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EFFECTS OF NEONATAL CAPSAICIN ON FORMALIN-INDUCED ATF3 EXPRESSION IN THE ADULT RAT

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A Thesis Approved on

June 10, 2016

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DEDICATION

This thesis is dedicated to my parents

who have continually supported me in reaching my goals

and encouraged me to push even further

ABSTRACT

EFFECTS OF NEONATAL CAPSAICIN ON FORMALIN-INDUCED ATF3 EXPRESSION IN THE ADULT RAT

Sarah Krupp

June 10, 2016

Acute and chronic pain can be extremely debilitating conditions, and a better understanding of their underlying pathology is necessary to provide effective treatment. C-Fibers are responsible for transmission of the majority of nociceptive signals, with TRPV1+ C-Fibers being specific to noxious heat. Formalin, an inflammatory agent, acts through TRPA1+ C-Fibers, which have a high degree of co-localization with TRPV1. Using a neonatal capsaicin preparation, which irreversibly ablates the majority of TRPV1+ C-Fibers, formalin-induced inflammation and subsequent ATF3 expression was investigated. Results provide evidence that in addition to a lack of thermal nociception, animals treated with neonatal capsaicin had a lower threshold for mechanical nociception. Furthermore, neonatal capsaicin treatment reduced formalin-induced allodynia and prevented formalin-induced hyperalgesia. Lastly, treatment with neonatal capsaicin was shown to produce less small- diameter and more intermediate and large-diameter neurons in the DRG compared to vehicle-treated animals, as well as less overall ATF3 expression.

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INTRODUCTION

Acute and chronic pain can be debilitating conditions, and they affect more people than diabetes, heart disease, and cancer combined (National Institute of Health, 2016). Thus a more in-depth understanding of the mechanisms involved in pain will be helpful in implementing new methods of treatment.

C-Fibers are one of four major types of primary afferent fibers, along with Aδ-, Aβ-, and Aα-Fibers, carrying sensory information from the peripheral nervous system into the central nervous system (Matsumoto et al., 2006). C-Fibers are small diameter, unmyelinated, and maintain a slow conduction velocity. A subset of C-Fibers and thinly myelinated Ad-fibers, often termed nociceptors, are responsible for the transmission of signals stemming from a variety of painful stimuli, including thermal, mechanical and chemical stimuli (Costigan & Woolf, 2000). Much of this noxious stimuli works by activating transduction channels located on terminal (i.e. peripheral) nerve endings of nociceptors and depolarizing the neurons, causing an action potential to be fired, and thus transmission of the signal to occur (Costigan & Woolf, 2000).

One specific ion channel on a subset of nociceptive primary afferents is the transient receptor potential vanilloid 1 (TRPV1) channel, a nonselective cation channel sensitive to extreme heat, and the receptor through which capsaicin

exerts its effects (Komaki & Esteky, 2005; Newson et al, 2014). In the adult, capsaicin application first activates these fibers, causing pain, followed by desensitization with continued or increased application (Komaki & Esteky, 2005). Neonatal injection of capsaicin however exerts a neurotoxic effect, irreversibly destroying almost all (~95%) C-fibers and sensory neurons expressing TRPV1 (Komaki & Esteky, 2005; Newson et al, 2014).

Activating transcription factor 3 (ATF3) is a reliable marker of nerve injury induced by a variety of noxious stimuli, including acute capsaicin (Braz & Basbaum, 2010). Capsaicin injections to adult rodents induce ATF3 in a large number of small, unmyelinated DRG neurons ipsilateral to the injection site, in both TRPV1 and non-TRPV1 expressing neurons (Braz & Basbaum, 2010). This is thought to occur through an ordered process; capsaicin directly induces ATF3 in TRPV1 expressing DRG neurons, which then indirectly induces ATF3 expression in neighboring non-TRPV1 expressing DRG neurons through a process called cross-excitation (Braz & Basbaum, 2010). Cross-excitation refers to the concept of mediators of the primary injury, here released from TRPV1 expressing neurons, being sufficiently neurotoxic to produce a secondary injury in nearby non-TRPV1 expressing neurons (Braz & Basbaum, 2010). TRPV1 knockout mice, which still have full use of sensory fibers but simply lack TRPV1 expressing neurons as cell bodies to those fibers, are very different than rodents treated with neonatal capsaicin, in which not only are the majority of TRPV1 expressing neurons eliminated, but their corresponding fibers are ablated. Despite these major differences the two models are often used as rationale and

supporting data for one another, when it is not truly known how neonatal capsaicin injections affect ATF3 expression in fully developed DRG neurons. To investigate this process and shed a greater light on changes in remaining DRG neurons and their ability to process painful stimuli following neonatal capsaicin, 2% formalin was injected subcutaneously into the hindpaw of adult rats treated neonatally with either capsaicin or a vehicle. An often used model of persistent pain, formalin injections induce inflammation lasting approximately 60-90 minutes, which can be separated into two distinct phases (Fu et al., 2001; Shields et al., 2010). The first, or acute, phase lasts for 5-15 minutes after injection, and reflects the activation of nociceptors (Fu et al., 2001; Shields et al., 2010). There is then a short quiescent phase before the second, or tonic, phase begins, lasting from 20 minutes to around 60-90 minutes, reflecting inflammation of the peripheral and central terminals, and central sensitization of the spinal cord (Fu et al., 2001; Shields et al., 2010). Each phase involves stereotypic behaviors; the first phase is mainly characterized by a lack of weight-bearing ability on the injected paw, while the second phase exhibits the majority of biting, licking, or shaking of the paw (Fu et al., 2001; Shields et al., 2010). Importantly formalin has been shown to induce ATF3 in a dose dependent manner (Shields et al., 2010; Braz & Basbaum, 2011). Low doses (0.5%) induce ATF3 in mostly TRPA1 positive neurons, another ion channel of the TRP family, with TRPA1 knockouts have significantly lower percentage of ATF3 induced, indicating this receptor as their major mechanism of action (Shields et al., 2010; Braz & Basbaum, 2011). Higher doses (2%-5%) induce ATF3 in a wide variety of neurons, both

myelinated and unmyelinated, and TRPA1 and TRPV1 knockouts had no significant effect on the percentage of neurons expressing formalin-induced ATF3 (Shields et al., 2010; Braz & Basbaum, 2011).

To ensure effectiveness of the neonatal capsaicin treatment, the current study utilized the cutaneous trunci muscle reflex (CTMR) to measure mechanical and thermal nociception. The CTMR manifests visually as a puckering of the skin in response to cutaneous stimulation, and in two commonly used laboratory rodents, rats and mice, this reflex is nociceptive specific (Petruska et al., 2014). The cutaneous trunci muscle responsible for this reflex is a thin subdermal muscle underling the majority of the back and flank (Pan et al., 2012). Early work utilizing the CTMR concluded that while thermal nociception is ablated, mechanical nociception remains unaffected by neonatal capsaicin (Doucette et al., 1987), and additional work using the von Frey and Hargreaves tests seem to support this conclusion (Nagy & van der Kooy, 1983; Ren et al., 1994).

