Published in final edited form as:

Dev Psychobiol. 2020 January ; 62(1): 88–98. doi:10.1002/dev.21889.

Inflammatory Neonatal Pain Disrupts Maternal Behavior and Subsequent Fear Conditioning in a rodent model

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Abstract

Infants spending extended time in the Neonatal Intensive Care Unit are at greater risk of developing a variety of mental health problems later in life, possibly due to exposure to painful/stressful events. We used a rodent model of inflammatory neonatal pain to explore effects on fear conditioning, somatosensory function and maternal behavior. Hindpaw injections of 2% χ -carrageenan on postnatal days 1 and 4 produced an attenuation in conditioned freezing during the postweaning period, similar to our previous work with acute pain, but did not cause lasting impacts on contextual freezing nor somatosensory function. Additionally, we assessed maternal behavior to observe dam-pup interactions during the neonatal period. Results showed dams of litters which experienced pain spent similar amounts of time with pups as undisturbed controls. However, the specific behaviors differed per condition. Dams of pain litters exhibited less time licking/grooming, but more time nursing than controls. These results suggest changes in maternal care following pain could be a contributing factor underlying the long-term effects of neonatal trauma. Furthermore, our lab has previously shown acute, but not inflammatory pain, disrupted conditioned freezing, the current experiment observed long-term effects of neonatal inflammatory pain on conditioned fear using a weak conditioning protocol.

Keywords

Inflammatory pain; maternal behavior; fear conditioning

Repeated painful procedures without the use of analgesics are often performed in neonatal intensive care units (NICUs) and the number of invasive procedures varies from an average of 55 up to 488 painful procedures having been recorded in one individual (Barker & Rutter, 1995). In humans, neonatal pain has been associated with numerous adverse outcomes later in life including anxiety, depression, and increased pain sensitivity as well as changes in brain development (Anand & Scalzo, 2000; Brummelte et al., 2012; Grunau, Holsti, & Peters, 2006; Mooney-Leber, Spielmann, & Brummelte, 2018; Taddio, Katz, Ilersich, & Koren, 1997). Understanding this relationship is complicated because it is often unclear whether it is the painful procedures performed in the NICU, or the condition that resulted in

an infant being admitted to the NICU, that leads to later-life adverse outcomes. Rodent models are therefore useful for understanding the conditions under which neonatal pain leads to lasting consequences and can provide insight into best practices in neonatal care (Anand et al., 1999).

There is an established literature regarding the effects of neonatal pain on later life affective and sensory behavior (for a recent review: (Kentner, Cryan, & Brummelte, 2019). Early seminal studies by Seymour Levine demonstrated enhanced resilience against stress in pups briefly separated from their mother and subjected to a footshock (Levine, 1967; Levine, Chevalir, & Korchin, 1956). More modern studies using repeated acute paw prick pain often produces heightened pain sensitivity and later anxiety-like behaviors (Anand, Coskun, Thirvikraman, Nemeroff, & Plotsky, 1999; Carmo, Sanada, Machado, & Fazan, 2016; Page, Hayat, & Kozachik, 2013), as opposed to resilience. In contrast, inflammatory pain has been shown to produce a *hypo*sensitivity in later-life somatosensory tests at baseline, although also an enhanced susceptibility to a subsequent injury (Bhutta et al., 2001; LaPrairie & Murphy, 2007; Wang, Ji, Lidow, & Traub, 2004). Although few studies have directly compared different types of neonatal pain on later behavior, our lab recently compared the effects of acute neonatal pain produced via hindpaw needle pricks with inflammatory neonatal pain on subsequent later-life fear and somatosensory responses (Davis et al., 2018). In that study we demonstrated a disruption in conditioned freezing following repeated neonatal acute pain and a marked mechanical hypersensitivity in post-weaning aged rats following neonatal pain or non-painful handling. However, following neonatal inflammatory pain, we did not observe any differences on subsequent fear or somatosensory function, leading to the conclusion that maternal separation and handling was a critical aspect causing the behavioral changes.

It's well known that adverse outcomes later in life may be produced by a lack of maternal care and/or exposure, which may exacerbate the effects of neonatal pain in NICU-like conditions (Mooney-Leber & Brummelte, 2017). For example, in humans, it has been demonstrated that as little as a 30 min separation from the mother can increase salivary cortisol levels in infants suggesting a direct link between maternal care and the HPA axis (Larson, Gunnar, & Hertsgaard, 1991). This effect has also been reported in rodents (McCormick, Kehoe, & Kovacs, 1998). Moreover, Liu et al., (1997) demonstrated that as adults, rats that were the offspring of mothers that performed less licking and grooming showed elevated ACTH and corticosterone levels compared to rats raised by mothers who performed high levels of licking and grooming. Offspring of low licking and grooming mothers also display a reduction of long-term potentiation in the hippocampus (Champagne et al., 2008) and a lack of maternal care causes later impairment in the Morris water maze (Liu, Diorio, Day, Francis, & Meaney, 2000). Taken together, it's apparent that changes in maternal interactions can greatly influence development and influence subsequent behavior in offspring.

