Effects of transdermal fentanyl treatment on acute pain and inflammation in the adjuvant-induced monoarthritis rat model

Mie S. Berke[1*¥], Pernille Colding-Jørgensen[1¥], Line G. Pedersen[1], Sara Hestehave[2], Otto Kalliokoski[1], Henrik E. Jensen[3], Dorte Bratbo Sørensen[3], Jann Hau[1] and Klas S.P. Abelson[1]

[1]Department of Experimental Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[2]Department of Cell and Developmental Biology, University College London, London, England
[3]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

** Corresponding author, email: mibe@sund.ku.dk*
¥ Equally contributed to this work

Running title: Transdermal fentanyl treatment as a refinement approach in the monoarthritis rat model

Abstract

Eliminating all unnecessary pain is of great importance when performing animal experimentation, as well as reducing and controlling pain when using animals in pain research. The aim of the present study was to refine an adjuvant-induced monoarthritis rat model by providing analgesia using a transdermal fentanyl solution (TFS). Male and female Sprague Dawley rats, single- or pair-housed were injected with 20 µl of complete Freund's adjuvant (CFA) into the left ankle joint. CFA-injected rats treated with a single dose of transdermal fentanyl solution (TFS) (0.33 mg/kg or 1 mg/kg), were compared with an untreated CFA-injected group and sham groups receiving either no treatment or TFS treatment (1 mg/kg) during 72 h. Transdermal fentanyl solution, administered in doses of 0.33 mg/kg or 1 mg/kg, resulted in decreased mechanical hyperalgesia, and improved the mobility, stance, rearing and lameness scores of subjects six hours post CFA-injection. Joint circumferences were not reduced by TFS treatment, and no significant differences were detected between various doses of TFS, or between single or pair housed rats. Fentanyl appeared not to interfere with the model development and characteristics. However, overall the analgesic effect was transient and several opioid-related side effects were observed.

Abbreviations: CFA, complete Freund's adjuvant; TFS, transdermal fentanyl solution

Introduction

Analgesics have the potential to reduce unnecessary suffering and to refine animal models of pain. Providing adequate and long-lasting analgesia, while keeping confounding factors at a minimum, is however challenging. Identifying novel or improved strategies is consequently of significant interest. Long-acting formulations that can be given as a single dose, may have the advantage of providing adequate analgesia while limiting stress due to frequent handling and repeated dosing.

Conventional analgesic drugs used in laboratory rodents (non-steroidal anti-inflammatory drugs (NSAIDs) or opioids), are commonly administered by repeated injections requiring repeated restraint of the animals. Therefore, improved analgesic strategies have been developed, *e.g.* voluntary ingestion of buprenorphine mixed in water, gel, nut paste or in feed pellets.[3, 15, 20, 21, 25, 26, 28, 30, 39, 42] These strategies are advantageous compared with repeated injections in being non-invasive and thereby minimizing stress which could affect the experiments. Subjects may, however, occasionally be reluctant to ingest the dosed medium, especially if the animal is unwell after surgery, why this type of dosing may be unreliable. A sustained release formulation of buprenorphine has been developed, where the drug is administered as a single subcutaneous injection, providing therapeutic plasma concentrations for up to 24 h in rodents.[11, 16 12, 29, 31, 47] The formulation is, however, not commercially available in Europe presently.

Fentanyl is a highly potent μ-opioid receptor agonist – 100 to 300 fold more potent than morphine.[40] It is highly lipophilic and passes the blood-brain barrier providing a rapid onset and short duration of effect under traditional formulations.[22] It is traditionally provided as a parenteral injection, or through constant-rate infusion, due to its poor bioavailability and rapid clearance. However, it possesses excellent skin flux properties and has been favored over other opioids for use in transdermal delivery systems pre-emptively and postoperatively, in humans and animals alike.[6, 9, 14, 17, 24, 41, 44, 46] A novel long-acting transdermal fentanyl solution (TFS) has been shown to provide adequate post-operative analgesia for up to four days when applied as a single topical administration of 2.6 mg/kg in dogs.[34, 37] A recent study examined the analgesic effect of TFS in male Sprague Dawley rats, using a paw incision model of post-surgical pain.[13] It was found that doses of 0.1, 0.33 and 1 mg/kg provided analgesic effects for 72 h. This has encouraged further investigations of this treatment strategy, since it – besides being non-invasive – is suggested to have the advantage of alleviating moderate to severe pain, avoiding first-pass metabolism, and providing long-acting effects.[18, 19, 34, 37]

The aim of this study was to examine the analgesic effects of TFS on acute pain and inflammation in male and female Sprague Dawley rats injected with complete Freund's adjuvant (CFA) to induce monoarthritis. Rats were either single or pair housed to test for whether pair-housed rats might accidentally ingest TFS through social grooming, which could have an impact on the analgesic effect for both parties. It was hypothesized that treatment with TFS would reduce pain-related behaviour while having little to no impact on the acute inflammatory response. The latter is needed for proper

development of the arthritis model, i.e. the analgesic regimen must not interfere with model development and characteristics. Moreover, it was hypothesized that TFS would reduce hyperalgesia and attenuate pain-associated body weight loss over 72 h post induction.

Materials and Methods

Ethical statement. The study was approved by the Animal Experiments Inspectorate under the Danish Ministry of Environment and Food (license number 2014-15-0201-00257). Animals were housed in an AAALAC accredited animal facility and experiments were carried out in accordance with the *Guide for Care and Use of Laboratory Animals* and the Directive 2010/63/EU.[2, 27]

Animals and housing. A total of 160 RjHan:SD male and female Sprague Dawley rats from Janvier Labs (Le Genest-Saint-Isle, France) were used. They were all aged seven weeks, weighing approximately 200 g (females) and 300 g (males), and apparently healthy on arrival. In Experiment 1, rats were housed in pairs where both received the same treatment throughout the experiment, whereas rats in Experiment 2 were housed in either pairs (receiving the same treatment) or single-housed from the day of arrival and throughout the experiment. All rats were housed in NextGen Rat 1800 (Allentown Inc., Allentown, USA) (size: 572 mm x 287 mm x 412 mm) individually ventilated cages (IVC) in the same room and on the same rack. The room had an artificial light-dark cycle of 12:12 hours with light from 6:00 to 18:00, with 15-30 min of twilight before and after each light cycle. Cage temperature was monitored and maintained at 22 °C (± 2 °C), a humidity of 55% (± 10%) and 75 air changes per hour. Pelleted food (Altromin 1314, Altromin BmhH & Co., Lage, Germany) and tap water was available *ad libitum*. The cages were equipped with aspen chip bedding (Tapvei, Harjumaa, Estonia), paper bedding (Enviro-Dri, Fibercore, Milford, USA), small wooden gnawing sticks (Tapvei), cardboard tunnels (Lillico Biotechnology, Surrey, UK) and red polycarbonate rat shelters (Molytex, Glostrup, Denmark). A small amount of pelleted food was placed on the bedding to ease access to food.