The current study aims to determine formalin-induced ATF3 expression following neonatal capsaicin injections with the hypothesis that there will be grossly reduced numbers of ATF3 expressing neurons in capsaicin treated rats due to the reduction of TRPV1 expressing neurons, and that this initial reduction will be accompanied by a reduction of ATF3 expressing neurons in both small and large non-TRPV1 expressing neurons as well. A secondary hypothesis concerns the rearrangement of the neuronal subtypes of the DRG, mainly that the lack of small diameter TRPV1 expressing neurons in neonatal capsaicin treated rats will allow for changes in the frequency of intermediate and large

diameter neurons, and furthermore that these changes in frequency will not correlate to frequency of formalin-induced ATF3 expression in those subtypes.

METHODS AND MATERIALS

Animals:

Six pregnant Sprague-Dawley females (E15) were obtained, individually housed, and given a week to recover from being shipped and adapt to their new housing environments, while being checked daily to identify the day or birth of their pups. At P2 liters were randomly assigned to experimental groups, and a total of 62 pups were given intraperitoneal (IP) injections of either capsaicin or a vehicle using sterile syringes. To complete injections the nest was first disturbed and pups scattered to distract the mother. Approximately half of the liter was removed at a time for injections. To anesthetize pups were individually wrapped in a gauze pad and placed on ice for approximately 3 minutes, or until movements ceased. Following this, injections were performed and pups were then placed on a towel covered heating pad and carefully monitored for the return of movements. To return pups to their home cages they were rubbed in both peanut oil and bedding removed from the home cage to cover any smell obtained throughout the procedure and limit their chances of rejection by their mother. All pups were successfully accepted by their mother. At P21 pups were weaned and separated by sex. Only males were used for the following experiments, yielding final experimental groups of neonatal capsaicin, n=10, and

vehicle treated, n=13. Males were initially triple housed, and finally double housed at the 2-month mark. There was a total 3-month maturation period following birth before beginning experiments.

Preparation of Capsaicin Injections:

Preparation of the capsaicin solution (50mg/kg) dissolved in 10% Tween-80 and 10% ethanol (v/v in 0.9% sterile saline) and vehicle solution 10% Tween-80 and 10% ethanol (v/v in 0.9% sterile saline) was performed under a fume hood, using protective equipment such as gloves, goggles, and masks as advised by the container labels.

Health Maintenance:

Over the duration of this study half of the capsaicin animals died, leaving a varying number of animals for each experiment with a minimum of n=5. Neonatal capsaicin-treated animals experienced bladder infections and all received 0.2 cc of cefazolin and 0.1 cc of baytril twice daily for 10 days as needed. Treatment with antibiotics did not overlap any injection or experimental procedure. Following antibiotics bladders were manually expressed as needed for the duration of the experiment.

Formalin Injections:

A 2% solution of formalin was prepared in saline using a formalin stock solution (37% formaldehyde) and injected in an intraplantar fashion to the left

hindpaw of all rats in 10µl volume. During the injection rats were placed under light isoflurane anesthesia and injections were performed using a sterile syringe with a 30-guage needle.

Nociception Assessments:

The following assessments were performed before (CTMR, von Frey, and Hargreaves) and after (von Frey and Hargreaves) formalin injections to both functionally confirm the ablation of C-fibers as well as assess the behavioral effects of intraplantar formalin injections.

Hargreaves: Thermal withdrawal threshold was determined using the Hargreaves device. Rats were placed in individual clear plexiglass chambers atop a glass platform kept at a basal temperature of 32^{o}, and allowed 30 minutes to acclimate. Radiant heat stimulation was applied to the medial aspect of the plantar surface of each hindpaw until a withdrawal reflex was observed, with a cutoff latency of 20 seconds to prevent tissue damage. Testing was repeated five times to produce an average, with a minimum of one minute between intervals.

Von Frey: Mechanical withdrawal threshold was determined using von Frey filaments. Rats were placed in individual clear plexiglass chambers on an elevated metal mesh and allowed 30 minutes to acclimate. Using an electrical von Frey filament mechanical force was applied to the medial aspect of the plantar surface of each hindpaw until a withdrawal reflex was observed. Testing was repeated five times to produce an average, with a minimum of one minute between intervals.

CTMR: All experiments were carried out under 40 mg/kg of pentobarbital anesthesia. The dorsal skin was shaved and beginning one inch below the C2 vertebrae a grid of dots was drawn on the skin. The grid consisted of four columns, two on either side of the midline, and eight rows of dots drawn 5mm apart using a fine tip permanent marker. The response to mechanical and thermal stimuli was tested with serrated Adson forceps constrained to deliver a consistent maximum pinch and a hot probe, respectively. The hot probe was maintained at 65-70^{0}C via a hot water bath. Cork handles were applied to the end of probes to prevent heat transfer to the experimenter throughout testing. Testing was repeated twice, fully completing the mechanical testing of one animal before moving onto thermal testing for that animal. Responses were recorded for a minimum of one minute following stimulation at a rate of 30 frames per second. Contractions with measured distances less than 0.737mm, 1 standard deviation of a control response, were qualified as a zero and omitted from statistical analysis.

Kinematic Analysis:

Following the recording of the CTMR responses, videos were analyzed via MaxTRAQ and the variables measured in CTMR were as follows:

Maximum pre-stimulus distance: Refers to the distance between points before any testing begins

Minimum stimulus distance: Refers to the minimum distance between points following application of the stimulus; the peak of contraction

Contraction distance: Calculated by subtracting the minimum stimulus distance from the maximum pre-stimulus distance

Time to minimum stimulus distance: Refers to the time between the application of the stimulus and the minimum stimulus distance

Speed to minimum stimulus distance: Refers to the speed at which the minimum stimulus distance is reached following application of the stimulus

Maximum post-stimulus distance: Refers to the maximum distance between points following application of the stimulus

Relax time: Refers to the time from minimum stimulus distance to maximum post-stimulus distance; return to 100% of the maximum pre-stimulus distance

Relax distance: Calculated by subtracting the minimum stimulus distance from the maximum post-stimulus distance

Animal Perfusion and Tissue Processing:

Upon completion of all experiments, two days post-formalin injections, animals were sacrificed with with an IP injection of 10 cc pentobarbital and transcardially perfused with paraformaldehyde (PFA) for tissue fixation as previously described (Xu et al., 2014). The L4 and L5 DRG were immediately dissected out and placed into 4% PFA for overnight fixation and transferred to 30% sucrose for a minimum of 4 days for cryoprotection. Tissue was then embedded using freezing medium and cut into 14μm sections for further analysis.