Although Davis et al 2018 demonstrated an effect of repeated paw pricks and not inflammatory pain, these two preparations differ in both the nature of pain and the amount of handling and maternal separation required, which might alter maternal interactions. Furthermore, we hypothesized that our lack of observed effect may have been due to using

sub-optimal testing methods which allowed for a “ceiling effect” on both the freezing and mechanical sensitivity measures. Therefore, in the current study we examined the effects of neonatal inflammatory pain on a modified fear conditioning protocol and sensory testing methods that were designed to be more sensitive to subtle disruption across the lifespan. We again chose three ages (postnatal days 24, 45, and 66) to observe effects at multiple developmental stages, roughly equating to childhood, adolescence and adulthood in humans. Moreover, although we have been unable to find any papers which observe the effects of neonatal inflammatory pain in conjunction with maternal behavior on subsequent fear and somatosensory function, there is also a suggestion in the literature that changes in maternal care following neonatal pain might serve to modulate the effects of pain, perhaps normalizing long-term behavior (Walker, Kudreikis, Sherrard, & Johnston, 2003). Thus, this study also differs from our previous work in that we examine the effects of neonatal pain on maternal behavior and dam-pup interaction as this may be a contributing factor in later-life development.

Methods

Subjects.

Male and female Sprague Dawley rats were either bred using timed-pregnant dams (Charles River) which arrived on gestational day (GD) 12 and gave birth on GD22, or rats were bred in-house using a protocol previously described in Davis et al., (2018). There was a total of 13 timed-pregnant litters and 13 in-house bred used for these studies. Among the timed-pregnant litters, seven served as experimental litters (i.e., received vehicle or carrageenan) and six served as undisturbed controls. Among the in-house bred litters six served as experimental litters and seven served as undisturbed controls (see Figure 1). As no differences in behavioral data were found between the pups on the two breeding protocols data were otherwise combined for analysis. A subset of all the litters (12 litters) were used for video scoring of maternal behavior, and also participated in the subsequent behavioral testing. All litters were housed in 43 cm x 44 cm x 20 cm closed-environment cages (Innovive, San Diego CA). On PND 1, pups were removed from their mother, placed on a heating pad, sexed, marked via crystal violet stain, and culled to no more than 10 rats per litter (5 males and 5 females when possible). In an effort to keep undisturbed litters the least disturbed as possible, body weights during the neonatal period were not collected. Pups were weaned on PND 21 and lived with their same-sex littermates (approximately 5 per cage). If the rats were tested beyond PND 45, rats were housed 2-3 per cage with same-sex littermates. No more than one same sex littermate was assigned to each experimental group (with experimental group defined as a combination of neonatal treatment, sex and age at testing). In the rare situation where that was violated, the data from the two subjects were averaged. All rats were maintained on a 12:12 light/dark cycle with lights on at 07:00. Food and water was available ad libitum, and at the end of experimentation, rats were euthanized via CO2 overdose.

Apparatus.

Fear conditioning was performed in four Startfear chambers (Harvard Apparatus/Panlab model #58722) with two separate contextual cues that differed in shape (square vs. circle),

color (black vs. white walls), and scent (70% ethanol vs. 1% ammonia). Tactile allodynia was measured by using the up/down technique with von Frey monofilaments (North Coast Medical, Gilroy CA) of varying gauges (equating to 0.4 g – 15 g pressure). Thermal hyperalgesia was assessed using a Hargreaves apparatus (Ugo Basile Plantar Test model #7371, Collegeville PA).

Drugs and Chemicals.

λ -Carrageenan (Sigma-Aldrich, St. Louis MO) was dissolved in 0.9% physiological saline in a 2% w/v concentration. Carrageenan or vehicle was administered *i.pl.* at a 25- μ l volume. 2.5% formalin was mixed in 0.9% physiological saline. Formalin was injected *i.pl.* at a 50- μ l volume.

Neonatal Pain.

The procedure was similar to previously described (Davis et al. 2018). Briefly, the thickness of each rat's left hindpaw was measured (in mm) via digital calipers daily until PND 7 (see Figure 2). This was performed as a confirmation that the carrageenan was producing edema, a measure of inflammation. On PNDs 1 and 4 rats received a 25- μ l injection of either vehicle (0.9% saline) or carrageenan into the plantar surface of the left hindpaw.

