SRC KINASE PLAYS A SEXUALLY DIMORPHIC ROLE IN THE MECHANISM UNDERLYING INTERMITTENT FASTING ENHANCED OPIOID ANTINOCICEPTION

By

CALEB ALAN SEEKINS

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Approved by:

Dr. John Streicher
Department of Pharmacology

Abstract

With its variety of positive reported effects, intermittent fasting was previously hypothesized to decrease negative outcomes associated with opioids. Ultimately, intermittent fasting combined with opioid therapy was shown to increase the antinociceptive effect of the opioids while decreasing the negative side effects, including abuse liability ([1]). In this study we thus attempted to elucidate the mechanism behind this enhancement of opioid antinociception by intermittent fasting. Proteomics was first performed in the spinal cord and significant differences in Src inhibitor 1 (Srcin1) was found between fasted and ad libitum mice, suggesting a role for Src kinase in this pathway. Western blotting was conducted, the results of which confirmed that in spinal cords Src kinase phosphorylation was increased by intermittent fasting. Next, Src inhibitor was administered to opioid treated fasted mice, which showed that enhanced opioid antinociception was abolished in male mice but not females. This sexually dimorphic effect was supported by immunohistochemistry, which showed that only in intermittent fasted opioid stimulated male mice, Src phosphorylation was greatly increased in the dorsal horn. Finally, colocalization was performed, though these results are preliminary, and no significant results have currently been found. Ultimately, these results suggest that decreased Srcin1 levels cause an increase in Src phosphorylation, regardless of sex, following intermittent fasting. Following opioid treatment, the dorsal horn of intermittent fasting male mice has increased Src phosphorylation that mediates enhanced antinociception. This has uncovered a role for Src kinase in intermittent fasting and opioid antinociception, as well as sexual dimorphism seen in both.

Introduction

Despite being one of the most powerful available treatments for pain, opioid drugs come with a variety of negative side effects. The most common of these side effects include constipation, respiratory depression, and sedation, though many others can be present ([2]). These negative effects are so common that the majority of patients will experience at least one during opioid use ([3]) and as a result have a decreased quality of life ([4]). Opioids have also been shown to have a high propensity for abuse which has an associated economic cost of 78.5 billion in the United States alone ([5]). Despite this, managing the negative effects of opioid has remained difficult and poorly understood ([6]).

In recent years intermittent fasting has become increasingly common for many of its reported benefits. The most direct of these benefits is seen in obesity and diabetes, both of which have seen significant improvement when intermittent fasting is implemented ([7], [8]). Diverse benefits in the body have also been observed, including decreased hepatic steatosis ([9]), decreased atherosclerosis ([7]), and increased cognitive performance ([10]). There has also been evidence that intermittent fasting can work in synergy with pharmaceuticals, such as in metformin based chemotherapy ([11]).

With the broad benefits of intermittent fasting and the goal of decreasing negative outcomes associated with opioids, our lab previously explored intermittent fasting as a potential pathway to this goal. Intermittent fasting in combination with opioid therapy was ultimately shown to increase the analgesic effect of the opioids while decreasing the negative associated side effects, including abuse liability ([1]). Research into the mechanism behind this effect is of keen interest, as a clear understanding could allow for the development of more effective opioids ([12]) or biased agonists which favor this pathway and its associated benefits ([13]). Furthermore, understanding this pathway could allow for the creation of drugs that could be taken in combination with opioids to replicate this effect. Similar effects have already been seen in the use of opioids in combination with cannabinoids ([14]), gabapentin ([15]), ziconotide ([16]) and α_2-adrenoreceptors agonists ([17]).

In this study we thus sought to understand the pathway behind intermittent fasting enhanced opioid antinociception. We ultimately found that inhibition of Src kinase eliminated the enhanced antinociception in male mice though not in females. Further exploration revealed Src phosphorylation to increase in both sexes after an intermittent fasting regimen. Despite this, the dorsal horn of fasted males treated with an opioid agonist had far more phosphorylated SRC than controls or their female counterparts. This ultimately indicates that intermittent fasting enhanced opioid antinociception is reliant on increased Src phosphorylation in the dorsal horn of male mice, but an alternative mechanism is responsible in females. These findings suggest new mechanisms for enhancing opioid antinociception while decreasing side effects, though further research is needed to investigate the remaining pathway and the sex differences described.

Materials and Methods

Materials:

DAMGO (#11711) and Src-inhibitor 1 (Src-I1) (#36-421-0R) were purchased from Fisher Scientific (Waltham, MA). Morphine sulfate pentahydrate was obtained through the National Institute on Drug Abuse Drug Supply Program and distributed through the Research Triangle Institute. Src-I1 was prepared as stock solutions in dimethyl sulfoxide (DMSO) and DAMGO was prepared as a 10 mM single use stock solution in water. Morphine was prepared fresh for each experiment in United States Pharmacopeia saline (USP Saline). Powders were stored as recommended by the manufacturer, and stock solutions were stored at -20^{o}C. Appropriate vehicle controls were used for each experiment: water for DAMGO and 10% DMSO, 10% Tween80 and 80% sterile water for Src-I1 intrathecal injections.