Study design. Two separate experiments were conducted to explore the TFS treatment on the model:

I. Experiment 1 was aiming at comparing different doses of TFS for up to 72 hours post induction. CFA-injected male and female rats received transdermal fentanyl solution (TFS) (0.33 mg/kg or 1 mg/kg) and were compared with an untreated CFA-injected group and a sham group receiving TFS treatment (0.33 or 1 mg/kg) (N = 8 in all groups; in total 64 rats).
II. Experiment 2 was designed to investigate TFS treatment and the potential effect of pair- vs single-housing on TFS-efficacy for up to 24 hours post induction. CFA-injected male and female rats – either single or pair housed – received 1 mg/kg TFS, and were compared with four sham groups either single- or pair-housed, with or without TFS treatment (N = 6 in all groups; in total 96 rats).

In each experiment, rats were randomly allocated to their cages on arrival. After one week of acclimatization, the rats were assigned their experimental groups (**Table 1**), stratified by body weight. Baseline testing was performed on all animals one day prior to CFA injection. At the end of the experiment, all animals were euthanized according to the Directive 2010/63/EU and FELASA recommendations.[2,1] They were stunned by blunt trauma to the head, followed by cervical dislocation.

Five rats were found dead in their cages, and one cage-mate was excluded from the study, reducing the sample size to 62 in Experiment 1 and 92 in Experiment 2 (Animal loss in Table 1). All the rats underwent necropsy. Details are explained in the result section.

Table 1: *Experimental groups in Experiment 1 and 2*

Experiment 1						
Groups	**Abbreviation**	**IA injection**	**Treatment**	**Housing**	**N**	**Animal loss**
TFS treated, no CFA-injection	Sham TFS	Saline	0.33 or 1 mg/kg TFS	Pair	8 males + 8 females	None
Untreated, CFA injected	CFA	CFA	No	Pair	8 males + 8 females	None
TFS treated (low dose), CFA-injected	CFA + TFS 0.33 mg/kg	CFA	0.33 mg/kg TFS	Pair	8 males + 8 females	None
TFS treated (high dose), CFA-injected	CFA + TFS 1 mg/kg	CFA	1 mg/kg TFS	Pair	8 males + 8 females	2 (males)

Experiment 2						
Groups	**Abbreviation**	**IA injection**	**Treatment**	**Housing**	**N**	**Animal loss**
Untreated sham	Sham	Saline	No	Pair Single	6 males + 6 females 6 males + 6 females	None None
TFS treated, no CFA-injection	Sham TFS	Saline	1 mg/kg TFS	Pair Single	6 males + 6 females 6 males + 6 females	None 1 (male)
Untreated, CFA injected	CFA	CFA	No	Pair Single	6 males + 6 females 6 males + 6 females	1 (female) None
TFS treated, CFA-injected	CFA + TFS 1 mg/kg	CFA	1 mg/kg TFS	Pair Single	6 males + 6 females 6 males + 6 females	1 (female) 1 (female)

IA = intra-articular injection
Animal loss: number of animal loss either found dead or euthanized

Drug administration. One day prior to CFA injection, an area of approximately 3 x 4 cm was shaved in the interscapular region while gently restraining the animal. On the following day, while the animal was awake and gently restrained, analgesic-treatment was initiated pre-emptively, 60 min before CFA injection. TFS (Recuvyra®, Elanco Animal Health, Indianapolis, USA) containing 50 mg/ml fentanyl combined with isopropanol and octyl salicylate,[4] was applied to the shaved area with a micropipette doses of 0.33 or 1 mg/kg. Volumes applied were depending on the weight of each animal, and thus varied from 1.4 to 3.0 µl for the lower dose, and from 4.3 to 9.1 µl for the higher dose. The analgesic regimen was based on a previous study where TFS was successfully used for post-operative pain treatment in rats.[13] Untreated sham animals were handled in the same way as the TFS treated groups, but without applying any liquid to the skin.

Induction of monoarthritis. Monoarthritis was induced by injecting 20 µl complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, USA), containing 1 mg/ml heat-killed and dried *Mycobacterium tuberculosis* (strain H37Ra, ATCC 25177), into the left ankle (tibio-tarsal) joint during

brief isoflurane anaesthesia (Attane Vet, Isoflurane 1000 mg/g, ScanVet) (3.5% isoflurane delivered in pure oxygen through an open face mask at a flow rate of 0.5 l/min). Sham animals were injected with 20 µL of saline into the ankle instead of CFA. The injection procedure was performed as previously described.[5]

Welfare assessment. Daily observations for adverse effects of fentanyl administration, such as administration-site reactions, sedative behaviour, or respiratory depression, were carried out.
A welfare assessment (WA) sheet, modified from Hampshire *et al.*,[23] was used to measure overall well-being of the animals (**Table 2**), similarly to a previous study.[5] Animals were evaluated in open home cages at baseline and at 4, 8, 24, 48 and 72 h post injection in Experiment 1, and at 6 and 24 h in Experiment 2. The rats were allowed to acclimatize for 2-5 minutes before being scored. The WA included attitude, gait and posture, porphyrin staining, changes in body weight, and wounds. Scores of 0 (no impact), 0.1 (mild impact) and 0.4 (severe impact) were assigned. Wounds were only scored as 0 or 0.4. The scores were pooled and if an overall score of 0.4 or higher was obtained, the animal was euthanized.

As a measure of welfare and possible adverse effects of TFS treatment, the animals were weighed on all experimental days.

Model-specific parameters. Scoring of arthritis was performed according to a modified scoring sheet from Butler *et al,* 1999 (**Table 3**), as used in previous studies.[5, 7] Animals were scored at baseline and at 24, 48 and 72 h in Experiment 1, and at 6 and 24 h in Experiment 2. Scoring was performed in their open cages after 2-5 minute of acclimatization with the cardboard tunnels and rat shelters removed. Each parameter was scored and compared separately.

Joint Circumference. Ankle joint circumference (left and right) was estimated as a parameter of the inflammatory response upon CFA injection, as performed in previous work.[5] Ankles were measured at baseline and at 24, 48 and 72 h in Experiment 1, and at 6 and 25 h in Experiment 2. The mediolateral (ML) and dorsoplantar (DP) radii were measured with callipers. The circumference (C) of the joint was estimated using the approximation for the perimeter of an ellipse: $\mathrm{C} = 2 \times \pi \times \sqrt{0{,}5 \times (\mathrm{ML}^2 + \mathrm{DP}^2)}$.[45]

Electronic von Frey test. Electronic von Frey testing (EVF) (model BSBIOEVF3, Bioseb, Vitrolles, France) was applied to assess mechanical hypersensitivity at baseline, at 24 h and 72 h post treatment in Experiment 1, and at 6 h and 24 h post treatment in Experiment 2. Rats were placed in individual Plexiglas chambers (size 16.5 x 24.2 x 14.6 cm) on an elevated metal grid platform. Rats were tested four at a time. After acclimatization for approximately 15 minutes, testing was commenced. The EVF tip was applied with a linearly increasing force, perpendicularly to the plantar surface of the hind paw, as close to the tibio-tarsal joint as possible. The test was repeated three times for each hind paw, starting with the right uninjured paw. The weight (g) required to elicit paw withdrawal, corresponding to the mechanical threshold, was recorded. The EVF tip was not applied, or the reading was discarded, if the animal was grooming, sleeping or performed locomotive behaviour not related to the EVF stimuli. The methodology was similar to what has previously been published by our group.[5]

Rat grimace scale. The Rat Grimace Scale (RGS) was assessed at baseline, 24 h and 72 h in Experiment 1, and at 6 h and 24 h in Experiment 2. RGS was used to assess non-evoked spontaneous pain-like behaviour and distress, based on four pain-related facial action units (orbital tightening, ear changes, nose/cheek flattening and whisker changes) originally described by Sotocinal *et al.*.[43] Pictures for RGS scoring were captured in the EVF setup after acclimatization, before EVF testing. Three images of each rat facing the camera were kept for assessments, the remainder were discarded. A score of 0, 1 or 2 was assigned for each action unit, with 0 = absent, 1 = moderately present and 2 = obvious present. In Experiment 1, three observers scored all the pictures and a mean value for each picture was calculated giving a total score between 0 and 8. In Experiment 2, only one observer scored the pictures and a total score between 0 and 8 was given for each picture. All observers were blinded and well-versed in the method.