Immunohistochemistry:

L4-L5 DRG were stained for TRPV1, TRPA1, and ATF3 using the following antibodies: guinea pig anti-TRPV1 (1:1000, Neuromics GP14100) with donkey anti-guinea pig 405 (1:100 Jackson Immuno 706475148), rabbit anti-TRPA1 (1:80,000, Neuromics RA14135) with donkey anti-rabbit 488 (1:100 Jackson Immuno 711545152), and mouse anti-ATF3 (1:250 Abcam, ab58668) with donkey anti-mouse Cy3 (1:100 Jackson Immuno 715165151) preadsorbed with 20 μl rat serum and 80 μl blocking serum to prevent non-specific binding.

RESULTS

Over the duration of this study half (n=5) of the capsaicin animals died during the maturation period, before formalin experiments could begin. The major underlying causes of these fatalities were as follows: Aortic aneurism (n=1), internal bleeding (n=1), and severe infection stemming from bladder issues, leading to hydronephrosis, kidney stones, and potential kidney failure (n=3). The remaining capsaicin animals which were utilized in the experiment all had bladder infections and received antibiotics.

Effects of neonatal capsaicin on CTMR

Neonatal capsaicin treatment ablates the majority of C-fibers, and has previously been shown to suppress thermal nociception while leaving mechanical nociception unaffected (Doucette et al., 1987). Consistent with this, CTMR contractions to thermal stimulation of the neonatal capsaicin treated animals fell below 0.737 mm contraction distance (equivalent to 1 standard deviation from the vehicle-treated group mean) and were therefore omitted. In contrast to previous work which lacked kinematic analysis and rather visually observed no change in mechanical nociception of neonatal capsaicin-treated animals, the current study found statistical differences in the mechanical responses of

neonatal capsaicin-treated animals compared to vehicle-treated animals in four measures of the CTMR; minimum stimulus distance, contraction distance, speed to minimum stimulus distance, and relax distance (table 1 and fig. 1). Additionally, there were no statistical differences in four measures of the CTMR; time to minimum stimulus and relax time, as well as two measures expected to show no difference, maximum pre-stimulus distance and maximum post stimulus distance (figure 1).

Thus neonatal capsaicin-treated animals exhibited a significantly ($p < .001$) greater minimum stimulus distance to mechanical (7.79mm ±1.36) and thermal (9.83 ± 0.68) stimulation compared to vehicles-treated animals mechanical (7.51mm ±1.02) and thermal (7.77mm ±1.01) minimum stimulus distance. These greater minimum stimuli distances were associated with a significantly ($p < .001$) shorter contraction distance for neonatal capsaicin-treated animals compared to those treated with a vehicle (mech. 2.5mm ±1.16 vs. 2.74mm±0.69; therm. 0.42 ±0.22 vs. 2.06mm ±0.74). The shorter contraction distance observed in neonatal capsaicin-treated animals corresponded to a shorter relax distances compared to vehicle-treated animals (mech. 2.45mm ±1.0 vs. 2.62mm ±0.76, $p < .001$; therm. 0.48mm ±0.18 vs. 1.83mm ±0.63, $p < .001$). In addition, the speed of contraction (i.e. speed to minimum stimulus distance) was slower in the neonatal capsaicin-treated group compared to vehicle-treated animals for mechanical (1.63mm/s ±0.87 vs. 1.92mm/s ±0.59, $p = .014$) stimulation. Taken together these results are consistent with previous data showing neonatal capsaicin-induced loss of TRPV1 fibers results in the loss of thermal nociception (Nagy & van der Kooy,

1983; Doucette et al., 1987; Ren et al., 1994). However, these results also indicate the novel finding that mechanical nociception is somewhat suppressed in animals treated neonatally with capsaicin, rather than unaffected as previously reported, indicating the CTMR combined with kinematic analysis in the current experiment provided an increased sensitivity necessary to capture those changes (Nagy & van der Kooy, 1983; Doucette et al., 1987; Ren et al., 1994)

Differences in mechanical and thermal nociception

Another novel finding of the current study was the observation of differences between mechanical and thermal nociception in the CTMR. Animals treated neonatally with a vehicle, rather than capsaicin, have been shown to be indistinguishable from control animals in regards to nociception (Nagy & van der Kooy, 1983) and thus the current results of the vehicle-treated animals can be seen as those of a control animal. When looking at differences in the CTMR to mechanical vs. thermal stimulation, the most interesting results are seen in vehicle treated animals, as the neonatal capsaicin-treated animals have a very different and well-characterized separation between mechanical and thermal nociception (Nagy & van der Kooy, 1983; Doucette et al., 1987; Ren et al., 1994). Animals treated with a vehicle showed significantly different responses to mechanical and thermal stimulation in six measures of the CTMR; minimum stimulus distance, contraction distance, time to minimum stimulus distance, speed to minimum stimulus distance, relax time, and relax distance (fig. 1 and table 2). The 2 additional measures showing no statistical differences were

maximum pre- and post-stimulus distance, which is expected given the relaxation of the contraction (fig. 1).

Vehicle-treated animals had a lesser minimum stimulus distance in response to mechanical than to thermal stimulation (7.51mm ±1.02 vs. 7.77mm ±1.01 , $p = .01$), which correlates to the greater mechanical contraction distance (2.74mm ±0.69 vs 2.06mm ±0.74, $p < .001$) and relax distance (2.62mm ±0.76 vs. 1.83mm ±0.63, $p < .001$) when compared to that of thermal stimulation. Interestingly the speed to reach that minimum stimulus distance was also greater for mechanical compared to thermal stimulation (1.92mm/s ±0.59 vs. 0.77mm/s ±0.56, $p < .001$), yielding thermal stimulation to produce a time course to minimum stimulus distance more than twice as long as that of the mechanical stimulation (3.33s ±1.49 vs 1.5s ±0.24, $p = .002$). Additionally, relax time was longer following mechanical stimulation compared to thermal (51.91s ±9.21 vs 48.75s ±11.79, $p = .011$). However, the differences seen in mechanical and thermal CTMR contractions here do not account for the severe differences seen between mechanical and thermal CTMR contraction in the neonatal capsaicin-treated animals, which instead result from a complete lack of contraction in response to thermal stimulation. Taken together these results indicate, for vehicle-treated animals, mechanical stimulation of the CTMR results in a larger and faster contraction than thermal stimulation, however there is also a slower post-contraction relaxation following mechanical stimulation compared to thermal stimulation.

