsal horn obtained 3d after CFA injection in the ankle joint. FKBP51 expression was never seen in astrocytes. Green, FKBP51; red, GFAP, a marker of astrocytes; blue, NeuN, a marker of neurons. Scale bar, 20μm.
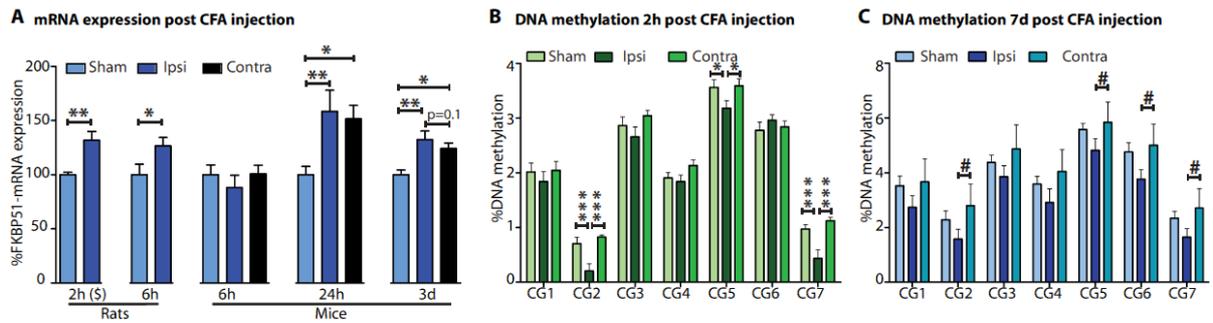
Fig. 2. After noxious stimulation, FKBP51 expression increased and *FKBP51* DNA methylation decreased. (A) FKBP51 mRNA expression (normalized to sham) in the rat dorsal horn 2h and 6h after CFA injection in the ankle joint and in the mouse ipsilateral and contralateral dorsal horn 24h and 3 days after CFA. Rat data: Student's t-test: 2h: P=0.007; 6h: P=0.033; N=4-5/group. Mouse data 1-way ANOVA, factor TREATMENT: 24h: $F_{(2,19)}$=7.73; P=0.004; Bonferroni post-hoc analysis: sham vs ipsi: P=0.009; sham vs contra: P=0.023; 3 d: $F_{(2,23)}$=9.7, P=0.001; Bonferroni post-hoc analysis: sham vs ipsi: P=0.002; sham vs contra: P=0.021; N=5-7/group. *P<0.05, **P<0.01 Bonferroni post hoc analysis. **$** data previously published *(10)*. **(B and C)** DNA methylation at CpG sites (CG1-7) in the promoter sequence of FKBP51 in spinal dorsal horn tissue, 2 h and 7 days after CFA injection in the ankle joint. Data represent mean percentage of DNA methylation at specific CpGs ± SEM. NESTED ANOVA (B) factor TREATMENT: CG2: $F_{(2,3)}$=142.1, P=0.001; Bonferroni post hoc analysis: ipsi vs sham: P<0.001, ipsi vs contra: P<0.001; CG5: $F_{(2,3)}$=59.1, P=0.004; Bonferroni post hoc analysis: ipsi vs sham: P=0.034, ipsi vs contra: P=0.021; CG7: $F_{(2,3)}$=370.9, P<0.001; Bonferroni post hoc analysis: ipsi vs sham: P<0.001, ipsi vs contra: P≤0.001; (N=6/group). (C) factor TREATMENT: CG2: $F_{(2,3)}$=154.9, P<0.001; Least Significant Difference (LSD) post hoc analysis: ipsi vs contra: P=0.031; CG5: $F_{(2,3)}$=19.4, P=0.016; LSD post hoc analysis: ipsi vs contra: P=0.050; CG6: $F_{(2,3)}$=107.4, P<0.001; LSD post hoc analysis: ipsi vs contra: P=0.026; CG7: $F_{(2,3)}$=85, P=0.001; LSD post hoc analysis: ipsi vs contra: P=0.031 (N=6/group). * and #: P≤0.05; ***P<0.001; * Bonferroni and # Least Significant Difference (LSD) post-hoc analysis.
