# Restraint stress exacerbates inflammation-induced facial and hind-limb hypersensitivity

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## Background

Stressful life events are known to exacerbate pain states, but the mechanisms linking pain with stress remain inadequately understood. Orofacial pain states are greatly affected by stress and are thus of particular interest when studying the link between stress and pain.

Recent research has shown that three short periods of restraint stress induces facial mechanical hypersensitivity in rodents[1]. Furthermore, restraint induces facial[1] and hind-limb[2] hyperalgesic priming; a long-lasting latent state of hyper-responsiveness of nociceptors.

The mechanisms that underlie the interactions between stress and pain are not fully understood. However, we believe that the common regulator of stress[3] (Fig1) and pain[4,5], FK 506 binding protein 51 (FKBP51: encoded by the gene *FKBP5)* is likely to contribute to these interactions.

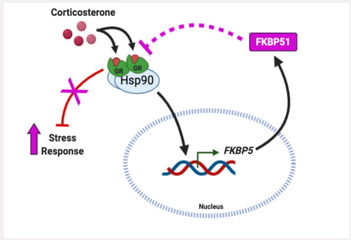



**Fig 1. FKBP51 is upregulated after stress, then modulates the binding of stress hormones with GR, prolonging the stress response**

## Materials and methods

**Restraint stress model:** The sub-chronic restraint stress paradigm involved restraint for 1 hour a day for three consecutive days.

**Pain models:** To induce an orofacial pain state the inflammatory agent Complete Freund's Adjuvant (CFA) was injected into the whisker pad. Hind-limb inflammation-induced hypersensitivity was induced by intra-plantar injection of CFA.

**Behavioual assessment:** Mechanical sensitivity was assessed using Von Frey filaments applied to the forehead, and/or to the hind-paw.

**Drug treatment:** Selective FKBP51 inhibitor, SAFit2 was given subcutaneously at 100mg/kg in a slow-release gel formulation 2 days prior to the onset of restraint. Injection at this time point provides inhibition for the duration of the restraint period.

**Tissue analysis:** Spinal cords of experimental mice were collected to investigate molecular pathways using RT-qPCR, and neuronal activation using IHC. Blood plasma corticosterone levels were measured using an ELISA.

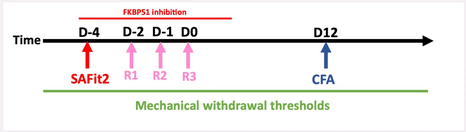



**Fig 2. Schematic of experimental design**

## Hypothesis and Aims

**Hypothesis:**

- Restraint stress induces facial and hind-limb hypersensitivity and leads to gene expression changes at the level of the spinal cord.
- Blocking FKBP51 during stress reduces the hyperalgesic priming caused by restraint stress.

**Aims:**

1) To study the effect of stress on facial and hind-limb sensitivity
2) To investigate gene expression changes in the spinal cord following restraint stress
3) To study the effect of FKBP51 inhibition during stress on stress-induced hyperalgesia and hyperalgesic priming, as well as neuronal activation in the spinal dorsal horn.

**References: 1.** Avona, A., *et al*., 2020 **2.** Alexander, J.K., et al., 2009 **3.** Stechschulte, L.A. & Sanchez, E.R., 2011 **4.** Maiarù, M., *et al*., 2016 **5.** Maiarù, M., Morgan, O.B., *et al.*, 2018. Images created with BioRender.com.

## 1. Restraint induces facial and hind-paw mechanical hypersensitivity in mice

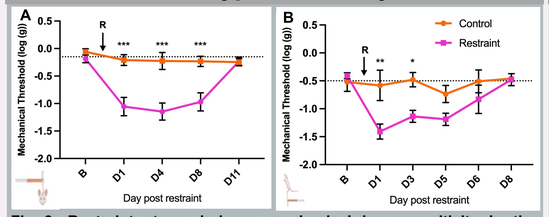



**Fig 3. Restraint stress induces mechanical hypersensitivity in the forehead (A) and hind-paw (B).** R: beginning of restraint stress procedure; D1 is first measure 24hrs after final RS. *p<0.05, **p<0.01,***p<0.001; (**A**) F (1, 21) = 31.40, p<0.0001; **(B)** F (1, 10) = 4.914, p<0.05. n=8/8 WP, =6/6 HP

## 2. Restraint leads to stress-related gene expression changes in the spinal dorsal horn

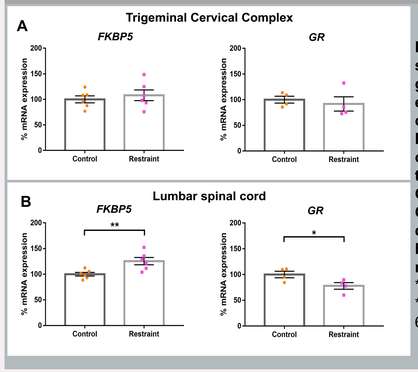



**Fig 4. Restraint stress induces gene expression changes in the lumbar spinal cord but not in the Trigeminal Cervical Complex dorsal horn 48 hours after restraint.** *p<0.05, **p<0.01; n= 6/6.