Maternal Behavior.

Prior to injection and/or paw thickness measurements (08:00) on PNDs 2-7, rat pup's home cage was removed from the rack and placed with a corresponding undisturbed litter on a cart for simultaneous maternal behavior recording. Four GoPro HD Hero Session (San Mateo, CA) cameras were placed on the side nearest the nests and on top of the two cages. All behavior was recorded for 1 hr. (Anseloni et al., 2005; Mooney-Leber et al., 2018) and scored using a modified snapshot method, (Rincel et al., 2016), with 10-s bins being scored for behavior for every 2 minutes. Videos were later scored by using Behavioral Observation Research Interactive Software (BORIS) (Friard, Gamba, 2016). Approximately 10% of videos were scored used 2 observers, and interrater reliability (IRR) calculations were performed. All IRR correlations were above 0.90. Behaviors scored included licking and grooming, nursing (both side and arched-back nursing), nest building (including burrowing), self-grooming, and maternal contact (any physical contact, excluding the tail, which may include licking/grooming and/or nursing with at least one pup). These behaviors were similar to those reported elsewhere (Caldji et al., 1998; LaPrairie & Murphy, 2007; Walker et al., 2003).

Fear Conditioning.

On PNDs 24, 45, or 66, subjects underwent a fear conditioning protocol, similar to that previously described (Burman, Simmons, Hughes, & Lei, 2014; Davis et al., 2018), with the exception that a weaker unconditional stimulus was used. For statistical reasons, separate pups from each litter were tested at each age. Briefly, on Day 1 rats were placed in their preassigned (counterbalanced) fear conditioning chamber (FCC) and the program was initiated. The percent of time spent freezing during the first 5 mins was recorded (Habituation). Following the habituation period, a 67-dB tone conditioned stimulus (CS) was

presented for 10 sec, immediately followed by a 2-s 0.3-mA foot shock serving as the unconditioned stimulus (UCS). There were 10 tone-shock pairings. The following day rats were placed in the same contextual FCC and the percent freezing for 5 mins was recorded (Context). The third day, rats were placed in the FCC with the opposite contextual cues than the FCC where the first initial day of fear conditioning took place, and the percent freezing for the first 5 mins was recorded (Novel Context). Subsequently, there were 10 presentations of the original CS (67-dB tone) every 30 sec, and the percent freezing for the 10-sec period during each CS presentation was recorded and averaged (AVG Tone). The change in freezing caused by the tone (Tone Difference Score) was calculated by subtracting novel context freezing from the AVG Tone score.

Formalin Injection.

Immediately after testing on the 3rd day of fear conditioning, all rats received a 50- μ l injection of 2.5% formalin into the plantar surface of the left hindpaw.

Somatosensory Testing.

24 hr after the Novel Context and Tone tests rats underwent somatosensory testing identical to that reported in Davis et al., (2018). Tactile allodynia of the left hindpaw was measured using the up-down method with von Frey monofilaments ranging from 2-15 g as previously described by (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). Upon completion, rats were subjected to the Hargreaves measure of thermal hyperalgesia. These methods were previously described in Davis et al., (2018).

Experimental Design and Analysis.

Data were analyzed with IBM SPSS version 22 mixed model MANOVAs or ANOVAs (when there were no more than 2 continuous variables) with Greenhouse-Geisser corrections where sphericity was violated. A Tukey post hoc analysis was performed when there were more than two levels of a variable to compare. No more than one same sex littermate was assigned to each experimental group. In the rare situation where that was violated, the data from the two subjects were averaged. Data are reported as mean $\pm$ SEM and a p -value of 0.05 was considered statistically significant.

Results

Body weight

Body weight expectedly differed between the sexes and increased as a function of ages but not within condition on the first day of behavioral testing. This was shown by a 2 (Sex: male, female) $\times$ 3 (Condition: vehicle, carrageenan, undisturbed) $\times$ 3 (Age: PND 24, 45, 66) ANOVA. There was a statistically significant main effect of age ($F(2, 103) = 789.15, p < 0.001$) with an age-dependent increase in body weight. There was also a statistically significant main effect of sex ($F(1, 103) = 174.35, p < 0.001$) with male rats weighing more than female rats when collapsed across age. There was no statistically significant main effect or interactions with neonatal pain condition as a factor (all p 's > 0.05) suggesting that neonatal pain and/or maternal interactions did not influence later-life body weight.