Animals:

Male and female CD-1 mice in age-matched controlled cohorts from 5 to 8 weeks of age were used for all experiments and were obtained from Charles River Laboratories (Wilmington, MA). Male and female mice were used in about equal numbers in each experiment; sex differences were observed, so sexes were not combined. CD-1 [also known as Institute for Cancer Research (ICR)] mice are commonly used in opioid research and in our previous work as a line with a strong response to opioid drugs ([1], [18]). Mice were allowed to recover for at least 5 days after shipment before being used in experiments. The mice were kept in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited vivarium at the University of Arizona under temperature control and 12-hour light/dark cycles with water available ad libitum. For control mice, standard laboratory chow was available ad libitum. Intermittent fasting (IF) mice were provided 5 g/mouse of laboratory chow from the periods of 8 AM to 2 PM (6 hours) ([1]). This feeding period is notably during the light cycle, the circadian inactive period for mice. Any leftover food was removed at the end of this period and repeated the next day for 1 week. No more than five mice were kept in a cage. The animals were monitored daily, including after surgical procedures, by trained veterinary staff. All experiments performed were in in accordance with Institutional Animal Care and Use Committee- approved protocols at the University of Arizona and according to the guidelines of the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* handbook.

Behavioral experiments:

Before any behavioral experiment or testing the animals were brought to the testing room in their home cages for at least 30 minutes for acclimation. Testing always occurred with the same approximate time of day between experiments, and environmental factors (noise, personnel, and scents) were minimized. All testing apparatus were cleaned between uses with 70% ethanol and allowed to dry. The experimenter was blinded to treatment group by another laboratory member coding the cages of mice, which were then decoded after collection of all data.

Tail-flick assay:

Mice were either subjected to fasting regime or control over 7 days. Tail-flick baselines were then determined in a 52^{o}C warm water tail-flick assay with a 10 second cutoff time ([18]). The mice were then injected intrathecally with Src-I1 or vehicle control. 10 minutes post injection mice were then injected with morphine (3.2 mg/kg subcutaneously), and tail-flick latencies were determined over a 2-hour time course. No animals were excluded from these studies.

Western Blotting and analysis:

Mice were either subjected to fasting regime or control over 7 days. These mice were randomly assigned to intrathecal injection of DAMGO or control. 10 minutes post injection mouse spinal cords were harvested and flash frozen. Spinal cord lysates were prepared using our previously published protocol ([18]) and quantified with a bicinchoninic acid protein quantification assay using the manufacturer's protocol (Bio-Rad). The protein was run on precast 10% bis-tris Bolt gels (Fisher Scientific, (NQ00100BOX) using the Bolt gel apparatus and following the manufacturer's instructions. The gels were transferred to a nitrocellulose membrane (Bio-Rad) using a wet transfer system (30 V, minimum of 2 hour on ice). The blots were blocked with 5% nonfat dry milk in tris-buffered saline (TBS and incubated with primary antibody in 1% (Src) bovine serum albumin (BSA) in TBS +0.1% Tween 20 (TBST) overnight rocking at 4^{o}C. The blots were then washed three times for 5 minutes in TBST, incubated with secondary antibody (see below) in 5% milk in TBST for 1 hour of rocking at room temperature, washed again, and imaged with an Azure Biosystems Sapphire biomolecular imager. Some blots were then stripped with 25 mM glycine HCl and 1% SDS (pH 2.0) for 30 to 60 min of rocking

at room temperature before being washed and re-exposed to primary antibody. The resulting image bands were quantified using imageJ (NIH). The pSrc signals were normalized to the total Src (tSrc). The intensities were normalized to a vehicle control present on the same blot.

Immunohistochemistry:

Mice were either subjected to fasting regime or control over 7 days. These mice were randomly assigned to intrathecal injection of DAMGO or control. 10 minutes post injection mice were perfused with cold phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde in PBS. Shortly after the perfusions were complete, fixed spinal cords were extracted and immediately placed in cold 4% paraformaldehyde for ~6 hours. Spinal cords were then placed in 15% sucrose in PBS overnight, followed by 30% sucrose in PBS overnight. Spinal cords were then flash-frozen in optimal cutting temperature (OCT) compound using liquid nitrogen and sectioned with an Epredia HM 525 cryostat at a thickness of 20 µm and mounted on Kimble microscope slides. Spinal cord sections were rehydrated in PBS in preparation for free-float staining. Samples were incubated in an endogenous peroxidase blocking buffer consisting of 60% methanol and 0.3% H_2O_2 in PBS at room temperature for 30 min and then washed with PBS + 0.1% Tween 20 (PBST). They were then incubated in 5% goat serum and 1% BSA in PBST at room temperature for 1 hour. Samples were then incubated with 1:50 primary pSrc antibody in 1.5% goat serum and 1% BSA in PBST at 4^{o}C overnight. Samples were then washed with PBST and incubated with a 1:400 biotinylated secondary goat anti-rabbit immunoglobulin G (IgG) antibody in 1.5% goat serum and 1% BSA in PBST at room temperature for 1 hour. Samples were prepared as instructed using the VECTASTAIN Elite ABC horseradish peroxidase kit (#PK-6101) from PerkinElmer and the TSA Fluorescein kit (#50-199-8281, 1:100) from Fisher Scientific. NeuN and IBA1 were used at 1:400 and 1:50 respectively during the pSrc primary incubation. The secondary for NeuN and IBA1 was Alexa Fluor goat anti-mouse IgG 594, which was used at 1:200 and was added during the pSrc secondary incubation mentioned above. Sections were then mounted onto slides with Fluoromount-G mounting medium (#50-187-88) from Fisher Scientific. Sections were imaged at 4x, 10x, and 20x using an Olympus BX51 microscope. Images were analyzed using ImageJ. Dorsal horn regions were selected, and average mean intensities were measured and normalized to vehicle controls within experimental groups.

Proteomics: In-gel digestion

Mouse spinal cord protein lysates (100 µg) were prepared as for Western blot from animals that were treated with either intermittent fasting or ad libitum control (*N* = 3 each) and were separated on a 10% SDS-PAGE gel and stained with Bio-Safe Coomassie G-250 Stain. Each lane of the SDS-PAGE gel was cut into six slices, and the gel slices were subjected to trypsin digestion. The resulting peptides were purified by C18-based desalting exactly as previously described ([19], [20]).