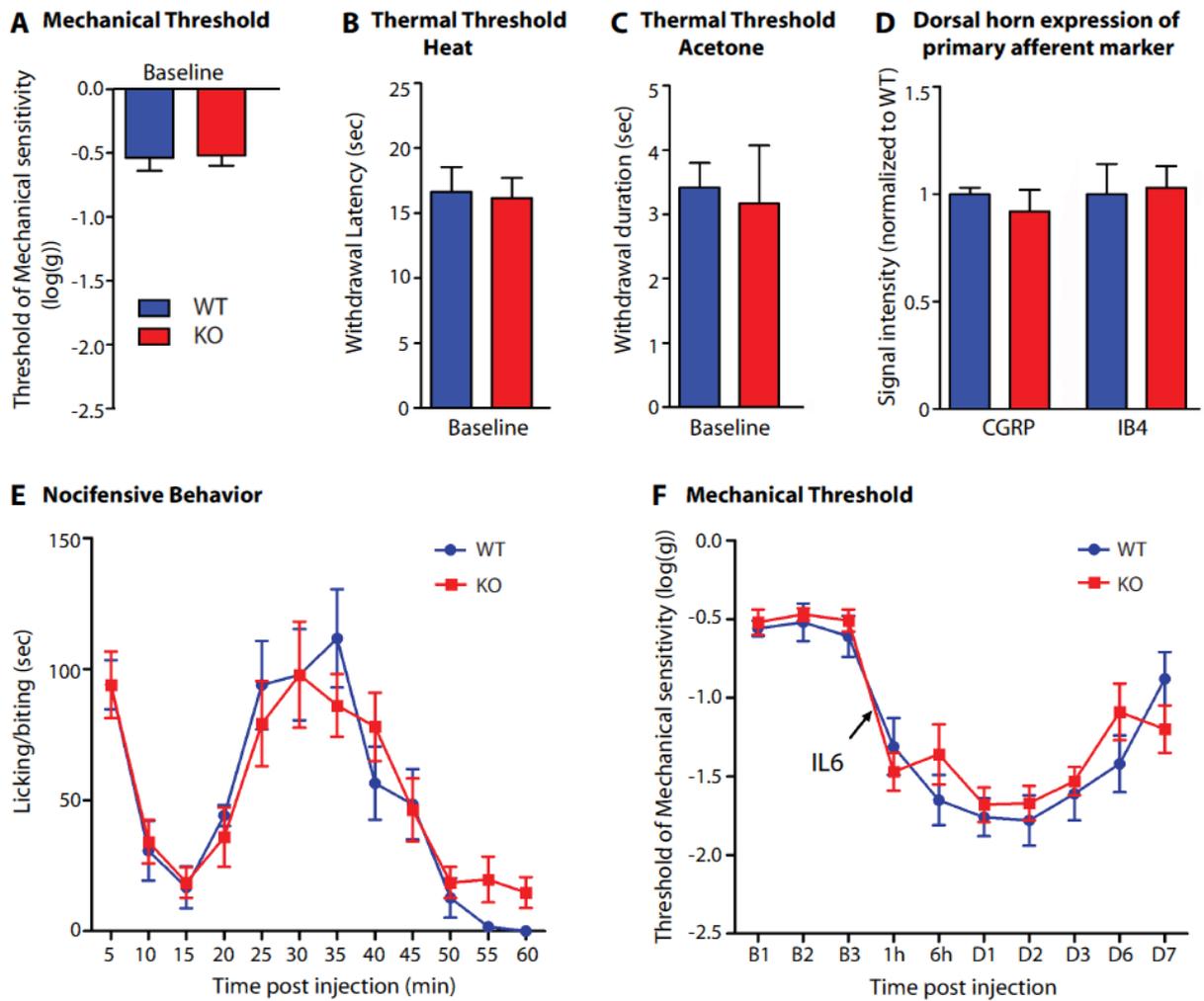
Fig. 3. Global deletion of FKBP51 has no influence on naïve thresholds or the response to short term inflammatory agents. (A-C) There was no difference in mechanical threshold (A), thermal heat threshold (B) and thermal cold threshold (C) between naïve KO and WT mice (N=8/group). **(D)** Measure of the immunohistochemical CGRP and IB4 signal intensity in tissue from untreated KO and WT mice. Signal intensity was normalized to WT expression levels. (N=3/group) **(E and F)** WT and KO mice showed equivalent **(E)** nociceptive behavior after intraplantar injection of formalin (N=7/group) and **(F)** mechanical hypersensitivity after intraplantar injection of IL6 (N=6-7/group). Data show mean ± SEM. B1, B2 and B3: baseline data taken on separate days.