Data analysis. In Experiment 1, we evaluated the effects of two different doses of fentanyl treatment during the first three days post induction. Sham TFS treated animals (0.33 and 1 mg/kg TFS) were combined to one sham TFS group because of no statistical differences in data. Findings in Experiment 1 led to the second experiment, where high-dose fentanyl was compared between single and pair housed rats, for the first 24 h. No statistical differences in data of some groups in both experiments led to the decision that certain groups were combined. In Experiment 2, single and pair housed rats receiving the same treatment as well as the two sham groups with or without TFS treatment were combined in the analyses.

Statistical analyses and graphical illustrations were performed and produced using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, USA) and SPSS (IBM SPSS statistics 27). The BW was analysed by repeated measure (RM) ANOVA to each sex. The effects of sex and treatment on EVF and circumference data were analysed by RM ANOVAs followed by Bonferroni's multiple comparisons *post hoc* test (comparing groups for each time point) or Tukey's multiple comparisons *post hoc* test (comparing groups to baseline values). The model-specific scores and RGS scores were compared using one-way non-parametric ANOVAs (Kruskal-Wallis tests) followed by a Dunn's multiple comparisons test to each time point (Experiment 1) or Mann-Whitney tests to each time point (Experiment 2). P values $p < 0.05$ were considered significant for all statistical analyses.

Results

Welfare assessment. In Experiment 1, one rat given 1 mg/kg TFS showed dyspnoea during anaesthesia and was found dead in its cage five hours later. Its cage-mate was also removed from the study. In Experiment 2, one male rat from the TFS sham group was found dead in its cage a few hours after TFS treatment. Three female rats were found with severe muscle rigidity and sedated attitude after TFS treatment, but before CFA injection. One of the rats had severe clinical manifestations and was euthanized. The two remaining rats went through anaesthesia as well as ankle joint injections however, three hours later, they were found dead in their cages. They were both in a pair-housed group, but it was decided to continue with their remaining partners. No obvious cause of death was detected during necropsy in any case. The remaining animals, that completed the experiments, remained healthy without a severe increase in welfare scoring during the study (scores never exceeded 0.4).

Body weight. Two-way ANOVAs for each sex detected no significant differences in body weight (BW) between groups at any time, and none of the rats suffered a weight loss of 5% or more.

Model specific parameters. In Experiment 1 (**Figure 1**), Kruskal-Wallis tests detected no significant differences in parameters in males, only in females. However, TFS-treated CFA males showed a tendency towards having greater impairment in all parameters compared to the untreated CFA male group. In females, no significant differences in mobility were detected between the groups (Figure 1B). In stance (Figure 1D), high-dose TFS-treated CFA females were significantly improved in scores compared to the untreated CFA group at 8 h ($p = 0.0052$) and low-dose TFS-treated CFA females were significantly improved at 48 h ($p = 0.0116$). In rearing (Figure 1F), TFS-treated CFA females showed significantly improved scores at 8 h (low-dose TFS: $p = 0.0224$ and high-dose TFS: $p = 0.0048$) and at 48 h (low-dose TFS: $p = 0.0119$) compared to the untreated CFA group. In lameness (Figure 1H), TFS-treated CFA females showed significantly improved scores at 4 h (low-dose TFS: $p = 0.0078$ and high-dose TFS: $p = 0.0078$), 8 h (low-dose TFS: $p = 0.0301$ and high-dose TFS: $p = 0.0022$), 24 h high-dose TFS: $p = 0.0196$) and at 48 h high-dose TFS: $p = 0.0401$) compared to the untreated CFA group (Dunn's multiple comparisons test).

In Experiment 2 (**Figure 2A-F**), Mann Whitney tests detected significant differences in model-specific parameters at 6 h, where the TFS-treated CFA animals showed significantly improved mobility (males: $p = 0.0396$ and females: $p = 0.009$, Figure 2A-B), stance (males: $p < 0.0001$ and females: $p = 0.0040$, Figure 2C-D) and rearing scores (males: $p = 0.0032$ and females: $p = 0.0019$, Figure E-F), compared to the untreated CFA group. At 24 h, no significant improvement in parameters were detected after TFS treatment, but in contrast, TFS-treated CFA females showed significantly impaired stance scores ($p = 0.0124$, Figure 2D).

Joint circumference. Left (Ipsilateral) ankle circumference measurements are presented in **Figure 3**. All CFA-injected groups showed a significant and profound increase of the left ankle joint from 4 until 24 h post induction in Experiment 1 and 2, and all CFA-injected groups differed significantly ($p < 0.0001$) from sham groups from 4 h to the end of experiment (two-way RM

ANOVA). A two-way RM ANOVA detected no significant differences between CFA groups or sham groups at any time.

Electronic von Frey test. In Experiment 1 (**Figure 4 A and B** for combined groups) (all groups are shown individually in Figure S1), an overall two-way RM ANOVA revealed only significant effects of sex ($F(1, 40) = 4.733$, $p = 0.036$), with females, on average, showing a lower mechanical threshold. No significant effects of TFS treatment, or any interactions, were detected.

In Experiment 2 (**Figure 4 C and D** for combined groups) (all groups are shown individually in Figure S1), an overall two-way RM ANOVA revealed significant effects of TFS treatment ($F(1, 41) = 71.612$, $p < 0.0001$) and sex ($F(1, 41) = 7.920$, $p = 0.007$). Significantly decreased thresholds compared to baseline were detected at 6 and 24 h post CFA-injection in the untreated CFA group ($p < 0.0001$) (Tukey's multiple comparisons test). TFS treatment resulted in significantly higher mechanical thresholds compared to no analgesic treatment, highest at 6 h (males: $p = 0.0001$, females: $p = 0.0004$; Bonferroni's multiple comparisons test). The TFS-treated CFA animals remained significantly different from the CFA group to some extent at 24 h in males ($p = 0.0115$) but not in females (Bonferroni's multiple comparisons test).

Rat grimace scale. Kruskal-Wallis tests (Experiment 1) and Mann Whitney tests (Experiment 2) detected no significant differences between CFA-injected groups or compared to the sham groups at any time in either sex and either experiment.