Proteomics: Mass spectrometry and database search

HPLC–electrospray ionization–MS/MS was performed in positive ion mode on a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer fitted with an EASY-Spray Source (Thermo Scientific, San Jose, CA). NanoLC was performed exactly as previously described ([19], [20]). Tandem mass spectra were extracted from Xcalibur "RAW" files, and charge states were assigned using the ProteoWizard 3.0 msConvert script using the default parameters. The fragment mass spectra were searched against the *Mus musculus* SwissProt_2018_01 database (16,965 entries) using Mascot (version 2.6.0; Matrix Science, London, UK) using the default probability cutoff score. The search variables that were used were as follows: 10–parts per million mass tolerance for precursor ion masses and 0.5 Da for-product ion masses; digestion with trypsin; a maximum of two missed tryptic cleavages; and variable modifications of oxidation of methionine and phosphorylation of serine, threonine, and tyrosine. Cross-correlation of Mascot search results with X! Tandem was accomplished with Scaffold (version Scaffold_4.8.7; Proteome Software, Portland, OR). Probability assessment of peptide assignments and protein identifications were made using Scaffold. Only peptides with ≥95% probability were considered.

Label-free quantitative proteomics

Progenesis QI for proteomics software (version 2.4; Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK) was used to perform ion intensity–based label-free quantification as previously described ([20]). In an automated format, RAW files were imported and converted into two-dimensional maps (*y* axis, time and *x* axis, *m/z*), followed by selection of a reference run for alignment purposes. An aggregate dataset

containing all peak information from all samples was created from the aligned runs, which was then further narrowed down by selecting only +2, +3, and +4 charged ions for further analysis. The samples were then grouped, and a peak list of fragment ion spectra from only the top eight most intense precursors of a feature was exported to a Mascot generic file (.MGF) format and searched using Mascot (version 2.4; Matrix Science, London, UK) with the same search variables as described above. The resulting Mascot.XML file was then imported into Progenesis, allowing for peptide/protein assignment, whereas peptides with a Mascot ion score of <25 were not considered for further analysis. Protein quantification was performed using only nonconflicting peptides, and precursor ion abundance values were normalized in a run to those in a reference run (not necessarily the same as the alignment reference run).

Antibodies:

The antibodies used were as follows: pSrc, Tyr 416 (1:500 for Western blots; Cell Signaling Technology, 6943S, lot 2341153, rabbit), tSrc (1:1000; Cell Signaling Technology, 2110S, lot 9, mouse), pSrc (1:50 for IHC; Cell Signaling Technology, 6943S, lot 10, rabbit), NeuN (1:400; Abcam, ab104224, lot GR3247200-1, mouse), IBA1 (1:50; Fisher Scientific, PIMA527726, lot XE3579184A, mouse), secondary GαM680 (1:10,000; LI-COR, 926-68020, lot C50721-02, goat), secondary GαR800 (1:5,000 to 1:10,000; LI-COR, 926-32211, lot C50602-05, goat), secondary Alexa Fluor goat anti-rabbit IgG 488 (1:200; Fisher Scientific, A11034, lot 2110499, goat) and secondary Alexa Fluor goat anti-mouse IgG 594 (1:200 to 1:400; Fisher Scientific, A21135, lot 1985396, goat).

Statistical analysis:

All data were reported as means +/- SEM and normalized where appropriate as described above to total protein and/or vehicle control groups. The behavioral data were reported raw without maximum possible effect or other normalization. Biological and technical replicates are described in the figure legends. Comparisons between groups were performed by one-way analysis of variance (ANOVA) (Western, IHC) or two-way ANOVA (behavior). In all cases, significance was defined as $P < 0.05$. All graphing and statistical analyses were performed using GraphPad Prism 9 (San Diego, CA).

Results

Intermittent fasting increases Src Kinase phosphorylation within mouse spinal cords

Our previous paper showed that intermittent fasting enhanced opioid antinociception cannot be explained by altered pharmacokinetics, alternate opioid receptor engagement, or increased opioid receptor expression ([1]). Thus, we hypothesized that the mechanism behind this effect must be through an alternate molecular signaling change. Proteomics was performed on intermittent fasted mice which showed a significant decrease in Src-inhibitor 1 (Srcin1) in IF mice when compared to control. Since Srcin1 is an endogenous inhibitor of Src Kinase, this suggested that intermittent fasted mice had increased Src phosphorylation when compared to control and could be responsible for the observed enhancement in opioid pain relief by intermittent fasting (Figure 1).

To confirm these results, Western blotting was performed on intermittent fasting mice treated with an opioid agonist (DAMGO), as well as appropriate controls (Figure 2). Similar results were seen in both male (Figure 2B) and female (Figure 2C) mice and thus the data was combined (Figure 2D). These results showed that fasting increased the ratio of phosphorylated Src to total Src in fasting mice, regardless of treatment with opioid agonists. These results confirm that intermittent fasting increases Src phosphorylation, indicating a potential role in the signaling pathway that ultimately leads to enhanced opioid antinociception. Interestingly, though not significant, ad libitum control mice treated with DAMGO showed a trend of increased ratio of phosphorylated Src to total Src, indicating a potential role for Src in the opioid signaling cascade at baseline (Figure 2).