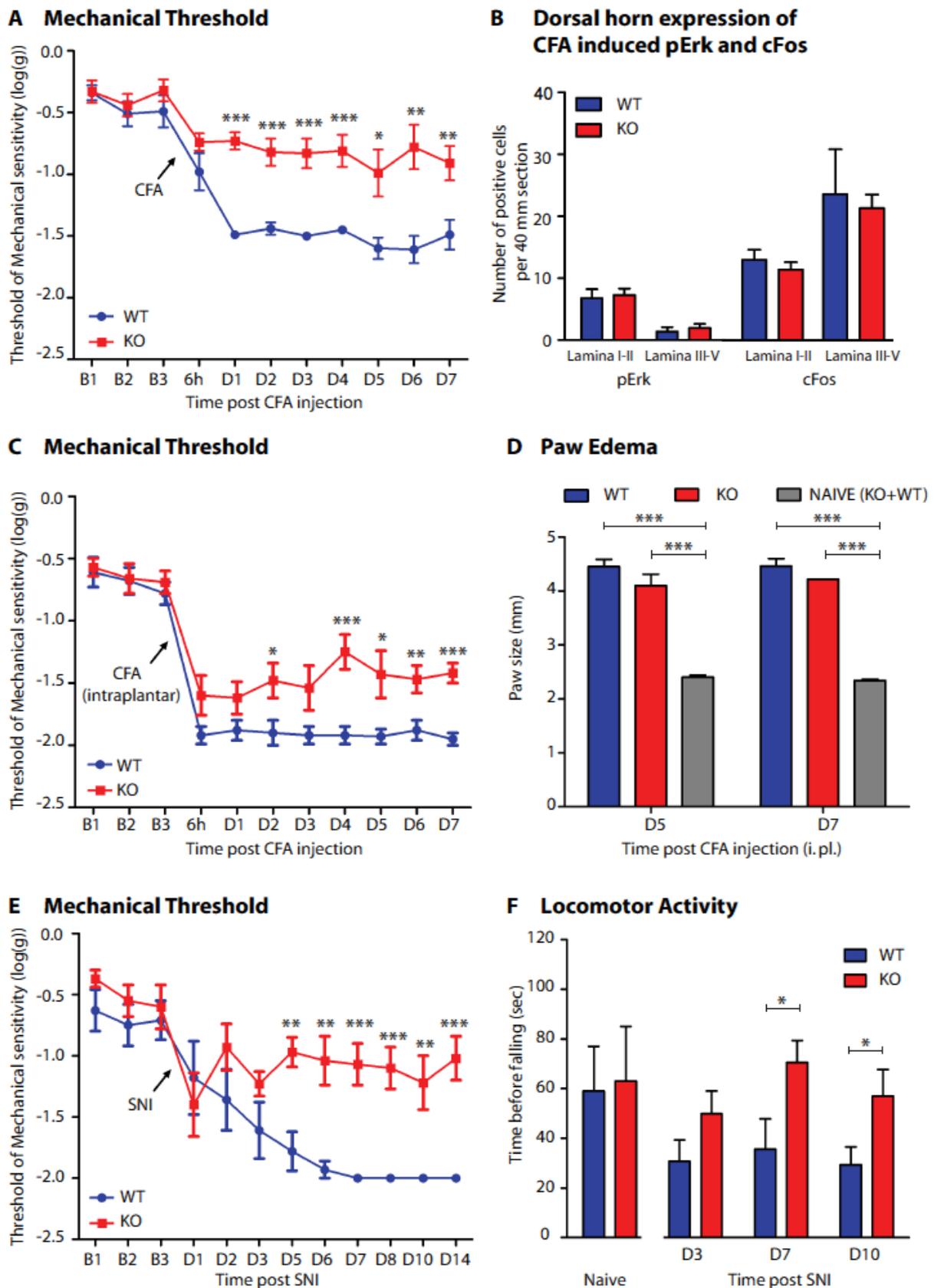
Fig. 4. Global deletion of FKBP51 reduces the mechanical hypersensitivity that develops in long term pain states. (A) Mechanical sensitivity in KO and WT mice after injection of CFA in the ankle joint (N=8/group): 2-way ANOVA, factor GENOTYPE D1 to D7: $F_{(1,13)}$=30.9; P<0.0001. KO vs WT: D1,

P<0.001 ; D2, P<0.001 , D3, P<0.001 , D4, P<0.001 , D5, P=0.012 , D6, P=0.002 , D7, P=0.01 .Although the sensitivity induced by CFA was reduced in KO compared to WT mice, it was nonetheless significantly different from baseline values (one way ANOVA factor TIME B3 to D4: $F_{(5,35)}$=3.7; P=0.008) **(B)** Number of pERK and cFos positive cellsin the superficial dorsal horn after CFA injection in the ankle joint, at 10 min and 2 h after injection respectively. Cells were counted on 40 µm sections stained by immunohistochemistry for pERK and cFOS.. **(C)** Mechanical sensitivity in KO and WT mice after intraplantar injection of CFA (N=8/group, 2-way ANOVA, factor GENOTYPE D2 to D7: $F_{(1,14)}$=20.3, P<0.0001; KO vs WT: D2, P= 0.024, D4, P= 0.001, D5, P= 0.022, D6, P= 0.010, D7, P<0.001.). Although the sensitivity induced by CFA was reduced in KO