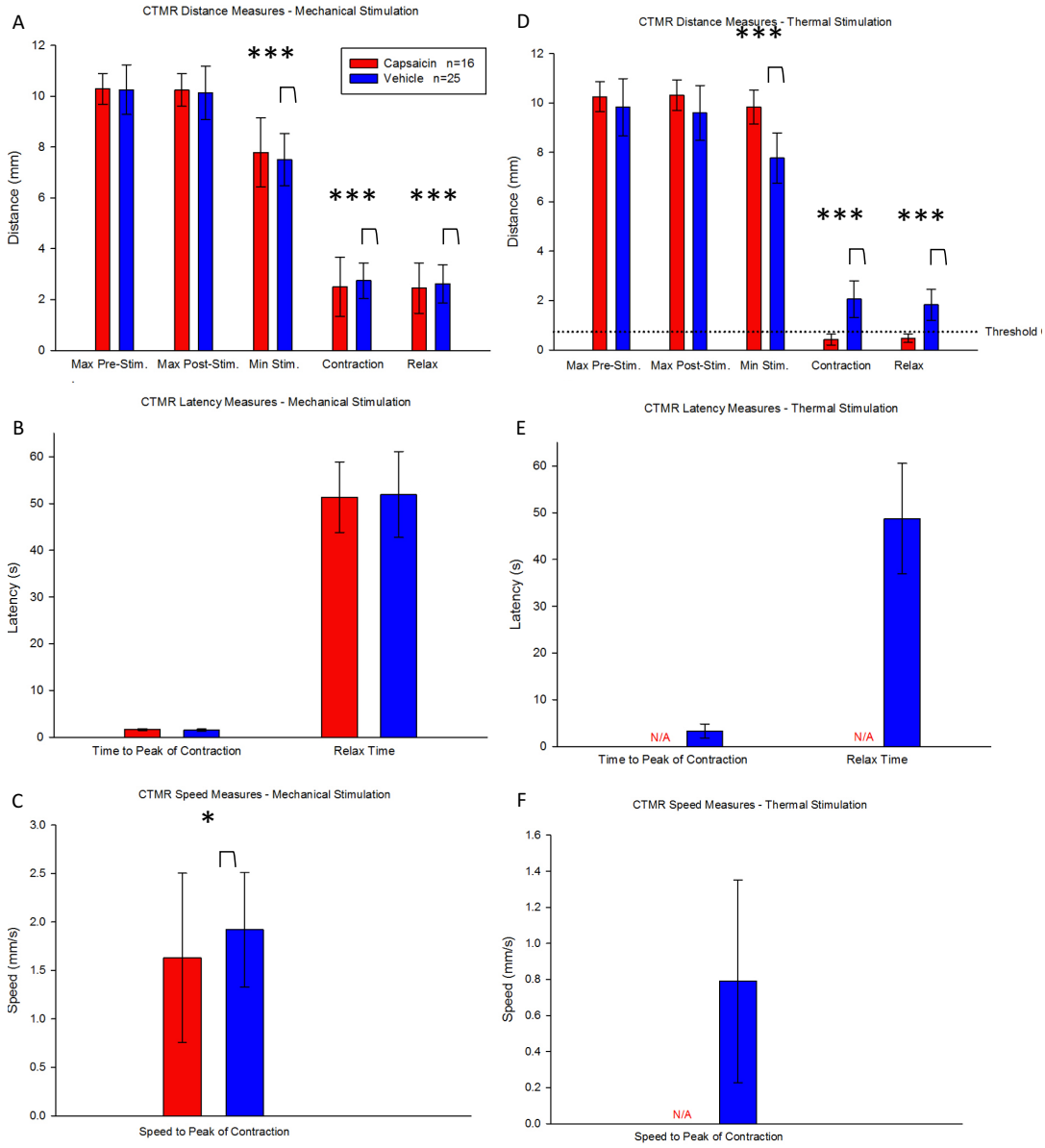

Figure 1. CTMR measurements for neonatal capsaicin- (red) and vehicle-treated animals (blue). ***A-C Mechanical Stimulation*** (A) Max pre- and post-stimulus distances, minimum stimulus distance (i.e. peak of contraction), contraction distance, and relax distance. (B) Time to peak of contraction and relax time. (C) Speed to peak of contraction. ***D-F Thermal Stimulation*** (D) Max pre- and post-stimulus distances, minimum stimulus distance (i.e. peak of contraction), contraction distance, and relax distance. Neonatal capsaicin-treated animals did not reach the threshold to be considered a contraction and were omitted from further results (labeled N/A) (B) Time to peak of contraction and relax time. (C) Speed to peak of contraction. * $p \leq .05$, *** $p \leq .001$

Stimulation	Group 1	Group 2	minimum stimulus distance	contraction distance	speed to minimum stimulus distance	relax distance
Mechanical	Capsaicin	Vehicle	$p < .001$	$p < .001$	$p = .014$	$p < .001$

Table 1. Statistical significance of minimum stimulus distance, contraction distance, speed to minimum stimulus distance, and relax distance of the CTMR for mechanical nociception of vehicle- vs neonatal capsaicin-treated animals

Group	Stimulation 1	Stimulation 2	minimum stimulus distance	contraction distance	time to minimum stimulus distance	speed to minimum stimulus distance	relax time	relax distance
Vehicle	Mechanical	Thermal	$p = .01$	$p < .001$	$p = .002$	$p < .001$	$p = .011$	$p < .001$

Table 2. Statistical significance of minimum stimulus distance, contraction distance, time to minimum stimulus distance, speed to minimum stimulus distance, relax time, and relax distance of the CTMR for mechanical vs thermal nociception of vehicle-treated animals

Effects of neonatal capsaicin on formalin-induced allodynia and hyperalgesia

Neonatal capsaicin treatment reduced formalin-induced mechanical allodynia as measured by the von Frey test. Prior to formalin injections there were no significant differences for the von Frey thresholds of vehicle treated animals (63.51 ±8.86) compared to those treated with neonatal capsaicin (60 ±4.5) (figure 2A). Following formalin injections however vehicle treated animal showed a significantly decreased threshold (48.85 ±12.46, $p< .05$), and while the neonatal capsaicin treated animals showed a slightly lower threshold (52.51 ±15.85), it was not significantly different from their baseline measurements

(figure 2A). These results indicate a reduced level of formalin-induced mechanical allodynia present in animals treated with neonatal capsaicin compared to those treated with a vehicle.