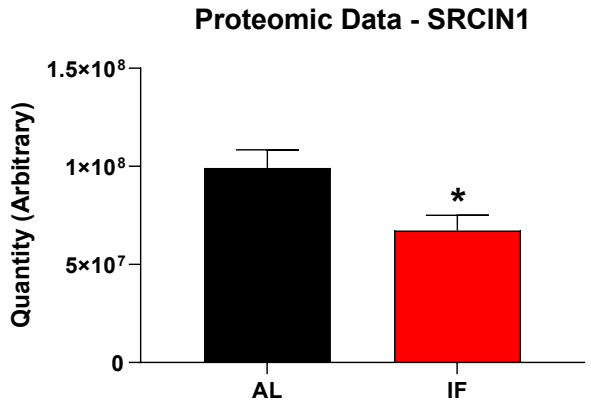

Figure 1: Intermittent Fasting Decreases Levels of Src Inhibitor 1 (Srcin1). Mice were treated with an 18/6 hour IF protocol for 7 days, along with ad libitum (AL)- fed controls. On day 8 spinal cords were harvested and flash frozen. Gel electrophoresis

was conducted with equal protein concentrations in each lane. These lanes were then separated and subjected to trypsin digestion. The peptides were then purified by C-18 based desalting. HPLC-electrospray-ionization—MS/MS performed in positive ion mode. Raw data was then extracted, analyzed, and compared between AL and IF groups. Data reported as the mean +/- SEM. Significant differences between the AUC data was determined via an unpaired two-tailed t *test. * = P , 0.05.*

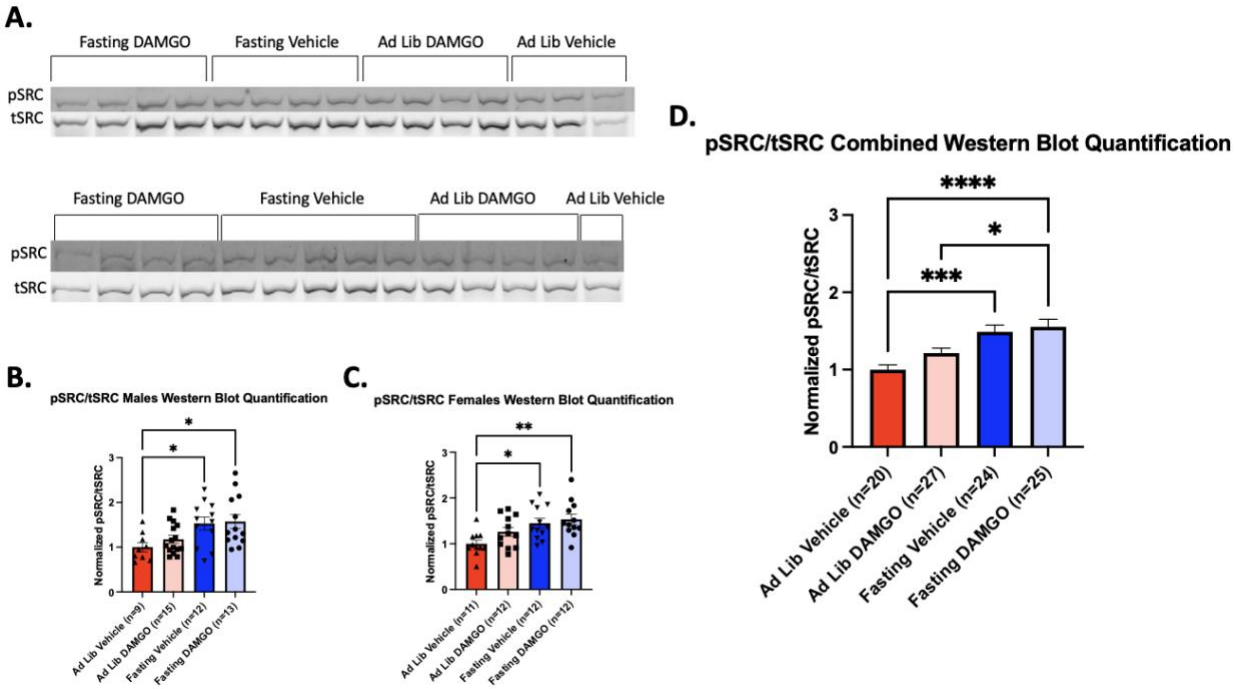

Figure 2: Intermittent Fasting Increases Src Phosphorylation Within Mouse Spinal Cords. Male and female CD-1 mice were treated with an 18/6 hour IF protocol for 7 days, along with ad libitum (AL)- fed controls. On day 8 mice were randomly assigned to intrathecal injection of DAMGO or control, and spinal cords were harvested. Resulting spinal cords were prepared following a standard western blotting protocol. Western blots were imaged using an Azure Biosystems Sapphire biomolecular imager (A). Quantification of the resulting images was conducted using Image J. The pSrc signals were normalized to the total Src (tSrc). The intensities were normalized to a vehicle control present on the same blot. This was done for both male (B) and female (C) mice. Similar results were seen for both sexes and thus results were combined (D). Significant differences between the data was determined via a One-way ANOVA with Sidak post hoc., **, ***, *** P , 0.05, 0.01, 0.001, .0001. Of note, regardless of opioid treatment, intermittent fasting significantly increased Src phosphorylation when compared to Ad Lib Vehicle.*

Inhibition of Src Kinase in the spinal cord inhibits intermittent fasting enhanced opioid antinociception in male mice.

To further investigate the role of Src Kinase we administered Src Inhibitor 1 (Src-I1) into the spinal cord of intermittent fasted mice. Src-I1 was administered intrathecally, 10 minutes later 3.2 mg/kg of morphine was given subcutaneously, and a tail flick assay was performed. Results show that the enhanced opioid antinociception normally induced by intermittent fasting was significantly decreased when Src inhibitor was given to male mice (Figure 3A, Figure 3C). Interestingly, female mice did not see a significant decrease in intermittent fasting enhanced opioid antinociception (Figure 3B, Figure 3D).

This indicates that this behavior is reliant on Src phosphorylation in male mice but not in females, indicating a potentially different signaling cascade.