compared to WT mice, it was nonetheless significantly different from baseline values (one way ANOVA factor TIME B3 to D7 $F_{(8,56)}$=5.23 P<0.0001). **(D)** Paw edema after intraplantar CFA--induced inflammation in KO, WT and naïve mice (N=8/group, ANOVA TREATMENT $F_{(1,21)}$=70.8; P<0.0001; D5, D7: Naïve vs WT: P<0.001 ; D5, D7: Naïve vs KO: P<0.001). **(E)** Mechanical sensitivity in KO and WT mice after SNI (2-way ANOVA, factor GENOTYPE: D5 to D14 $F_{(1,13)}$=31.6; P<0.0001; KO vs WT: D5, P=0.001 ; D6, P=0.01, D7, P<0.001, D8, P<0.001, D10, P=0.005; D14, P<0.001. (N=7-8/group). Although the sensitivity induced by CFA was reduced in KO compared to WT mice, it was nonetheless significantly different from baseline values. (one way ANOVA factor TIME B3 to D1: $F_{(1,6)}$=8.1; P=0.029). **(F)** Time on the rotarod apparatus after SNI surgery in KO and WT mice (N=8-9/group; Student's t-test; D7 KO vs WT: P=0.030; D10 KO vs WT P=0.020). Animals were not tested at baseline to minimise the number of tests on the rotarod. Data show mean ± SEM. *P<0.05, **P≤0.01, ***P≤0.001, WT *vs* KO in A, C, E and F and KO or WT CFA *vs* naïve in D. Injection or surgery time indicated by arrows.