Neonatal capsaicin treatment also prevents formalin-induced hyperalgesia as measured by the Hargreaves test. Baseline measures showed a significantly ($p = .001$) higher threshold for neonatal capsaicin treated animals (13.04s ±2.73) compared to that of vehicle treated animals (9.17s ±1.48) (figure 2B). Following formalin injection neonatal capsaicin treated animals again showed a significantly ($p < .001$) higher threshold (17.1s ±1.89) than vehicle treated animals (6.8s ±0.67), additionally there was a significant ($p < .05$) decrease in thresholds between baseline and terminal for vehicle treated animals, and surprisingly a significant ($p < .05$) increase in baseline to terminal thresholds for capsaicin treated animals (figure 2B). Not taken into account in these averages were the number of times an animal “maxed out”, or hit the 20 second mark where the heat source automatically stops to prevent tissue damage. Figure 2C shows that between baseline and terminal testing, capsaicin animals had a total of 62 maxs, 31 at baseline and 31 at terminal time points, while vehicle animals only showed 4 maxs, all during baseline testing. When looked at over the total number of trials (i.e. possible maxs), capsaicin animals maxed out significantly more than vehicle animals (51.67% vs 2.22%, $p<.001$). Taken together these results indicate neonatal capsaicin treatment prevented formalin-induced thermal hyperalgesia as compared to vehicle treated animals.

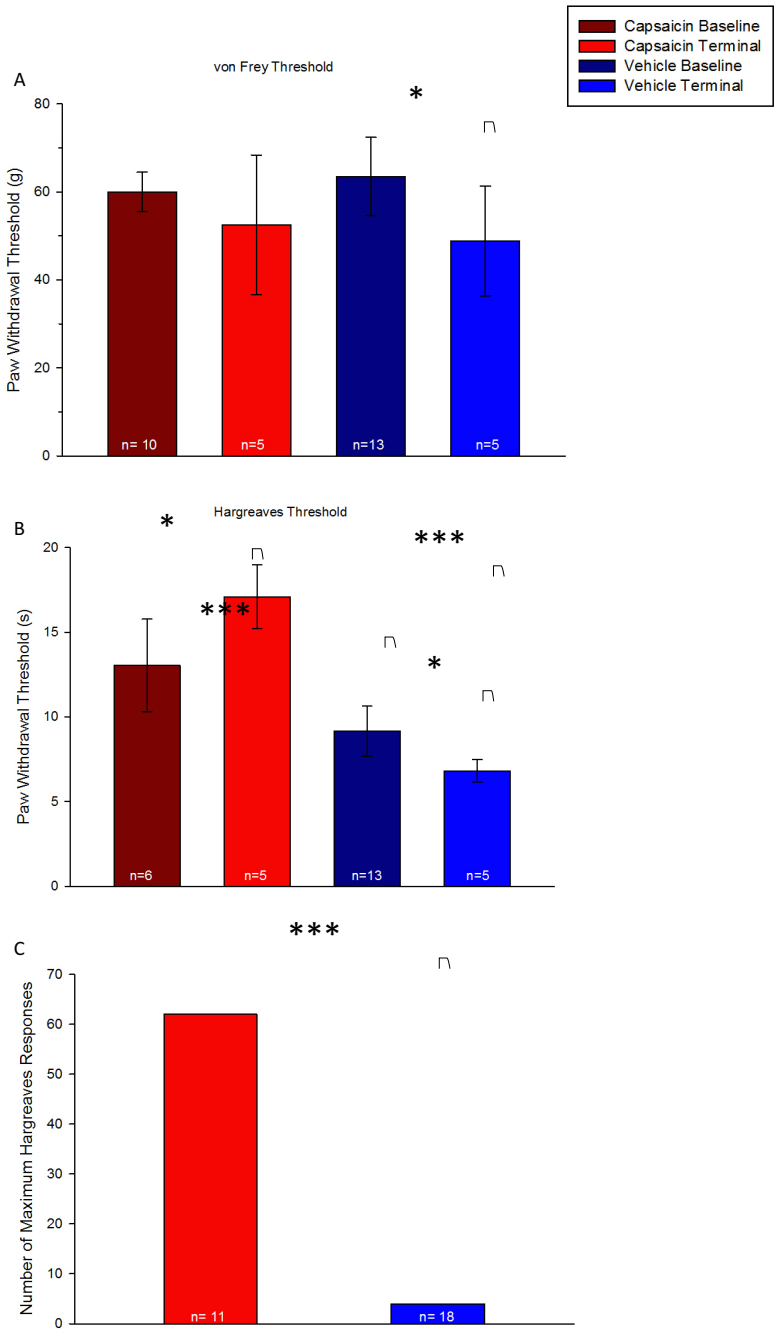

Figure 2. Paw withdrawal thresholds of neonatal capsaicin- (red) and vehicle-treated animals (blue) before (baseline) and after (terminal) 2% formalin injections for both the (A) von Frey and (B) Hargreaves assessments. (C) number of max threshold responses (20s) on the Hargreaves test for neonatal capsaicin- (red) and vehicle-treated animals (blue)

** p≤ .05, *** p≤ .001*

Effects of neonatal capsaicin on formalin-induced ATF3 expression

Neonatal capsaicin had an effect on the overall makeup of the L5 DRG. Figure 3A shows the distribution and cell counts for different cross-sectional areas of cell bodies, while figure 3B shows the number of cells qualified as small (< 800 μm^2) intermediate (800-1200 μm^2) and large (>1200 μm^2) neurons. Vehicle-treated animals had more small neurons than neonatal capsaicin-treated animals (1390 vs 807), consistent with the elimination of TRPV1 neurons, as well as fewer intermediate (223 vs 422) and large (229 vs 366) neurons when compared to neonatal capsaicin-treated animals (fig 3B).

Vehicle-treated animals had 537 TRPV1+ neurons (536 small, 1 intermediate), 226 TRPA1+ neurons (175 small, 49 intermediate, 2 large) and 283 ATF3+ neurons (189 small, 49 intermediate, 45 large), significantly ($p \leq .001$) more than animals treated with neonatal capsaicin, which had 21 TRPV1+ neurons (18 small, 3 intermediate), 86 TRPA1+ neurons (48 small, 35 intermediate, 3 large), and 55 ATF3+ neurons (18 small, 14 intermediate, 23 large) (table 3).