Discussion

The aim of this study was to evaluate effects of a transdermal fentanyl solution ("Recuvyra") on model-specific parameters, inflammation and pain-related behavior during the immediate post-induction period in the complete Freund's adjuvant-induced monoarthritic rat model.

Recuvyra was developed for post-operative analgesic treatment in dogs, and has been reported to sustain effective analgesia for up to four days in this species.[34, 37] A recent study from our group suggested that the analgesic coverage of TFS in a hind-paw incisional model of post-operative pain in male rats was 72 h.[13] However, to our surprise, the analgesic effect of fentanyl was shown to be reduced at 24 h, although some improvement in model-specific scores was detected in females for up to 48 h. Beside some improvement of model-specific scores in females, no prolonged analgesic effect could be detected, indicating that the pharmacokinetic profile of TFS is different between dogs and rats, and that efficacy could be dependent on the type of injury explored. High-dose TFS appeared to provide effective analgesia up to six hours in Experiment 2, demonstrated by a significant decrease in mechanical hyperalgesia by EVF testing and improved model-specific scores. However, in Experiment 1, males showed a tendency towards being more mechanically hypersensitive and more impaired in all model-specific parameters compared to the untreated CFA group, regardless of dose. This may be due to random variation but it is also possible that fentanyl paradoxically activates both pain inhibitory (analgesia) and pain excitatory (hyperalgesia) systems in a sex-related manner. An opioid-induced hyperalgesia is a recognized complication to opioid use and has been reported in fentanyl-treated male rats in a previous study.[10] However, why this tendency appears to be sex-related and only seen in Experiment 1 is dubious. In a previous study investigating opioid-induced hyperalgesia after morphine treatment,[25] it was found that the opioid-induced hyperalgesia was enhanced in a dose- and sex-related manner in rats, where males showed enhanced analgesic efficacy of treatment and females enhanced hyperalgesia, which is opposite findings compared to the present study. It should be noted that no increase or decrease was detected in the sham TFS groups and further investigations is needed to elucidate this.

All CFA-injected animals developed progressive joint swelling, as well as redness and increased temperature located to the left ankle, observed from 6 h post induction. No differences were detected between CFA-injected groups, suggesting that TFS treatment, regardless of dose, did not affect the model-relevant inflammation in the immediate post induction period. No differences in effectiveness between doses were found, the lower dose of 0.33 mg/kg may be favourable in alleviating pain during the first 24 h, without affecting the relevant model development, and also presumably causes less side-effects. However, additional studies are needed to verify these effects.

Single housing was applied because of the concern that the rats would groom each other, and thereby accidentally ingesting the skin-applied analgesic of their cage mate. However, nothing indicated that this happened. This suggests that rats do not find the application interesting or tasty or, most likely, that the solution rapidly absorbs and dries from the skin. Thus, taken several known negative consequences of single housing into account, it seems reasonable not to implement single housing in studies exploring or applying TFS treatment.[32]

Despite a few promising findings in this study, several concerns of using TFS needs to be addressed. It is known that fentanyl can cause adverse effects including dose-dependent sedation, and respiratory-, CNS- and circulatory depressions.[22] Severe adverse reactions were noticed in both experiments with administration of 1 mg/kg TFS. Five rats (male and females) died in their cage or had to be euthanized due to what appeared to be an overdose of fentanyl. The clinical manifestations were dyspnea, severe muscle rigidity and sedated attitude. This raises significant concerns and indicates that a dose of 1 mg/kg TFS was higher than necessary, especially as the higher dose showed no additional effect on the pain-related parameters. No adverse effects was reported using this dose in the previous study in rats,[13] however other studies with dogs have reported some opioid-related adverse effects and death using TFS treatment.[35, 38] Increasing the monitoring is recommended, but it remains unknown whether it would have been possible to predict death with the welfare monitoring protocol followed in the present study. It would likely be necessary to identify novel behavioral signs for this purpose, which should be attempted in future studies. In addition, it is likely that adverse effects of fentanyl treatment had some impact on behavioural readouts in this study (EVF, model-specific parameters and RGS).

Species-specific and individual variation has been reported in other studies using transdermally administered fentanyl.[8, 34, 36, 37] When TFS is applied, it accumulates in skeletal muscle and adipose tissue, and is slowly released into the blood stream. Differences in skin thickness, amount of body fat, as well as location of application, can contribute to absorption variability.[33] The lower body-fat percentage seen in rodents is likely to be the primary explanation for the transient analgesic effect of TFS seen in this study. It may also be the reason for the severe side effects, due to rapid release to the blood stream leading to opioid overdoses. Findings in this study suggest that TFS treatment for rats is ideally kept below 1.0 mg/kg and perhaps with more frequent dosing intervals for example every 24 h. Serum concentration measurements of fentanyl would be of great support here, gauging absorption, distribution, and elimination of the compound,[48] and will be considered for future studies.

In conclusion, transdermal fentanyl solution, administered through a single topical application, showed an analgesic effect for six hours in arthritic male and female Sprague Dawley rats. The effect was independent of animals being single- or pair-housed. This is inconsistent with previous studies, where sustained analgesia could be demonstrated for 72 h in rats. The severe adverse effects seen in this study should be considered in the risk-benefit assessment when selecting TFS for pain management in rats.

Acknowledgements

We thank Helle Runchel Porsdal, Trine Marie Ahlman Glahder and Daniel Kylmann Hansen for their invaluable assistance.

This work was supported by Centre for Applied Laboratory Animal Research (CALAR) and the Danish 3R-Center.

References

1. Recommendations for euthanasia of experimental animals: Part 2, 1997. Laboratory Animals **31**:1-32.
2. Directive 2010/63 of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union.* L246, 33-77.
3. **Abelson KS, Jacobsen KR, Sundbom R, Kalliokoski O, Hau J**. 2012. Voluntary ingestion of nut paste for administration of buprenorphine in rats and mice. Laboratory animals **46**:349-351.
4. **Annex I**. 1999. Summary of product characteristics. Committee for Proprietary Medicinal Products The European Public Assessment Report (EPAR) Stocrin London: The European Agency for the Evaluation of Medicinal Products.
5. **Berke MS, Abelson KSP**. 2020. The adjuvant-induced rat model of monoarthritis: welfare implications and possible refinement strategies. Scandinavian Journal of Laboratory Animal Science **46**:39-50.
6. **Bowdle TA**. 1998. Adverse effects of opioid agonists and agonist-antagonists in anaesthesia. Drug Safety **19**:173-189.
7. **Butler SH, Godefroy F, Besson JM, Weil-Fugazza J**. 1992. A limited arthritic model for chronic pain studies in the rat. Pain **48**:73-81.
8. **Carlson AM, Kelly R, III, Fetterer DP, Rico PJ, Bailey EJ**. 2016. Pharmacokinetics of 2 Formulations of Transdermal Fentanyl in Cynomolgus Macaques (Macaca fascicularis). Journal of the American Association for Laboratory Animal Science : JAALAS **55**:436-442.
9. **Carroll G, Hooper R, Boothe D, Hartsfield S, Randoll L**. 1999. Pharmacokinetics of fentanyl after intravenous and transdermal administration in goats. American journal of veterinary research **60**:986-991.
10. **Célèrier E, Rivat C, Jun Y, Laulin J-P, Larcher A, Reynier P, Simonnet G**. 2000. Long-lasting Hyperalgesia Induced by Fentanyl in Rats: Preventive Effect of Ketamine. Anesthesiology **92**:465-465.
11. **Chum HH, Jampachairsri K, McKeon GP, Yeomans DC, Pacharinsak C, Felt SA**. 2014. Antinociceptive effects of sustained-release buprenorphine in a model of incisional pain in rats (Rattus norvegicus). Journal of the American association for laboratory animal science **53**:193-197.
12. **Clark TS, Clark DD, Hoyt RF**. 2014. Pharmacokinetic comparison of sustained-release and standard buprenorphine in mice. Journal of the American Association for Laboratory Animal Science **53**:387-391.
13. **Clemensen J, Rasmussen LV, Abelson KSP**. 2018. Transdermal Fentanyl Solution Provides Long-term Analgesia in the Hind-paw Incisional Model of Postoperative Pain in Male Rats. In Vivo **32**:713.
14. **Egger CM, Glerum LE, Allen SW, Haag M**. 2003. Plasma fentanyl concentrations in awake cats and cats undergoing anesthesia and ovariohysterectomy using transdermal administration. Veterinary anaesthesia and analgesia **30**:229-236.
15. **Flecknell P, Roughan J, Stewart R**. 1999. Use of oral buprenorphine ('buprenorphine jello') for postoperative analgesia in rats-a clinical trial. Laboratory animals **33**:169-174.
16. **Foley PL, Liang H, Crichlow AR**. 2011. Evaluation of a sustained-release formulation of buprenorphine for analgesia in rats. Journal of the American Association for Laboratory Animal Science **50**:198-204.