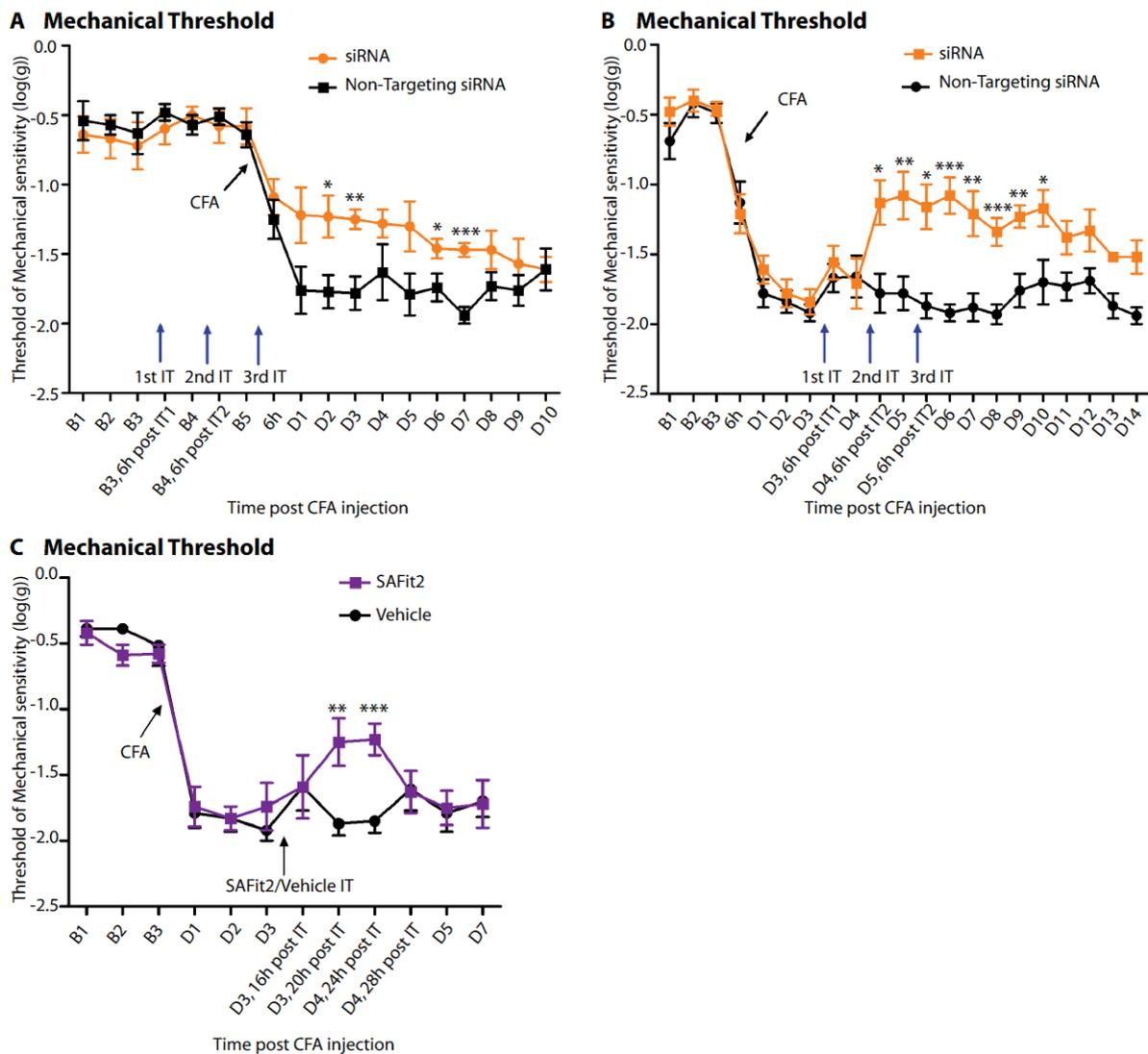
Fig. 5. Spinal FKBP51 not only contributes to the full development of CFA induced hypersensitivity but is also critical to the maintenance of established pain states. (A and B) Effect of local silencing of FKBP51 in the lumbar spinal cord, before (A) and after (B) CFA injection in the ankle joint, on CFA-induced mechanical hypersensitivity. (N=7-8/group). (A) 2-way ANOVA, factor TREATMENT D1 to D8 $F_{(1,14)}$=13.4; P=0.003; KO vs WT D1, P= 0.056; D2, P=0.013; D3, P=0.002; D5, P=0.057; D6, P=0.037; D7, P<0.001. (B) 2-way ANOVA, factor TREATMENT D4, 6h post IT2 to D14 $F_{(1,13)}$=50.6; P<0.0001. KO vs WT D4 6h post IT2, P=0.025; D5, P=0.002; D5 6h post IT3, P=0.011; D6, P=0.001; D7, P=0.005; D8, P<0.001; D9, P=0.006; D10, P=0.023. **(C)** Effect of intrathecal administration of SAFit2 3days after injection of CFA in the ankle joint on mechanical hypersensitivity. 2-way ANOVA, factor TREATMENT 20h to 24h $F_{(1,14)}$=16.042; P=0.001. KO vs WT D3, 20h post IT P=0.007, KO vs WT D4, 24h post IT

P=0.001 .(N=8/group). IT, intrathecal injection. Data show mean ± SEM. *P<0.05, **P≤0.01, ***P≤0.001, WT *vs* KO.