Colocalizations can be seen in figure 4A for vehicle-treated animals and 4B for neonatal capsaicin-treated animals, as well as in table 4. Briefly, out of the 537 TRPV1+ neurons in vehicle-treated animals, 37 colocalized with TRPA1 only, 58 colocalized with ATF3 only, and 57 colocalized with both TRPA1 and ATF3, additionally 45 TRPA1 neurons colocalized with ATF3 only. (figure 4A and table 4). In the neonatal capsaicin-treated animals, out of 21 TRPV1+ neurons, 5 colocalized with TRPA1 and 2 colocalized with both TRPA1 and ATF3, and

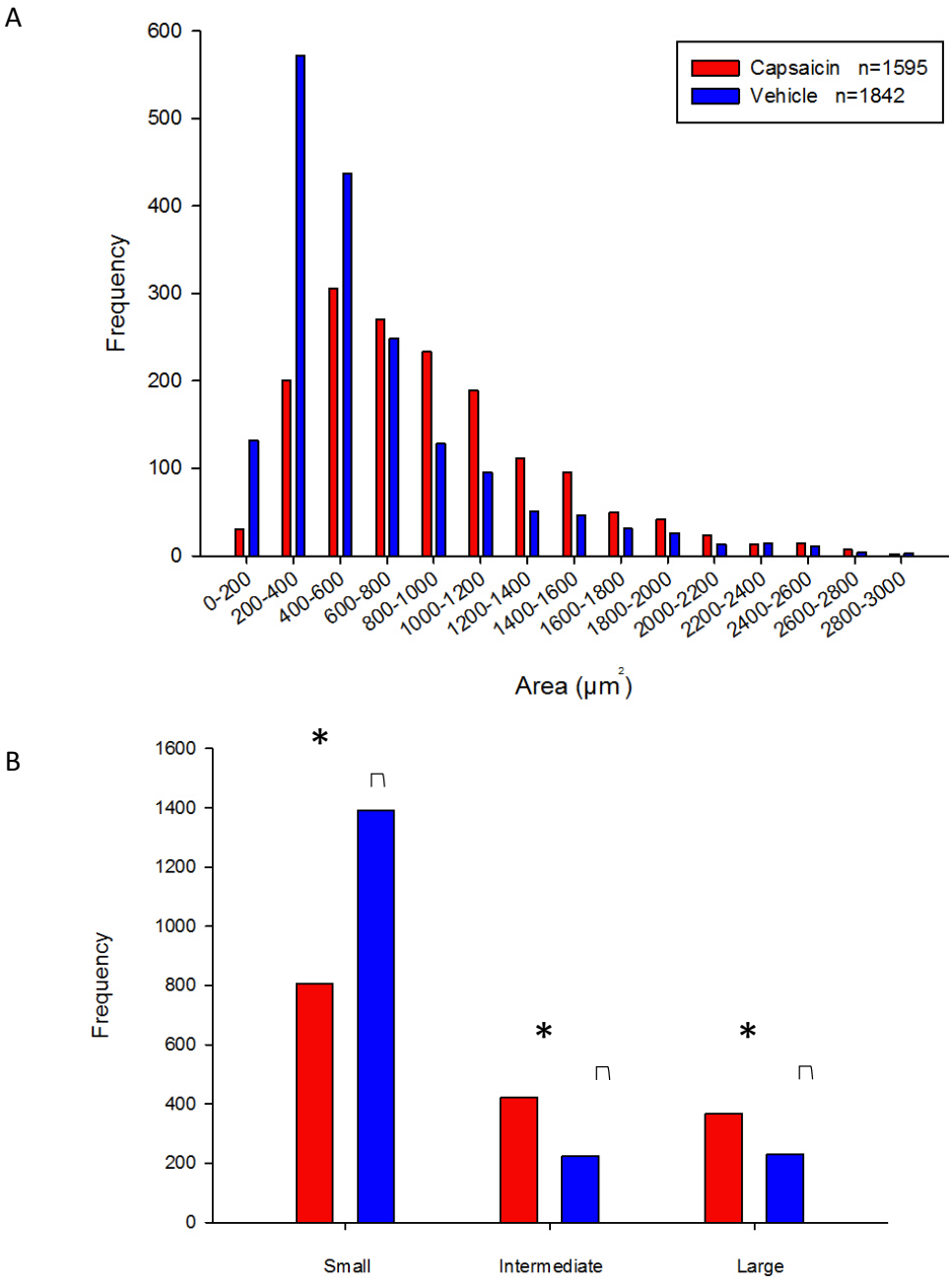

Figure 3. Frequency of cells present in the L5 DRG of neonatal capsaicin- (red) and vehicle-treated (blue) animals (A) by ranges of 200 μm^2 in area and (B) by small (< 800 μm^2) intermediate (800-1200 μm^2) and large (>1200 μm^2) areas.
* $p \leq .05$

	Vehicle	Capsaicin
TRPV1	537 *(29.15%)*	21 *(1.32%)***
TRPA1	226 *(12.27%)*	86 *(5.93%)***
ATF3	283 *(15.36%)*	55 *(3.45%)***

Table 3. Number of TRPV1, TRPA1, and ATF3 neurons counted in vehicle- and neonatal capsaicin-treated animals. In parenthesis are their percentage of total neurons counted in vehicle- (n=1595) and neonatal capsaicin-treated (n-1842) animals. *** $p \leq .001$ compared to vehicle-treated animals

5 TRPA1+ neurons colocalized with ATF3 (figure 4B and table 4). Within these colocalizations there was no significant difference in cell body cross-sectional area between capsaicin and vehicle treated animals, however there were significant differences in the number of colocalizations. Additionally, there were a total of 399 TRPV1 only neurons, 161 TRPA1 only neurons, and 171 ATF3 only neurons (figures 4A-B and table 4).

Taken together these results indicate not only the effectiveness of neonatal capsaicin treatment in ablating TRPV1+ neurons, but also the ability of neonatal capsaicin to change the frequency of size distribution in the DRG, as well as the way these non-TRPV1 expressing neurons of all sizes process and respond to nociceptive signals.

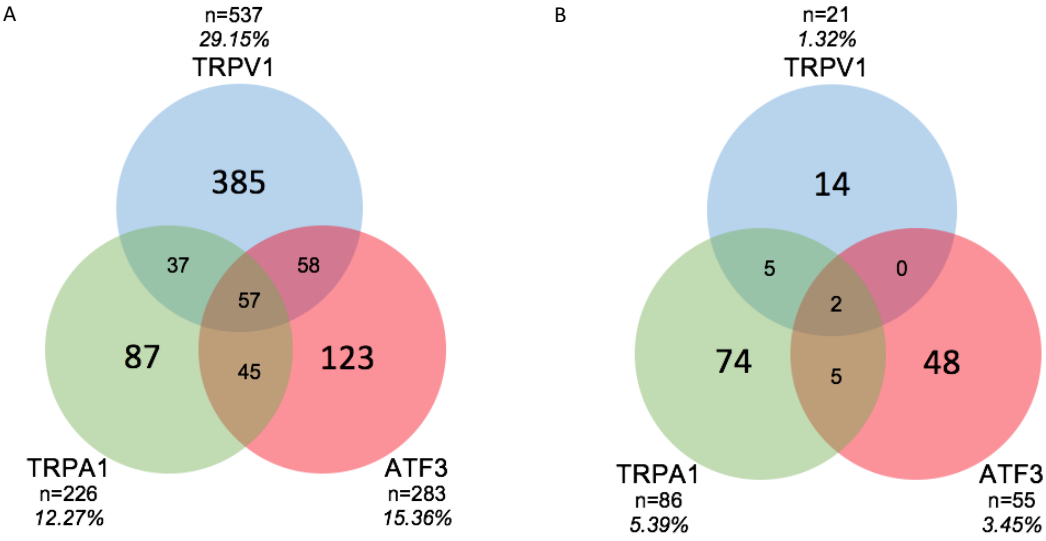

Figure 4. TRPV1 (blue), TRPA1 (green), ATF3 (red), and colocalizations present in the L5 DRG of (A) vehicle-treated animals and (B) neonatal capsaicin-treated animals