17. **France C, Ahn S, Brockunier L, Bagley J, Brandt M, Winsauer P, Moerschbaecher J**. 1998. Behavioral effects and binding affinities of the fentanyl derivative OHM3507. Pharmacology Biochemistry and Behavior **59**:295-303.
18. **Freise KJ, Newbound GC, Tudan C, Clark TP**. 2012. Pharmacokinetics and the effect of application site on a novel, long-acting transdermal fentanyl solution in healthy laboratory Beagles. J Vet Pharmacol Ther **35 Suppl 2**:27-33.
19. **Freise KJ, Savides MC, Riggs KL, Owens JG, Newbound GC, Clark TP**. 2012. Pharmacokinetics and dose selection of a novel, long-acting transdermal fentanyl solution in healthy laboratory Beagles. J Vet Pharmacol Ther **35 Suppl 2**:21-26.
20. **Goldkuhl R, Hau J, Abelson KS**. 2010. Effects of voluntarily-ingested buprenorphine on plasma corticosterone levels, body weight, water intake, and behaviour in permanently catheterised rats. in vivo **24**:131-135.
21. **Goldkuhl R, Jacobsen KR, Kalliokoski O, Hau J, Abelson KS**. 2010. Plasma concentrations of corticosterone and buprenorphine in rats subjected to jugular vein catheterization. Laboratory animals **44**:337-343.
22. **Grape S, Schug SA, Lauer S, Schug BS**. 2010. Formulations of Fentanyl for the Management of Pain. Drugs **70**:57-72.
23. **Hampshire VA, Davis JA, McNickle CA, Williams L, Eskildson H**. 2001. Retrospective comparison of rat recovery weights using inhalation and injectable anaesthetics, nutritional and fluid supplementation for right unilateral neurosurgical lesioning. Laboratory animals **35**:223-229.
24. **Harvey-Clark C, Gilespie K, Riggs K**. 2000. Transdermal fentanyl compared with parenteral buprenorphine in post-surgical pain in swine: a case study. Laboratory Animals **34**:386-398.
25. **Hestehave S, Munro G, Pedersen TB, Abelson KS**. 2017. Antinociceptive effects of voluntarily ingested buprenorphine in the hot-plate test in laboratory rats. Laboratory animals **51**:264-272.
26. **Hovard A, Teilmann A, Hau J, Abelson K**. 2015. The applicability of a gel delivery system for self-administration of buprenorphine to laboratory mice. Laboratory animals **49**:40-45.
27. **Institute of Laboratory Animal Research**. 2011. Guide for the Care and Use of Laboratory Animals, 8th ed. Washington DC: The National Academies Press.
28. **Jessen L, Christensen S, Bjerrum O**. 2007. The antinociceptive efficacy of buprenorphine administered through the drinking water of rats. Laboratory animals **41**:185-196.
29. **Jirkof P, Tourvieille A, Cinelli P, Arras M**. 2015. Buprenorphine for pain relief in mice: repeated injections vs sustained-release depot formulation. Laboratory animals **49**:177-187.
30. **Kalliokoski O, Abelson KS, Koch J, Boschian A, Thormose SF, Fauerby N, Rasmussen RS, Johansen FF, Hau J**. 2010. The effect of voluntarily ingested buprenorphine on rats subjected to surgically induced global cerebral ischaemia. in vivo **24**:641-646.
31. **Kendall LV, Hansen RJ, Dorsey K, Kang S, Lunghofer PJ, Gustafson DL**. 2014. Pharmacokinetics of sustained-release analgesics in mice. Journal of the American Association for Laboratory Animal Science **53**:478-484.
32. **Krohn TC, Sørensen DB, Ottesen JL, Hansen AK**. 2006. The effects of individual housing on mice and rats: A review. Animal Welfare **15**:343-352.
33. **Kuip EJM, Zandvliet ML, Koolen SLW, Mathijssen RHJ, van der Rijt CCD**. 2017. A review of factors explaining variability in fentanyl pharmacokinetics; focus on implications for cancer patients. British journal of clinical pharmacology **83**:294-313.