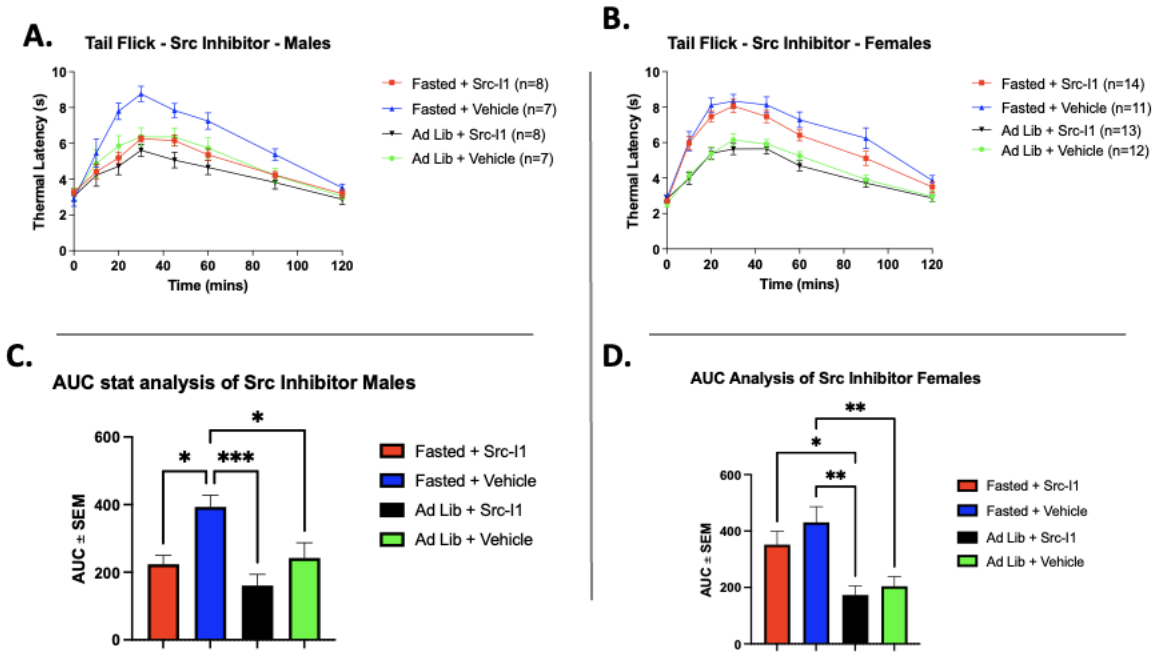

Figure 3: Intermittent Fasting Enhanced Opioid Antinociception is Src Mediated in Male Mice. Male (A) and female (B) CD-1 mice were treated with an 18/6 hour IF protocol for 7 days, along with ad libitum (AL)- fed controls. On day 8 a baseline tail flick was performed, and intrathecal injection of 10 nM Src Inhibitor 1 (Src-I1) or corresponding vehicle was administered. 10 minutes post Src Inhibitor administration, 3.2 mg/kg of morphine was administered subcutaneously, and a two-hour tail-flick time course was performed. Data reported as the mean +/- SEM with the sample size of mice/group noted in each graph. Data plotted as the area under the curve (AUC) +/- SEM for male (C) and female (D) groups. Two-way ANOVA was used to determine differences between groups for tail flick with a Sidak post hoc. Significant differences between the AUC data was determined via a One-way ANOVA with Sidak post hoc., **, ***, P , 0.05, 0.01, 0.001. Of note, a significant difference is seen between intermittent fasting mice and ad libitum mice administered Src inhibitor 1 in males (C), though this difference is not seen within female mice (D). Src kinase thus mediates intermittent fasting enhanced opioid antinociception for males but not females. ANOVA, analysis of variance; AUC, area under the curve.*

Intermittent fasting in combination with opioid stimulation selectively phosphorylates Src Kinase in the spinal dorsal horn of male mice.

As seen in Figure 2, intermittent fasting alone significantly increases Src Kinase phosphorylation in both male and female mice. Despite this, as seen in Figure 3, inhibition of Src only significantly inhibits intermittent fasting enhanced opioid antinociception in male mice. We thus hypothesized that this may be due to selective phosphorylation of Src Kinase within specific regions of the spinal cord. To determine this, immunohistochemistry was performed on perfused spinal cord tissue from mice treated with DAMGO as in our previous Western blotting.

Immunohistochemistry showed a significant increase in phosphorylated Src Kinase in the dorsal horns of male mice that had undergone intermittent fasting and been given DAMGO (Figure 4A, Figure 4C). In the remaining controls and female mice, there was not a significant increase in phosphorylated Src Kinase when compared to non-fasted mice given vehicle (Figure 4B, Figure 4D). The dorsal horn plays an important role in processing sensory information, including pain. This suggests that the intermittent fasting enhanced opioid antinociception in males is due to increased Src Kinase phosphorylation within the dorsal horn. This is in-line with the previous results showing that inhibiting Src phosphorylation will inhibit enhanced opioid pain relief in male mice (Figure 2).