Paw thickness

Carrageenan produced pronounced edema in rat pup's hindpaw on PND's 2-7, as measured in mm thickness. This was shown by a 2 (Sex: male, female) x 2 (Condition: vehicle, carrageenan) repeated measures ANOVA with day serving as the repeated variable. This resulted in a statistically significant day by condition interaction ($F(6, 71) = 46.79, p < 0.001$). Pup's paw thickness did not differ on PND 1 for vehicle-treated rats and carrageenan-treated rats. On PND's 2-7, carrageenan-treated rats had greater paw thickness than their vehicle-treated counter parts (see Figure 2). There was no sex difference in paw thickness measurements (all p 's > 0.05).

Effect of inflammatory pain on maternal behavior.

Dams of litters that experienced inflammatory pain provided less overall licking and grooming than dams of undisturbed litters (see Figure 3). This was shown by using a 2 (Condition: carrageenan, undisturbed) x 6 (Day: 2-7) repeated measures ANOVA with testing day serving as the repeated variable. This resulted in a statistically significant main effect of condition ($F(1, 8) = 6.49, p < 0.05$) with undisturbed litters receiving more licking and grooming collapsed across all 6 testing days compared to pain litters (see Figure 3A).

Additionally, dams of litters exposed to inflammatory pain spent more time nursing on days 2-6 than dams of undisturbed litters. This was shown by a 2 (Condition: carrageenan, undisturbed) x 6 (Day: 2-7) repeated measures ANOVA with testing day serving as the repeated variable, resulting in a statistically significant day by condition interaction ($F(5, 40) = 2.50, p < 0.05$). There were variations across the observation period, with pain litters experiencing more nursing on Days 3-7 (but not Day 2) than undisturbed litters (see Figure 3B).

There were no statistically significant main effects or interactions on nest building, maternal contact, nor self-grooming. (all p 's > 0.10).

Behavioral testing

The effects of neonatal pain on fear conditioning and sensory testing was analyzed using several 2 (Sex: male, female) x 3 (Age: PND 24, 45, 66) x 3 (Condition: undisturbed, vehicle, carrageenan) ANOVA or MANOVA tests. First, we looked at our contextual freezing variables (habituation, contextual freezing upon replacement into the conditioning chamber and generalization to a novel context) using a repeated measures MANOVA. Second, we examined conditioned freezing as the AVG tone variable and the tone difference score measure using two separate one-way ANOVAs. Third, we examined the 10 conditioned tone presentations using a repeated measures MANOVA to look for effects on extinction. Finally, we examined sensory function using separate ANOVAs on von Frey and Hargreaves test data. Given the complexity of these analyses, we've divided our results by the effects of each independent variable, rather than dependent variable or analysis.

Effect of neonatal inflammatory pain on later-life fear conditioning and sensory function.—

Neonatal inflammatory pain did not produce any statistically

significant main effects or interactions on any of the three contextual freezing variables (Habituation, Context, or Novel Context tests; all p 's > 0.05: see Figures 4).

Inflammatory pain did produce a modest attenuation in freezing to the conditioned stimulus (tone) presentations following the Novel Context test, when collapsed across the other variables (see Figure 5). This effect can best be represented using the AVG tone measure where the univariate ANOVA resulted in a statistically significant main effect of condition ($F(2, 95) = 3.44, p < 0.05$). Post hoc analyses revealed, undisturbed rats had higher freezing than carrageenan rats, $p = 0.023$.

When observing behavioral responses to each of the 10 tone presentations separately, freezing percentage increased after the presentation of the first tone (see Figure 6). This was shown by a repeated measures MANOVA with percent freezing during each of the 10 tones serving as the repeated measures variable. This resulted in a statistically significant effect of the tone presentations ($F(9, 92) = 7.14, p < 0.001$). In addition there was a trending 4-way interaction between the tone presentations, age, sex, and condition ($F(36, 380) = 1.42, p = 0.059$) therefore we further analyzed the data between sex and age. This effect is likely driven by a pronounced attenuation of the freezing response in later trials in PND 24 carrageenan-treated males compared to older rats; a similar effect appears to persist to PND 45 in female rats.

Neonatal inflammatory pain did not produce any statistically significant main effects or interactions on either the von Frey mechanical sensitivity or Hargreaves thermal hyperalgesia measures (all p 's > 0.10: see Figures 7).