## 3. Restraint increases blood plasma corticosterone levels

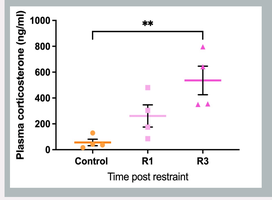
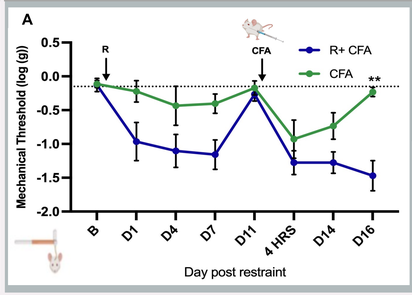



**Fig 5. There is an incremental increase in blood plasma CORT levels following restraint.** F(2,9) =8.619, p<0.01; **p<0.01; n= 4/4/4.

## 4. Restraint exacerbates and prolongs facial hypersensitivity induced by injection of CFA in the whisker pad



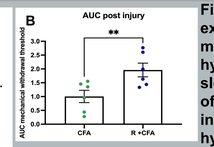
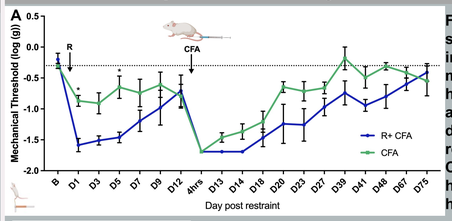




**Fig 6. Restraint stress exacerbates mechanical hypersensitivity and slows down the rate of recovery from CFA-induced facial hypersensitivity.**

Hypersensitivity induced by restraint stress had returned to baseline by day 11, with some groups receiving a whisker pad CFA injection on day 12. B-D11: $F_{treatment}$ (1, 10) = 7.369, p<0.05; 4HRS – D16: $F_{treatment}$ (1, 10) = 8.606, p<0.01. **p<0.01. AUC analysis, 4hrs-D16 (**B**). n=6/6

## 5. Restraint exacerbates and prolongs mechanical hypersensitivity induced by intra-plantar CFA injection



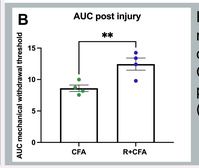




**Fig 8. Restraint stress increases mechanical hypersensitivity and slows down the rate of recovery from CFA induced hind-paw hypersensitivity**

Hypersensitivity induced by restraint stress had returned to baseline by day 9, both restraint and control groups received intra-plantar injections of CFA on day 12 (**A**). B-D12: $F_{treatment}$ (1, 6) = 8.816, p<0.05; 4hrs-D75: $F_{treatment}$ (11,66) = 21.65, p<0.05 (**A**). *p<0.05. AUC analysis, 4hrs-D75 (**B**). n=4/4.

## 6. Restraint primes neuronal spinal activation after injury

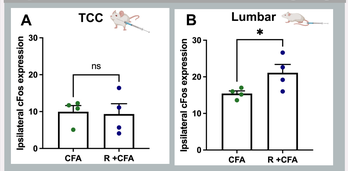



**Fig 7. Restraint stress increases cFos expression in layer i-ii of the lumbar spinal dorsal horn (B), but not the Trigeminal Cervical Complex dorsal horn (A), ipsilateral to CFA-induced inflammation, at 2 hours post CFA injection.** *p<0.05, n=4/4 WP & HP

## 7. Inhibition of FKBP51 during restraint does not alter restraint-induced hypersensitivity in the face, but reduces subsequent CFA-induced facial hypersensitivity

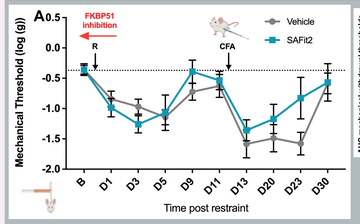



**Fig 9. Inhibition of FKBP51 during restraint does not alter stress-induced hypersensitivity in the face but reduces stress-induced priming.** Hypersensitivity induced by restraint stress had returned to baseline by D11, with both groups receiving whisker pad CFA at day 12 (**A**). B-D9: $F_{treatment}$ (1, 9) = 0.01, p=0.91; D13-D23: $F_{treatment}$ (1, 9) = 3.075, p=0.11. AUC analysis, D13-D23 (**B**). n=6/5

**Conclusions:**

In line with our aims, we have found that restraint stress:

1) induces hypersensitivity in the face and the hind-paw
2) exacerbates and prolongs orofacial and hind-paw mechanical hypersensitivity induced by CFA
3) increases blood plasma CORT levels
4) leads to the upregulation of stress-related genes at the level of the lumbar spinal cord, including FKBP51 and the glucocorticoid receptor.
5) alters neuronal activation at the level of the spinal dorsal horn, as measured with immediate early gene, cFos.

**We have also found that** inhibition of FKBP51 using the specific inhibitor SAFit2, during the restraint period, does not alter stress-induced hypersensitivity in the face, but reduces stress-induced hyperalgesic priming.

**Overall, this study provides further understanding on the interactions between stress and pain, with a focus on the role of FKBP51.**

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