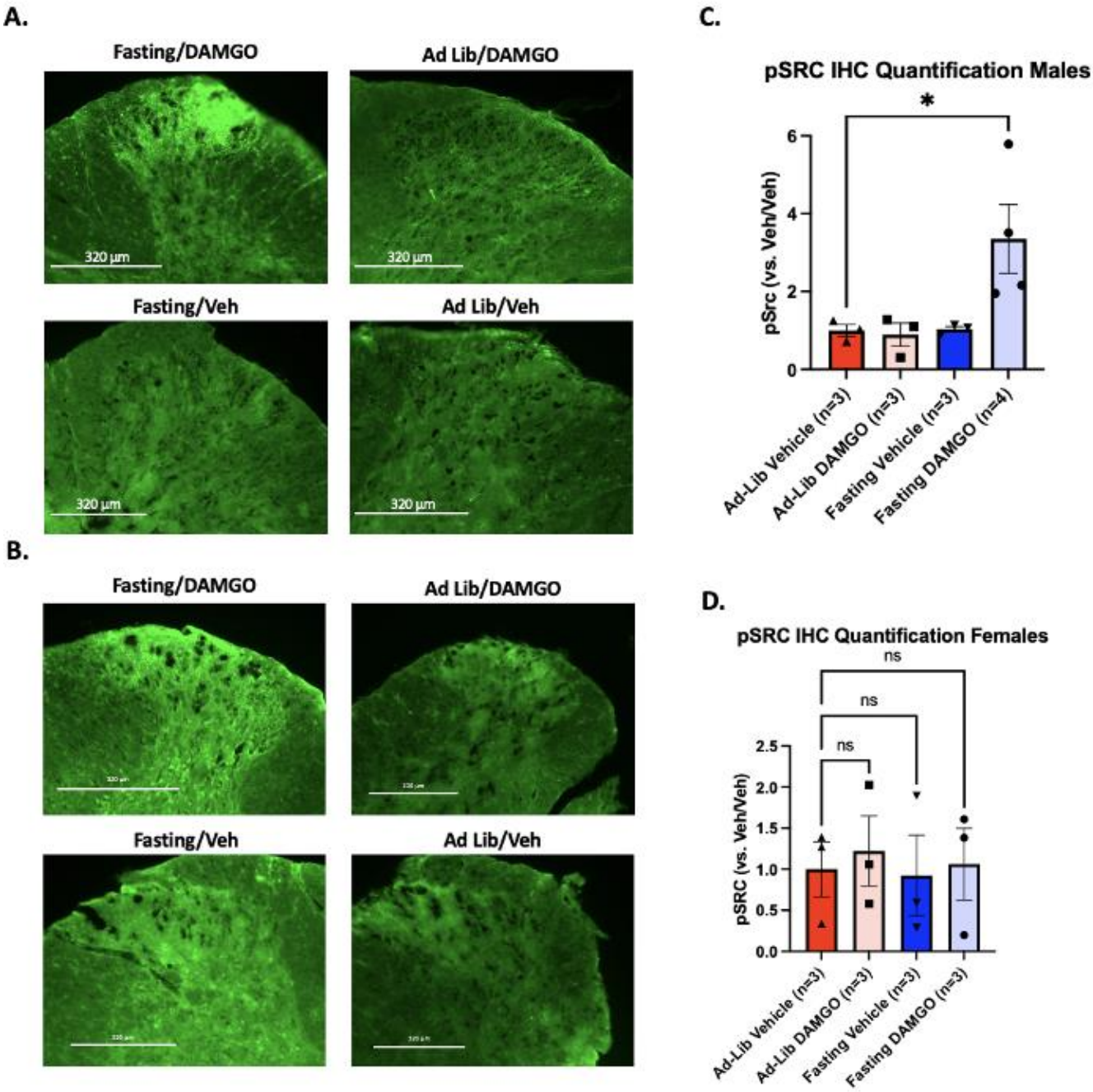

Figure 4: Intermittent Fasting Increases pSrc in Dorsal Horn of Male Mice. Male (A) and female (B) CD-1 mice were treated with an 18/6 hour IF protocol for 7 days, along with ad libitum (AL)- fed controls. On day 8 mice were randomly assigned to intrathecal injection of DAMGO or control, perfusion with PFA was performed, and spinal cords were harvested. Resulting spinal cords were cut using a cryostat to a thickness of 20 um and rehydrated in PBS. Slices were treated with antibodies for phosphorylated Src (pSrc) and with the TSA Fluorescein kit. Slices were mounted and imaged using an Olympus BX51 microscope. Quantification of the resulting images was conducted using Image J. The mean intensity within the dorsal horn was measured and subtracted from background. This was then normalized to the Ad-Lib Vehicle group for males (C) and females (D) and reported as the mean +/- SEM. Significant differences between the data was determined via a One-way ANOVA with Sidak post hoc., **, ***, *** P , 0.05, 0.01, 0.001, .0001. Of note fasting combined with opioid stimulation significantly increased Src phosphorylation in the dorsal horn of male mice but not female mice.*

Preliminary intermittent fasting induced Src Kinase stimulation localization.

To further investigate differences in intermittent fasting-induced Src kinase stimulation between male and female mice localization of this stimulation to specific cell types was attempted. Co-staining was first performed with the neuron marker neuronal nuclei (NeuN), in both male (Figure 5A) and female (Figure 5B) mice. Comparison of average Pearson correlation coefficients indicate that pSrc appears to colocalize with NeuN to a greater extent in females than males, though significance is not achieved with a p value of only .0883. Next, we co-stained with ionized calcium binding adaptor molecule 1 (IBA1), a marker for microglia, in both male (Figure 6A) and female (Figure 6B) mice. Comparison of average Pearson correlation coefficients indicate that pSrc appears to colocalize with IBA1 to an equal extent in males and females, though sample size for males is too low to determine if this is significant. These preliminary results indicate that differential Src Phosphorylation in neurons may partially be responsible for the observed difference in Src phosphorylation in male and female mice, though significance has not been obtained and thus conclusions cannot be drawn.