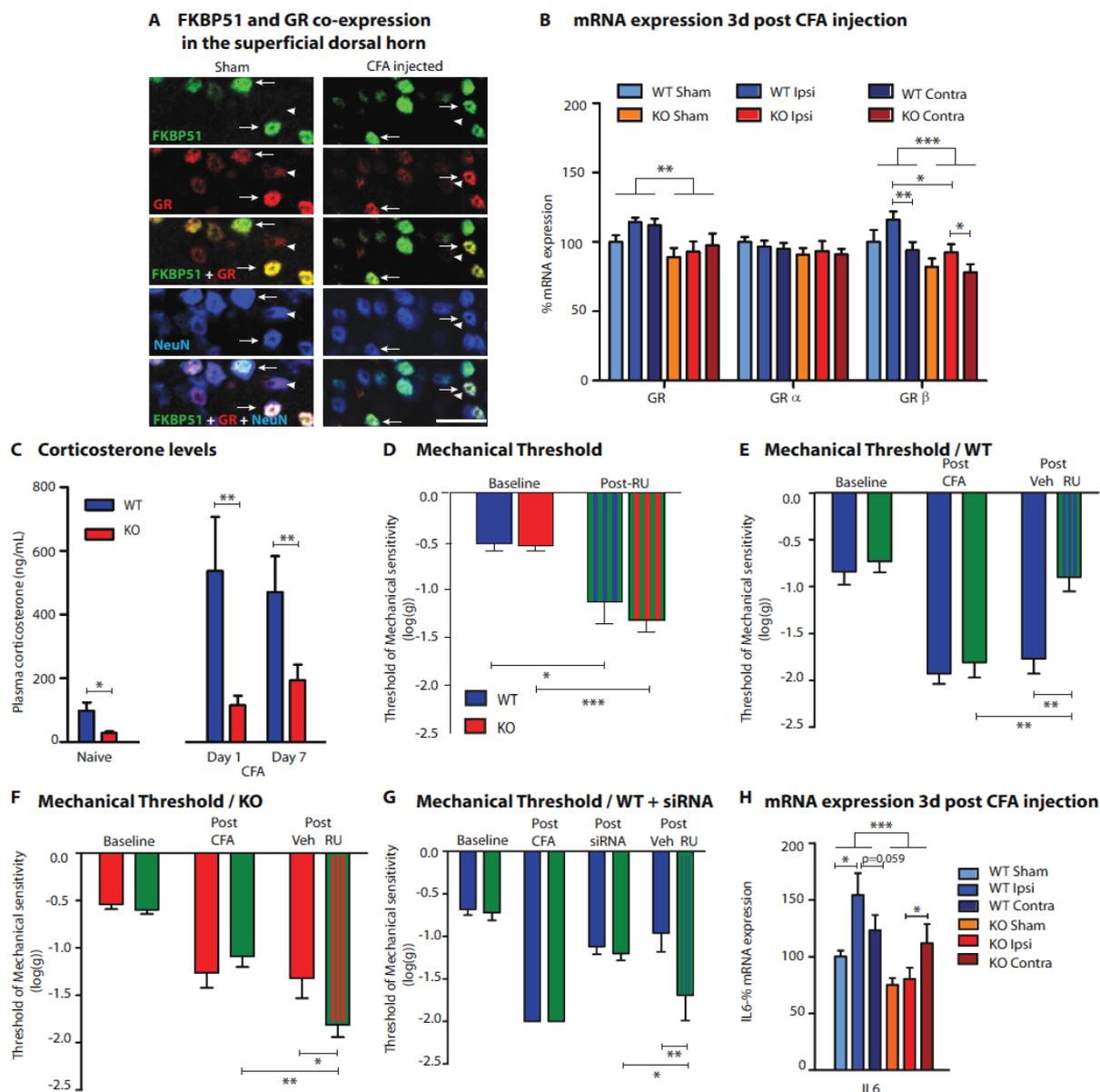

Fig. 6. FKBP51 regulates long term pain states by modulating glucocorticoid signaling. (A) Typical images of FKBP51 and GR immunoreactivity in the superficial dorsal horn of WT sham mice and mice after CFA injection in the ankle joint. FKBP51, green; GR: red; NeuN: blue. Arrows, neurons expressing both FKBP51 and GR. Arrowheads, GR positive neurons that do not express FKBP51. Scale bar, 20 μm. **(B)** RT-qPCR analysis of GR expression in the dorsal horn 3 d after CFA injection in the ankle joint; data normalized to WT sham. For GR data: 2-way ANOVA, factor GENOTYPE $F_{(1,66)}$=9.84,

P=0.003. For GRβ data: 2-way ANOVA, factor GENOTYPE $F_{(1,66)}$=12.94; P=0.001; TREATMENT $F_{(2,66)}$=4.07; P=0.0022. Post hoc analysis: univariate analysis, ipsi KO vs ipsi WT: $F_{(1,21)}$=7.72, P=0.011; paired Student's t-test: WT ipsi vs WT contra, P=0.001; KO ipsi vs KO contra, P=0.029. (N=11-12/group). **(C)** Concentration of plasma corticosterone in KO and WT mice before and 1 and 7 days after CFA injection in the ankle joint (N=4/group). CFA injected animals were not sampled at baseline to minimise the number of blood withdrawals. Statistics show the results of a nested ANOVA. Factor GENOTYPE Naïve $F_{(1,2)}$=75.6, P=0.013; Day 1: $F_{(1,2)}$=45.5, P=0.001; Day 7, $F_{(1,2)}$=18.5, P=0.005. **(D)** Effect of mifepristone [RU38486 (RU)] administered to naïve WT and KO mice on the mechanical threshold. Data collected 1h after RU38486. (N=4/group) *P<0.05, ***P<0.001, results of paired Student's t-test. KO, baseline vs post-RU38486: P= 0.007; WT, baseline vs post-RU38486: P=0.048. **(E)** RU38486 (Mifepristone, GR