	TRPV1 only	TRPA1 only	ATF3 only	TRPV1/TRPA1	TRPV1/ATF3	TRPA1/ATF3	TRPV1/TRPA1/ATF3
Vehicle	385	87	123	37	58	45	57
Capsaicin	14***	74	48***	5***	0***	5***	2***

Table 4. Cell counts for vehicle- (n=1842) and neonatal capsaicin- (n=1595) treated animals for TRPV1 only, TRPA1 only, ATF3 only, and all possible colocalizations. *** $p \leq .001$ compared to vehicle-treated animals

DISCUSSION

Health issues

The most unexpected finding of the current study was the large number of health problems of animals treated with neonatal capsaicin. Upon review of the literature, its evident most studies using a neonatal capsaicin model did not carry out their experiments past 3 months, the exact time point at which the current studies rapid number of fatalities began. The major underlying causes of these fatalities were (I) an inability to empty the bladder leading to bladder infection, hydronephorsis, kidney stones, and ultimately kidney failure, and (II) to a lesser extent, tissue structural integrity. Furthermore, the remaining capsaicin animals utilized for the duration of the experiment all experienced bladder infections, meaning these issues affected 100% of the capsaicin treated group.

The bladder ultimately has only two modes of operation; it is either storing urine with the detrusor relaxed or eliminating it via detrusor contraction (Fowler et al., 2008). While some argue that Ad fibers are responsible for relaying afferent information about distension and contraction while c-fibers are silent in the control of bladder function (Fowler et al., 2008), this idea is inconsistent with the current results. In fact, TRPV1 is present in bladder epithelial cells and the

terminals of afferents innervating the bladder are TRPV1 positive, and animals lacking TRPV1 have shown decreased contractions normally stimulating elimination (Birder et al., 2002). Additionally, drugs acting through a variety of mechanisms to ultimately produce an excitatory effect of the micturition reflex do so by stimulating c-fiber afferents, while certain inhibitors of c-fiber afferent activity improve detrusor over activity (Yokoyama et al., 2006; Yokoyama et al., 2007). These results suggest that inhibiting c-fiber activity would improve bladder storage, and are consistent with our results that ablating c-fibers has a severe impact in preventing normal contractions and subsequent elimination of urine.

In addition to the bladder problems seen in capsaicin treated animals, there were an additional 2 fatalities due to an apparent lack of structural integrity of internal tissues. One of these fatalities resulted from a ruptured aortic aneurysm, the other presented significant internal bleeding within the chest cavity, although a source of the bleed was not easily identifiable, leading to the hypothesis it was the result of a ruptured aneurysm within the heart itself. Previous research identifies TRPV1+ C-fibers as widely distributed throughout the entire cardiovascular system, including innervating the myocardium and coronary vasculature (Wang, 2005; Zhong & Wang, 2007; Peng & Li, 2010; Xu et al., 2011; Earley & Brayden, 2015). Within the cardiovascular system, particularly during an ischemic event, these fibers work to release calcitonin gene-relate peptide (CGRP) and substance P (SP), both of which work as endothelial dependent vasodilators through their release of nitric oxide (NO), and ultimately serve to protect the heart through their release of nitric oxide (NO), and

ultimately serve to protect the heart and its surrounding vasculature from injury or damage (Zhong & Wang, 2007; Peng & Li, 2010; Xu et al., 2011; Torres-Narváez et al., 2012). A recent clinical study found a correlation between aneurysm size and vasodilation, such that larger aneurysms (i.e. those closer to rupture) were associated with significantly lower levels of endothelial dependent vasodilation in an unrelated artery (Medina et al., 2010). Taken together these results indicate that decreased vasodilation due to a lack of TRPV1-dependent NO release may be responsible for the aneurysms experienced by a subset of the capsaicin treated animals in the current study. Ultimately, if the goal of research in line with that of the current experiment is to provide new targets for therapeutics in the treatment of pain disorders, more research needs to go into the secondary and long term effects of acting on c-fibers or their receptors, specifically TRPV1.

Differences in Mechanical and Thermal Nociception, and the Effects of Neonatal Capsaicin

Consistent with previous literature the current study showed ablation of thermal nociception as measured by the CTMR following treatment with neonatal capsaicin (Nagy & van der Kooy, 1983; Doucette et al., 1987; Ren et al., 1994¬). However, the current study also found evidence that neonatal capsaicin treatment suppressed mechanical nociception as measured by the CTMR. This is potentially a result of the polymodality of C-fibers, of which ~70% of these unmyelinated afferents can actually be activated by a diverse set of nociceptive stimuli, including mechanical force and heat (Perl, 1996; Cain et al., 2001;

Cavanaugh et al., 2009) These results may explain the differences in mechanical nociception as measured by the CTMR seen in vehicle- vs neonatal capsaicin-treated animals. Potentially, by ablating TRPV1+ neurons and fibers responsible for the transmission of thermal nociceptive signals, neonatal capsaicin treatment may actually be ablating a subset of polymodal TRPV1+ positive neurons and fibers, in addition to those responding only to thermal nociceptive signals, resulting in a slight reduction in mechanical nociception as well.

In addition, the current found differences in mechanical and thermal nociception as measured by the CTMR in vehicle-treated animals. While there may be a reason behind these differences, potentially surrounding different receptors and fiber-types, there is also a relatively simple explanation; differences in methodology. To conclusively state there is a difference in mechanical and thermal nociception-induced CTMR contractions, additional studies would need be performed with this as their sole focus, using different forceps and probe sizes, as well as different forces and temperatures, to investigate if there is indeed a specific intensity for each type of stimuli for which the results are then indistinguishable. If this proves impossible, it is then more likely a result of the different neurons, receptors, and fibers involved in the transmission of various nociceptive signals.