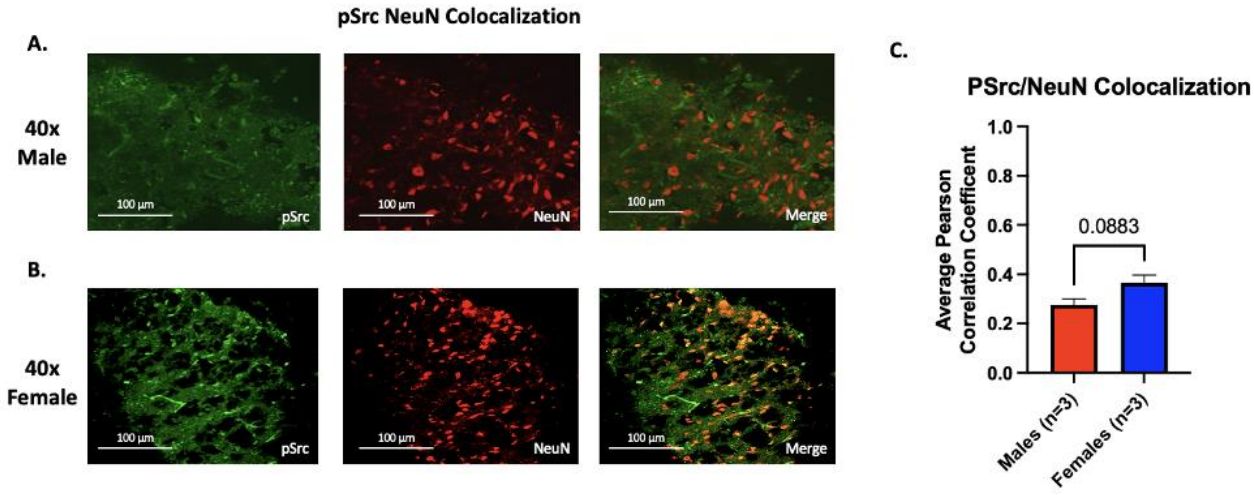

Figure 5: pSrc Colocalizes with Neurons to a Greater Extent in Females then Males. Male (A) and female (B) CD-1 mice were treated with an 18/6 hour IF protocol for 7 days. On day 8 mice were administered intrathecal injection of DAMGO, perfusion with PFA was performed, and spinal cords were harvested. Resulting spinal cords were cut using a cryostat to a thickness of 20 um and rehydrated in PBS. Slices were treated with antibodies for phosphorylated Src (pSrc), NeuN (neuronal marker), and with the TSA Fluorescein kit. Splices were mounted and imaged using a Confocal microscope at 40x magnification and Pearson correlation coefficient obtained. Pearson correlation was averaged for a minimum of 3 slices per mouse. Results are reported as the mean +/- SEM for males and females (C). Significant differences between the data was determined via an unpaired 2-tailed t *test. *, **, ***, *** P , 0.05, 0.01, 0.001, .0001. Of note pSrc appears to colocalize with NeuN to a greater*

extent in females than males, though significance is not achieved with a p value of .0883. Further experimentation is required to determine if this trend is significant.

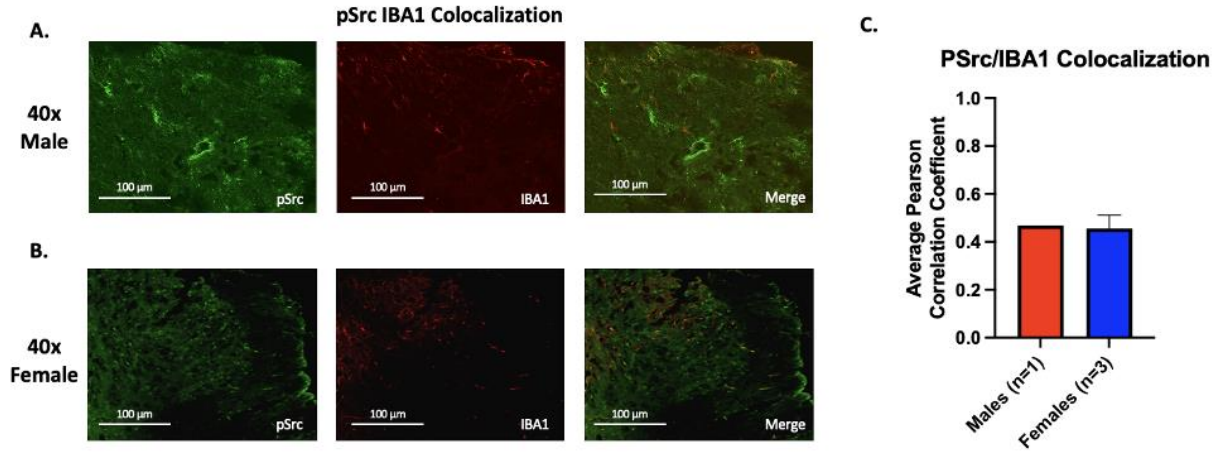

Figure 6: Preliminary Results Indicate pSrc Colocalizes with IBA1 to an Equal Extent in Males and Females. Male (A) and female (B) CD-1 mice were treated with an 18/6 hour IF protocol for 7 days. On day 8 mice were administered intrathecal injection of DAMGO, perfusion with PFA was performed, and spinal cords were harvested. Resulting spinal cords were cut using a cryostat to a thickness of 20 um and rehydrated in PBS. Slices were treated with antibodies for phosphorylated Src (pSrc), IBA1 (microglial marker), and with the TSA Fluorescein kit. Slices were mounted and imaged using a confocal microscope at 40x magnification and Pearson correlation coefficient obtained. Pearson correlation was averaged for a minimum of 3 slices per mouse. Results are reported as the mean +/- SEM for males and females (C). Further analysis was not conducted, due to a sample size of 1 in males. Of note pSrc appears to colocalize with IBA1 to an equal extent in males and females, though sample size for males is too low to determine if this is significant. Further, experimentation is required to determine if this trend is significant.