34. **Linton D, Wilson M, Newbound G, Freise K, Clark T**. 2012. The effectiveness of a long-acting transdermal fentanyl solution compared to buprenorphine for the control of postoperative pain in dogs in a randomized, multicentered clinical study. Journal of veterinary pharmacology and therapeutics **35**:53-64.
35. **Linton DD, Wilson MG, Newbound GC, Freise KJ, Clark TP**. 2012. The effectiveness of a long-acting transdermal fentanyl solution compared to buprenorphine for the control of postoperative pain in dogs in a randomized, multicentered clinical study. Journal of Veterinary Pharmacology and Therapeutics **35**:53-64.
36. **Malavasi LM, Augustsson H, Jensen-Waern M, Nyman G**. 2005. The effect of transdermal delivery of fentanyl on activity in growing pigs. Acta Vet Scand **46**:149-157.
37. **Martinez S, Wilson M, Linton D, Newbound G, Freise K, Lin TL, Clark T**. 2014. The safety and effectiveness of a long-acting transdermal fentanyl solution compared with oxymorphone for the control of postoperative pain in dogs: a randomized, multicentered clinical study. Journal of veterinary pharmacology and therapeutics **37**:394-405.
38. **Martinez SA, Wilson MG, Linton DD, Newbound GC, Freise KJ, Lin T-L, Clark TP**. 2014. The safety and effectiveness of a long-acting transdermal fentanyl solution compared with oxymorphone for the control of postoperative pain in dogs: a randomized, multicentered clinical study. Journal of Veterinary Pharmacology and Therapeutics **37**:394-405.
39. **Molina-Cimadevila M, Segura S, Merino C, Ruiz-Reig N, Andrés B, de Madaria E**. 2014. Oral self-administration of buprenorphine in the diet for analgesia in mice. Laboratory animals **48**:216-224.
40. **Peng PW, Sandler AN**. 1999. A review of the use of fentanyl analgesia in the management of acute pain in adults. The Journal of the American Society of Anesthesiologists **90**:576-599.
41. **Pettifer GR, Hosgood G**. 2004. The effect of inhalant anesthetic and body temperature on peri-anesthetic serum concentrations of transdermally administered fentanyl in dogs. Veterinary anaesthesia and analgesia **31**:109-120.
42. **Seymour TL, Adams SC, Felt SA, Jampachaisri K, Yeomans DC, Pacharinsak C**. 2016. Postoperative analgesia due to sustained-release buprenorphine, sustained-release meloxicam, and carprofen gel in a model of incisional pain in rats (Rattus norvegicus). Journal of the American Association for Laboratory Animal Science **55**:300-305.
43. **Sotocinal SG, Sorge RE, Zaloum A, Tuttle AH, Martin LJ, Wieskopf JS, Mapplebeck JC, Wei P, Zhan S, Zhang S, McDougall JJ, King OD, Mogil JS**. 2011. The Rat Grimace Scale: a partially automated method for quantifying pain in the laboratory rat via facial expressions. Mol Pain **7**:55.
44. **Szeitz A, Riggs KW, Harvey-Clark C**. 1996. Sensitive and selective assay for fentanyl using gas chromatography with mass selective detection. Journal of Chromatography B: Biomedical Sciences and Applications **675**:33-42.
45. **Tag HM, Khaled HE, Ismail HA, El-Shenawy NS**. 2016. Evaluation of anti-inflammatory potential of the ethanolic extract of the Saussurea lappa root (costus) on adjuvant-induced monoarthritis in rats. J Basic Clin Physiol Pharmacol **27**:71-78.
46. **Thomasy SM, Slovis N, Maxwell L, Kollias-Baker C**. 2004. Transdermal fentanyl combined with nonsteroidal anti-inflammatory drugs for analgesia in horses. Journal of veterinary internal medicine **18**:550-554.

47. **Traul KA, Romero JB, Brayton C, DeTolla L, Forbes-McBean N, Halquist MS, Karnes HT, Sarabia-Estrada R, Tomlinson MJ, Tyler BM**. 2015. Safety studies of post-surgical buprenorphine therapy for mice. Laboratory Animals **49**:100-110.
48. **Varvel J, Shafer S, Hwang S, Coen P, Stanski D**. 1989. Absorption characteristics of transdermally administered fentanyl. The Journal of the American Society of Anesthesiologists **70**:928-934.

Figure legends

Figure 1. Model-specific parameters (Experiment 1). Mobility in **A.** Males and **B.** Females, stance in **C.** Males and **D.** Females, rearing in **E.** Males and **F.** Females and lameness in G. Males and H. Females. All data are presented as means ± SEM. Data were analysed by a Kruskal-Wallis test, followed by Dunn's multiple comparisons test to each time point. The asterisks (*) represents statistically significant differences from the CFA group at *p < 0.05 and **p < 0.01. Groups: N = 8 in all groups (except N = 6 in males CFA + TFS 1 mg/kg).

Figure 2. Model-specific parameters at 6 and 24 h (Experiment 2). Mobility in **A.** Males and **B.** Females, stance in **C.** Males and **D.** Females and rearing in **E.** Males and **F.** Females. All data are presented as means ± SEM. Data were analysed by a Mann Whitney test to each time point. The asterisks (*) represents statistically significant differences from the CFA group at *p < 0.05, **p < 0.01 and ****p < 0.0001. Groups: N = 23 in Sham/Sham TFS, N = 12 in CFA (N = 11 in females) and N = 12 in CFA + TFS (N = 10 in females).

Figure 3. Left (ipsilateral) ankle circumference measurements in millimetre (mm). A. Males and **B.** Females in Experiment 1, and **C.** Males and **D.** Females in Experiment 2. Data were analysed by a two-way RM ANOVA followed by Tukey's multiple comparisons tests. Values are expressed as means ± SEM. In experiment 1: N = 8 in all groups (except N = 6 in males CFA + TFS 1 mg/kg). In Experiment 2: N = 12 in CFA (N = 11 in females), N = 12 in CFA + TFS (N = 10 in females) and N = 23 in Sham/Sham TFS.

Figure 4: Left (Ipsilateral) paw withdrawal thresholds (g) as an indication of mechanical hyperalgesia. **A.** males and **B.** females in Experiment 1 and **C.** males and **D.** females in Experiment 2. All data are presented as means ± SEM. Data were analysed by a two-way RM ANOVA followed by a Tukey's multiple comparisons test. The asterisks (*) represents statistically significant differences from the untreated CFA group at *p < 0.05 and ***p < 0.001. Groups: N = 8 in sham TFS and CFA groups, N = 8 in both CFA + TFS groups (except N = 6 in CFA + TFS 1 mg/kg males) in Experiment 1. N = 12 in sham group, N = 12 in Sham TFS (N = 11 in males), N = 12 in CFA (N = 11 in females) and N = 12 in CFA + TFS (N = 10 in females) in Experiment 2.

Figure S1. Left (ipsilateral) paw withdrawal thresholds in gram (g) (Experiment 2). A. males and **B.** females single ("single") or pair ("pair") housed in Experiment 2. Data were analysed by a two-way RM ANOVA followed by a Tukey's multiple comparisons test. All data are presented as means ± SEM. N = 6 in most groups (N = 5 in Sham TFS single males and Sham TFS pair, CFA + TFS pair, CFA + TFS single and CFA pair in females).

Tables

Table 1: *Experimental groups in Experiment 1 and 2*

Experiment 1						
Groups	**Abbreviation**	**IA injection**	**Treatment**	**Housing**	**N**	**Animal loss**
TFS treated, no CFA-injection	Sham TFS	Saline	0.33 or 1 mg/kg TFS	Pair	8 males + 8 females	None
Untreated, CFA injected	CFA	CFA	No	Pair	8 males + 8 females	None
TFS treated (low dose), CFA-injected	CFA + TFS 0.33 mg/kg	CFA	0.33 mg/kg TFS	Pair	8 males + 8 females	None
TFS treated (high dose), CFA-injected	CFA + TFS 1 mg/kg	CFA	1 mg/kg TFS	Pair	8 males + 8 females	2 (males)

Experiment 2						
Groups	**Abbreviation**	**IA injection**	**Treatment**	**Housing**	**N**	**Animal loss**
Untreated sham	Sham	Saline	No	Pair Single	6 males + 6 females 6 males + 6 females	None None
TFS treated, no CFA-injection	Sham TFS	Saline	1 mg/kg TFS	Pair Single	6 males + 6 females 6 males + 6 females	None 1 (male)
Untreated, CFA injected	CFA	CFA	No	Pair Single	6 males + 6 females 6 males + 6 females	1 (female) None
TFS treated, CFA-injected	CFA + TFS 1 mg/kg	CFA	1 mg/kg TFS	Pair Single	6 males + 6 females 6 males + 6 females	1 (female) 1 (female)