antagonist) IT effect on CFA-induced mechanical hypersensitivity in WT mice (N=4-5/group) 2-way ANOVA, interactions TIME x TREATMENT: Post-CFA − Post-Veh/RU38486, $F_{(1,7)}$=11.00; P=0.013. Data collected 3d after CFA and 1h after RU38486 administration. Post RU38486 vs post vehicle: P=0.010, Post CFA vs post RU38486: P=0.0015. **(F)** RU38486 IT effect on CFA-induced mechanical hypersensitivity in KO mice (N=6-8/group) 2-way ANOVA, interactions TIME x TREATMENT Post-CFA − Post-Veh/RU38486, $F_{(1,11)}$=12.9; P=0.004. Post RU38486 vs post vehicle: P=0.043, Post CFA vs post RU38486: P=0.0055. Data collected 3d after CFA and 1h after RU38486. **(G)** RU38486 IT effect on CFA-induced mechanical hypersensitivity in mice that had received two injections of anti-FKBP51 siRNA. (N=5-6/group). 2-way ANOVA factor TREATMENT post siRNA vs post RU38486 $F_{(1,9)}$=5.63, P=0.043; Post siRNA vs post RU38486, P=0.050. Post RU vs post vehicle: P=0.005. Data collected 5d after CFA, 48h after 1st siRNA and 1h after RU38486. **(H)** RT-qPCR analysis of IL6 expression in the dorsal horn 3 d after CFA injection in the ankle joint; data normalized to WT sham. 2-way ANOVA, factor GENOTYPE $F_{(1,65)}$=19.01, P<0.0001; factor TREATMENT $F_{(2,65)}$=3.72, P=0.030. Post-hoc analysis for WT animals: factor TREATMENT: $F_{(2,34)}$=4.19, P=0.024. Bonferroni post hoc analysis WT ipsi vs WT sham, P=0.020; paired Student's t-test: WT ipsi vs WT contra P=0.059; KO ipsi vs KO contra P=0.020. (N=11-12/group). Data show mean

± SEM. *P<0.05, **P≤0.01, ***P≤0.001. (**E, F** and **G**: *P≤0.05, ** P≤0.01, results of univariate analysis or paired analysis as relevant).