Nociceptive Testing

The von Frey and Hargreaves tests, or some version of them, are commonly seen in pain-related behavioral research. These tests however are

subject to some inherent problems; those surrounding the animal and those surrounding the experimenter. When it comes to the animal, one question that is present throughout these types of tests is what is the effect of successive trials or multiple testing time points on the results. A recent study found mean nociceptive thresholds significantly increased over testing on days 1, 3, 7, 10, and 24, indicating that accounting for changes between testing intervals may be necessary to place results in context (Raundal et al., 2010). The current study found an increase in thermal threshold of capsaicin treated animals following formalin injections, which could be a coincidence, but could also be an effect similar to that seen in previous research, where because formalin did not affect the thermal nociception in these animals, thresholds are actually increasing over time. Additionally, there are potential gender differences involving nociception, including differences in the melanocortin-1 receptor (Mogil et al., 2003) and NMDA receptor activity (Nemmani et al., 2004) between genders. While the majority (~80%) of pain research involving animals is performed on males (Mogil & Chandra, 2005) and thus unlikely to be a cause of inconsistency across a particular subset of literature, it is something to bear in mind when reviewing pain research. Looking at the experimenter, previous research has also noted challenges with inter-observer agreement, meaning some observers may qualify a behavior as representing the nociceptive reflex expected from these types of tests, while others may not (Raundal et al., 2010). One way to limit experimenter bias and ensure the activation of nociceptors would be to combine these behavioral tests with simultaneous EMG recordings.

These challenges, along with the high degree of variability seen in the results of these tests, begs for a more standardized and precise way to measure nociception. The CTMR here proved to be an accurate measure, echoing the results of the other behavioral tests, but with far less variability, room for experimenter bias or error, and increased accuracy, as this reflex in rodents is nociceptive specific. However, it is also necessary to point out the fundamental differences in what these tests; von Frey and Hargreaves testing measures threshold, while the CTMR instead assesses amplitude and duration, and cannot measure threshold, at least not in the manner it is currently used. Additionally, recognizing the sensation of pain is different than the activation of nociceptors, the addition of simultaneous EMG recordings and the CTMR to these already commonly used behavioral measures could prove useful in providing accurate, reproducible results that, in the care of the CTMR, due to the use of kinematic analysis rather than experimenter observation, would be comparable between studies regardless of experimenter.

Effects of neonatal capsaicin on formalin-induced allodynia and hyperalgesia

The reduced formalin-induced allodynia, and ablated formalin-induced hyperalgesia, are relatively consistent with the majority of previous literature (Nagy & van der Kooy, 1983; Ren et al., 1994¬; Fu et al., 2001; Cavanaugh et al., 2009; Shields et al., 2010). However, potentially due to the inherent flaws of these nociceptive measures, there is also a large degree of variability seen within

these generally agreed upon results. For instance, one group found a difference between the injected and contralateral hindpaws, such that the injected paw actually exhibited hypoalgesia, which was attributed to formalin-induced damage to peripheral nociceptors (Fu et al., 2001). The current study found no statistical differences between sides (data not shown) and data for each paw was therefore combined. Another study observed an increase in mechanical threshold following treatment neonatal capsaicin, but found this increased threshold was long after application of complete freunds adjuvant, another commonly used inflammatory agent, which presents almost the opposite of what the current paper found, a similar starting mechanical threshold but decreased mechanical allodynia. (Cavanaugh et al., 2009). These differences are unlikely due to some variety within the methodology, but rather to a difference in experimenter and/or the criteria for a withdrawal. Again the use of simultaneous EMG recordings when performing these tests may limit some of this variability seen across the literature and provide more reproducible results.

Effects of neonatal capsaicin on formalin-induced ATF3 expression

There is little in the way of previous literature regarding the effects of neonatal capsaicin on ATF3 expression in the adult rat. The current study however provides evidence that animals treated with neonatal capsaicin show increases in the frequency of intermediate and large area neurons, and that these neurons do not respond to high doses of formalin as they would in a vehicle treated animal, despite the fact that a lack of TRPV1+ neurons and fibers

should not affect the way non-TRPV1-expressing neurons process the signal (i.e. no “cross-excitation” as is seen with capsaicin injections). The increased frequency seen in animals treated with neonatal capsaicin could simply be a results of the increased space present in the DRG following ablation of TRPV1+ neurons, and similar experiments with ablation of varying neurons and fibers at a developmentally critical window could support or refute that hypothesis. The differences in processing and responding to nociceptive signals of intermediate and large area neurons seen with neonatal capsaicin-treatment is somewhat interesting when put in a clinical context. Though fundamentally different from the ablation that occurs with neonatal capsaicin, there is a current clinical environment looking towards TRPV1 antagonists in the treatment of chronic pain in both animals (Kim et al., 2014) and humans (Szallasi et al., 2007). With that in mind it is prudent to understand what, if any, implications such drugs may have on the responsiveness of other neurons to nociceptive signals.

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2014 - Current — Masters of Science in **Anatomical Science and Neurobiology**, School of Medicine, University of Louisville, Louisville, Kentucky. GPA 3.9

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Research Experience

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2013 — **Research Assistant** at Aggression Lab, Dr. Ricci, Boston, MA.

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Poster Presentations

2014 — Klug, S., Krupp, S., **Effect of Maternal Responsive and Directive Behavior on Children's Cognitive Development and Performance** *National Association of School Psychologists Annual Convention, Washington, D.C.*

2013 Krupp, S., **Examining the Effects of Older Sibling on Cognitive Developmental Domains in Children**. *Research, Innovation, and Scholarship Exposition, Boston, MA*

Honors & Awards

2014 - Current **Out-Of-State Merit Scholar Award**, University of Louisville, tuition subsidy award for Masters students with an undergraduate GPA above a 3.2, GRE scores above the 50th percentile, and strong evidence of scholarly ability, GPA must be maintained above a 3.0

2010 **Deans List**, Northeastern University, awarded to student with a GPA above 3.5 and no grade below a C

2009 - 2013 **Merit Scholarship**, Northeastern University, scholarship awarded to students in the top 25% of incoming freshman, GPA must be maintained above a 3.0

Conferences

2015 The 21st Annual Kentucky Spinal Cord and Head Injury Research Trust Symposium, Louisville, KY, USA.

2015 The 25th Annual Neuroscience Day, Louisville, KY, USA.

2013 The 2nd Annual Research, Innovation, and Scholarship Exposition, Boston, MA USA.

Lab Techniques

Animal Care (rodent)

Behavioral Testing

Cryostat Tissue Sectioning

Dissection (rodent)

Enzyme-Linked Immunosorbent Assay

Immunohistochemistry

Perfusion (rodent)

Reflex Testing

Solution Preparation

Professional Memberships

2015	Society for Neuroscience
2015	International Brain Research Organization
2015	Kentucky Academy of Science
2012	PSI CHI The International Honor Society in Psychology

Community Service

2015	Louisville Youth Science Summit
2015	Kentucky Science Center's NanoDays
2010 - 2013	Animal Rescue League
2009	Salvation Army