IA = intra-articular injection
Animal loss: number of animal loss either found dead or euthanized

Table 2. *Modified welfare assessment score sheet from Hampshire et al., 2001*[23]

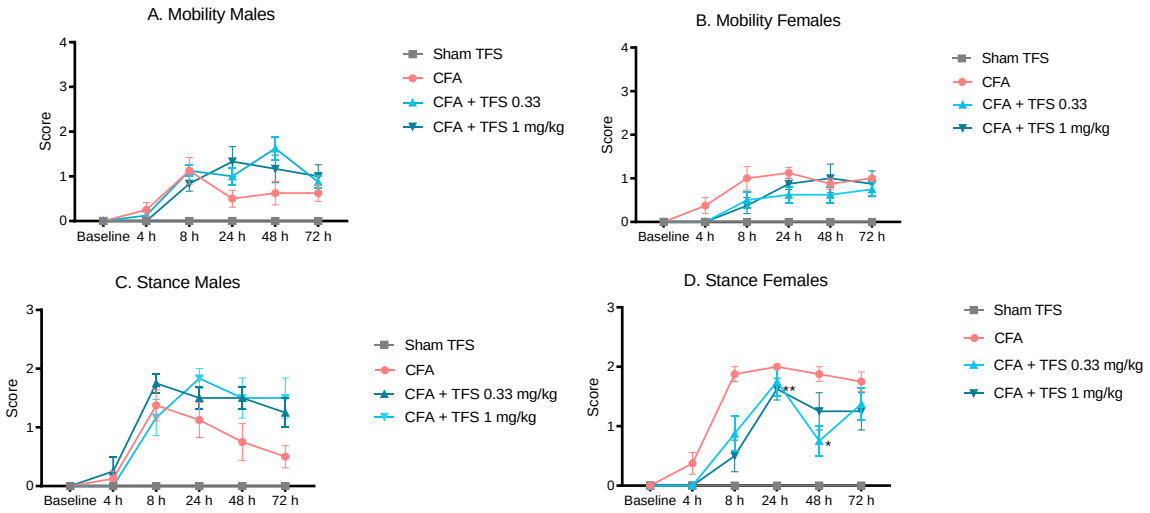
General appearance	Reference score
Bright and alert	0
Burrowing or hiding, quiet but rouses when touched	0.1
Burrowing or hiding, quiet but rouses when touched. No exploration when lid off, burrows, hides, head presses. Might be aggressive when touched	0.4
Porphyrin staining	
None	0
Mild	0.1
Obvious on face or paws	0.4
Gait and posture	
Normal	0
Mild incoordination when stimulated, hunched posture, mild piloerection	0.1
Obvious ataxia or head tilt, hunching, severe piloerection	0.4
Body weight loss compared to baseline weight (pre-CFA-injection) and controls	
<5%	0
5-10%	0.1
10-20%	0.4
Self-injury	
None	0
Bites or scratches itself, leading to wounds	0.4

Table 3. *Model-specific parameters (modified from Butler et al, 1992)* [8]

Mobility	Reference score
The rat walks and runs normally	0
The rat walks and runs with difficulty	1
The rat walks with difficulty	2
The rat crawls using front legs only	3
The rat lies down only	4
Stance	
The rat stands bearing weight equally on all four limbs	0
The rat stands bearing some weight on the arthritic limb	1
The rat stands with the arthritic paw touching floor, toes curled under	2
The rat stands on three paws only	3
Rearing	
The rat is equally bearing weight on both hind limbs	0
The rat is bearing some weight on the arthritic limb	1
The rat is only bearing weight on the non-arthritic hind limb	2
Lameness	
Normal ambulation	0
Mild, slight lameness	1
Moderate, toe touching ground	2
Severe, limb carried	3

Figures

Figure 1



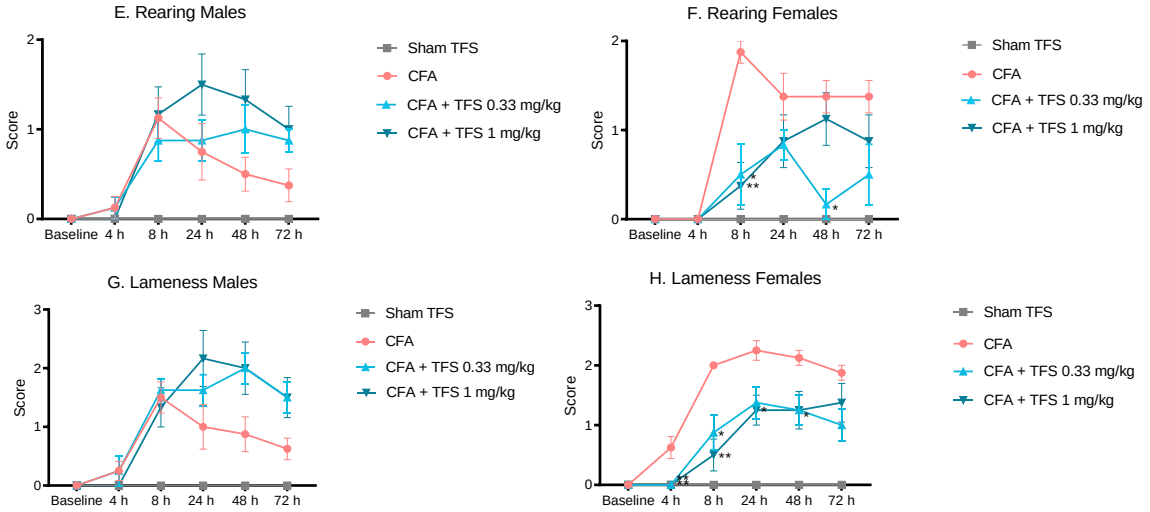

E. Rearing Males
Score
Baseline
4 h
8 h
24 h
48 h
72 h
Sham TFS
CFA
CFA + TFS 0.33 mg/kg
CFA + TFS 1 mg/kg
F. Rearing Females
G. Lameness Males
H. Lameness Females