Discussion

Within this paper a sexually dimorphic response was observed within the intermittent fasting enhanced opioid antinociception signaling pathway. Specifically, decreased levels of Src Inhibitor 1 (Srcin1) after intermittent fasting caused greater overall Src kinase phosphorylation in both male and female mice. Despite this, after combination of intermittent fasting and opioid treatment male mice specifically have greatly increased Src kinase phosphorylation in the dorsal horn of the spinal cord. As the dorsal horn of the spinal cord plays a key role in pain signaling, this indicates a potential sexually dimorphic role for Src kinase in the intermittent fasting enhanced opioid antinociception pathway. This is in line with behavioral data that indicates that inhibition of Src kinase will abolish this enhanced opioid antinociception in male but not female mice. Finally, preliminary results show that Src

stimulation may be colocalized to a greater extent with neuronal bodies in female mice than males, providing further evidence for the sexually dimorphic role of Src in this pathway. Combined, these results highlight a sexually dimorphic key role for Src kinase in the intermittent fasting enhanced opioid antinociception signaling pathway.

While this is the first description of this specific effect, it is not necessarily without precedent, as intermittent fasting has repeatedly been shown to have sexually dimorphic responses. As the most studied conditions in relation to intermittent fasting, these sex differences are most often seen in diabetes and obesity research. This includes sexual differences in plasma insulin ([21], [22]), anabolic and catabolic pathways ([23]), and musculoskeletal improvements ([24]) following intermittent fasting protocols. Interestingly, there has also been reports of sexually dimorphic effects on the nervous system. These effects range from increased sympathetic axonal fiber density ([21]), increased spatial memory ([24]), decreased development of anxiety related behaviors ([24]), and increased production of neuroprotective H_2S ([24]) following intermittent fasting. Furthermore, this sexually dimorphic effect can be seen within the immune system as inflammatory markers were seen to have unique changes between sexes post intermittent fasting ([21], [24]). Our paper shows that Src kinase may play an important role in the sexual differences in intermittent fasting. Pertinent future research could include investigating Src kinase's role in modulating the other sex dependent effects of intermittent fasting, outside of our described effect.

Further support for our sexually dimorphic response to intermittent fasting enhanced opioid antinociception is the repeated observations of sex dependent responses to opioids. Most obviously, it has repeatedly been observed that opioids are more effective within males ([25], [26]). One major cause of this effect is sexual hormones, as male sexual hormones have generally been shown to increase opioid efficacy, while female sex hormones have had negative or mixed results ([25], [26]). An alternative cause can be found in structural differences in major pain circuitry such as the PAG-RVM, whose sexual differences have been shown to modulate opioid effectiveness ([25], [26]). Finally, various other effects such as the MOR/KOR heterodimer ([27])([28]) and the modulation of microglia ([25]) have also been shown to have sex dependent effects on opioid antinociception. Ultimately, while a single one of these observations cannot account for the sexual differences observed within our experiment, the repeated sexual dimorphism found in opioid signaling supports our observation of a similar effect. Furthermore,

our paper adds to this body of research by showing that Src kinase may play an important role in sexual differences in opioid antinociception. Further research into how Src contributes to these differences is necessary to connect it to the various other effects described above.

Within this paper it was uncovered that the intermittent fasting enhanced opioid antinociception is dependent on Src kinase and this effect can be inhibited through the administration of a Src inhibitor. This would suggest that Src kinase plays a role in enhancing antinociception, though there is significant evidence suggesting the opposite. For one, administration of a Src inhibitor in a cancer induced bone pain model has been shown to attenuate mechanical ([29]) and thermal hypersensitivity ([30]). Furthermore, decreasing Src phosphorylation indirectly, through the inhibition of protein tyrosine phosphatases, was also shown to decrease hypersensitivity and inflammatory pain ([31]). Finally, Src activation has been to show to upregulate NMDARs, whose activation plays a key role in the development of hypersensitivity in formalin induced pain ([32]), sensitization of neurons ([33]), and activation of microglia to a pro-inflammatory state ([34]). Interestingly, inhibition of Src within these models seem to only be effective in decreasing chronic pain. When measurements of acute pain were attempted, Src inhibition was shown to have no effect ([30], [31]). In fact, there is evidence that in models of acute pain, Src activation may play a key role in antinociception, and its inhibition prevents this effect ([35]). Furthermore, there is evidence that the inhibition of Src may increase inflammatory cytokines and decrease anti-inflammatory cytokines ([36], [37]). Combined, these results suggest that Src kinase activation may play a unique role by being antinociceptive in acute pain while being pronociceptive in chronic pain. Further research into this effect could provide greater insight into the mechanism behind this, as well as how this could be targeted to create medications that target chronic/acute pain specifically.

The pathway uncovered within this paper shows a sexually dimorphic role for Src kinase in intermittent fasting enhanced opioid antinociception signaling. Despite this, further research is required to fully understand this pathway. Particularly, at present this Src phosphorylation has not shown significant difference in cell type colocalization and thus further experimentation is necessary. Furthermore, while Src kinase does not modulate the effect in female mice, a similar enhanced opioid antinociception is seen following intermittent fasting. This would suggest further research into the pathway that modulates this effect in females is necessary. Research into the remaining signaling

pathway for this effect in males is also still not well understood. As Src plays a key role in a variety of signaling pathways, understanding this mechanism could allow for greater understanding of its role within the body. Finally, future research into the role of Src kinase in intermittent fasting and pain signaling is necessary. As intermittent fasting has been shown to have a variety of benefits, understanding Src Kinases role could allow for development of new drugs that exploit its effect. Furthermore, Src kinase is shown to play a key sexually dimorphic role here, thus understanding the role of Src kinase could allow for greater understanding of the difference in the treatment of pain between males and females. Finally, the difference in Src Kinases role in chronic/acute pain should be investigated, to determine how to treat these pain states more effectively.

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