Figure 2

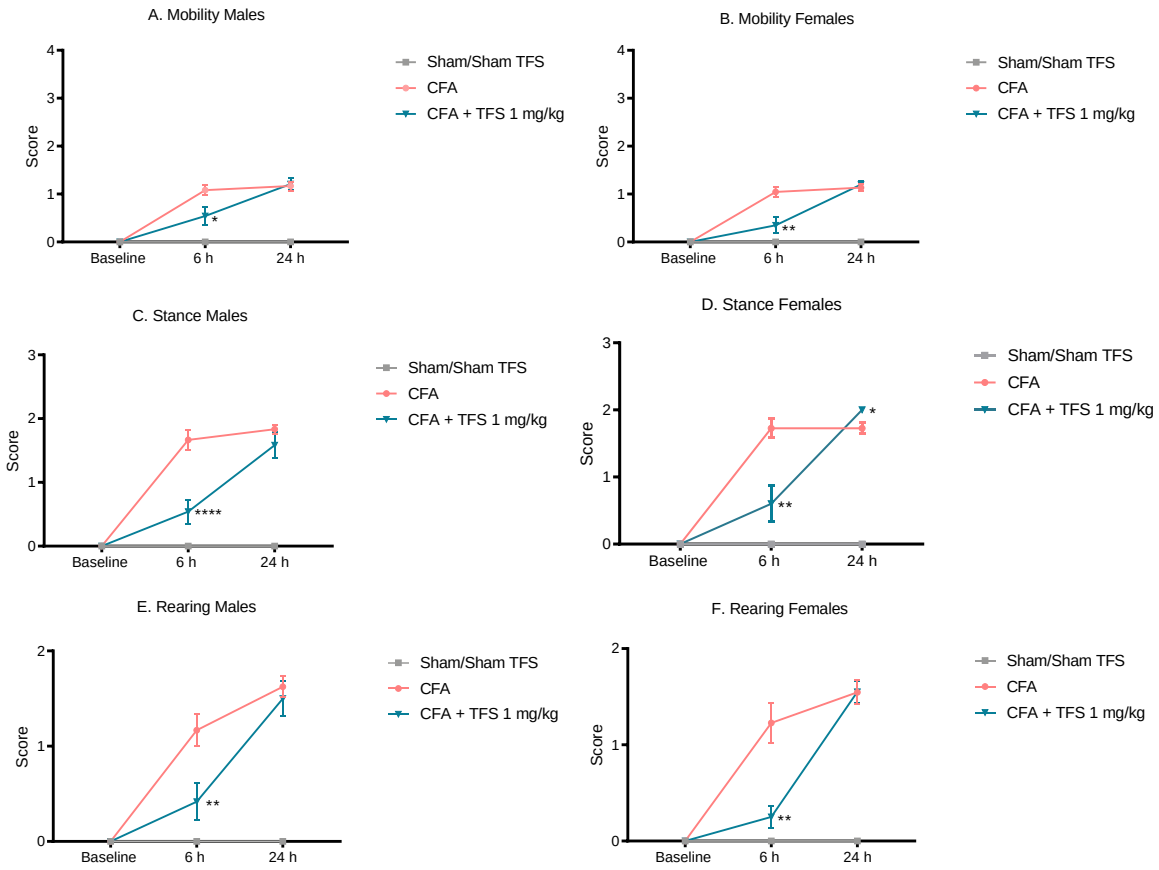
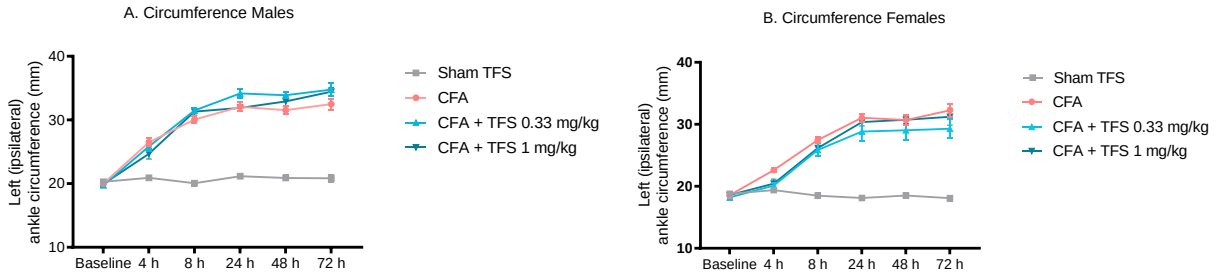

A. Mobility Males
Score
Baseline
6 h
24 h
*
Sham/Sham TFS
CFA
CFA + TFS 1 mg/kg
B. Mobility Females
**
C. Stance Males
****
D. Stance Females
E. Rearing Males
F. Rearing Females

Figure 3

Experiment 1



Experiment 2

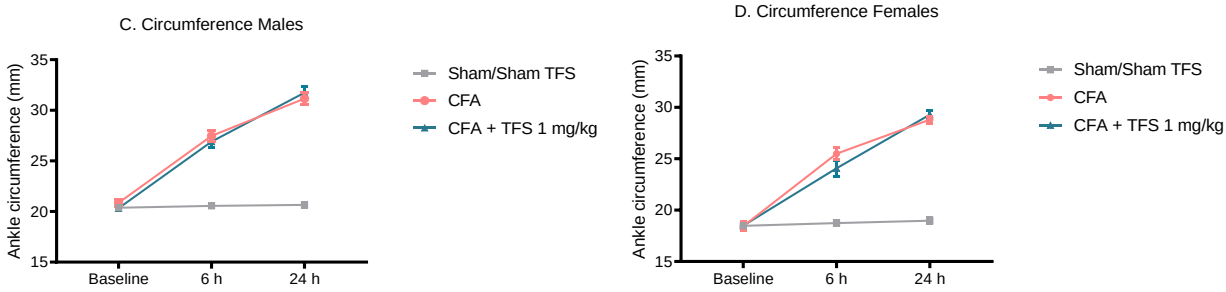
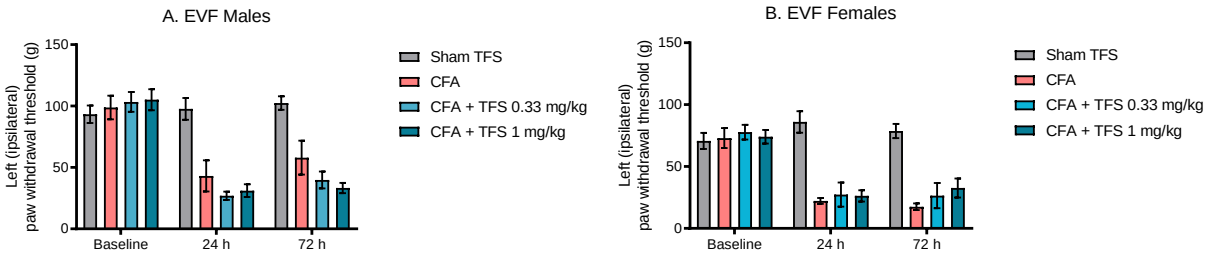
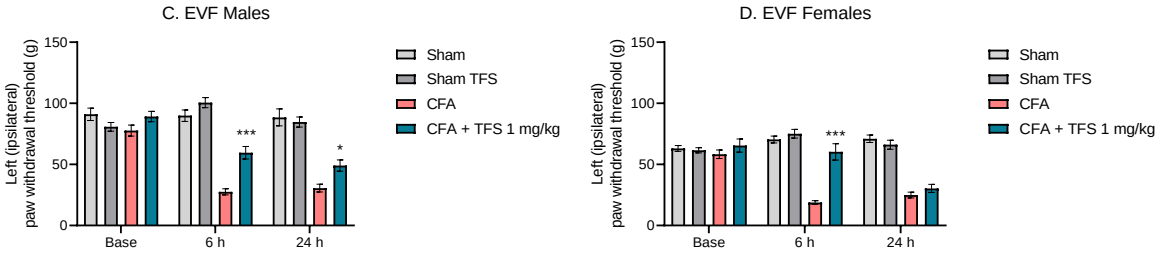


Figure 4

Experiment 1



Experiment 2



Supplementary materials

Figure S1. All data from EVF testing in Experiment 1 and 2.