Effects of sex on later-life fear conditioning and sensory function.—There was a sex difference in two of the three contextual freezing variables that was dependent on the age of the rat. This was shown by a statistically significant 3-way interaction between the contextual freezing variables, age, and sex ($F(4, 200) = 2.72, p < 0.05$). To determine where the individual effects were found, further analyses were conducted at each separate age. These analyses revealed a statistically significant Condition x Sex interaction in the PND 66 rats ($F(2, 36) = 3.44, p < 0.05$), but not the PND 45 ($F(2, 33) = 0.77, n.s.$) nor PND 24 rats ($F(2, 28) = 1.97, n.s.$). Subsequent analyses for each of the PND 66 contextual freezing variables showed a statistically significant sex difference during the Habituation test ($F(1, 37) = 12.81, p < 0.01$) with females ($M = 6.91\%$, $SEM = 0.73$) freezing more than males ($M = 3.33\%$, $SEM = 0.60$: see Figure 4). Additionally, there was a statistically significant sex difference during the Context test ($F(1, 37) = 4.31, p < 0.05$) with females ($M = 62.35\%$, $SEM = 5.28$) also spending more time freezing than males ($M = 42.26\%$, $SEM = 7.30$: see Figure 4). There was no observed sex difference in PND 66 rats during the Novel Context test ($F(1, 42) = 0.08, n.s.$).

Effect of age on tone freezing and other measures.—PND 24 rats demonstrate a lower AVG tone freezing than PND 45 and PND 66 rats. This was showed by a statistically significant main effect of age ($F(2, 95) = 3.98, p < 0.05$). Post hoc analyses revealed that when collapsed across condition and sex, PND 24 rats had an attenuated freezing response to the tone presentations ($M = 68.57\%$, $SEM = 3.85$) compared to PND 66 rats ($M =$

83.31%, $SEM = 3.20$), $p = 0.01$, but not PND 45 ($M = 75.26\%$, $SEM = 3.71$) rats, $p > 0.10$ (see Figure 4).

PND 24 rats also showed an attenuated freezing response to the Tone Difference variable, a measure of fear response to the original CS less freezing from the Novel Context, than both PND 45 and PND 66 rats regardless of sex or condition, as shown by a statistically significant main effect of age ($F(2, 100) = 5.66$, $p < 0.01$). Post hoc analyses revealed that PND 24 rats froze less to the tone itself ($M = 37.07\%$, $SEM = 5.02$) than PND 45 ($M = 54.12\%$, $SEM = 4.12$), $p = 0.014$ and PND 66 ($M = 58.88\%$, $SEM = 4.27$), $p < 0.001$ rats (see Figure 4).

Discussion

We had three specific hypotheses. First, it was hypothesized that inflammatory neonatal pain would disrupt later-life fear conditioning. This was supported in that carrageenan-treated rats showed an attenuation of tone freezing (when collapsed across age and sex). This effect is particularly important because it is similar to the results following repeated tactile pain in Davis et al., (2018) and indicates that our modification to our fear conditioning protocol was successful in detecting long-term effects of neonatal inflammatory pain. This suggests that inflammatory pain produces effects similar to, but perhaps less robust than, repeated acute pain. Second, it was hypothesized that neonatal inflammatory pain would produce a later-life hyposensitivity on somatosensory tests. This hypothesis was not supported as there were no observed group differences on either the von Frey or Hargreaves measures. Finally, it was hypothesized that dams of litters that experienced inflammatory pain would alter their nurturing behaviors. This hypothesis was supported. Specifically, dams spent overall less time licking and grooming and more time nursing litters that experienced pain compared to undisturbed litters.

There were two notable differences in maternal behavior between litters that experienced pain compared to undisturbed litters. First, pain litters received *less* licking and grooming than their undisturbed counterparts. This is different from results found in other literature (Walker et al., 2003), in which pups in the neonatal pain treatment group receive a marked increase in licking/grooming. One explanation for these differences is that Walker et al. (2003) examined the effects of repeated tactile pain, such as that used in Davis et al. (2018), where in the current study we used an inflammatory pain stressor. Different types of pain may induce different changes in maternal behavior. These findings are important as it has been demonstrated that a lack of pup care can result in oxidative stress in pups resulting in a wide range of developmental problems including anxiety and learning/memory deficits later in life (De Moura, Brito, Porawski, Saffi, & Giovenardi, 2017). Second, in the current study, litters that experienced neonatal pain demonstrated a modest *increase* in nursing later in the week. This might suggest that the dams effectively supplemented one nurturing behavior with another in response to neonatal pain and stress, consistent with our observation that there was no difference in overall pup contact. In other words, these behaviors were not necessarily mutually exclusive, an increase in nursing may result in a lack of another behavior (in this case licking and grooming).

Another possible explanation for the increase in nursing behavior could be related to the “protective” effects of nursing. For example, one study looked at thermal withdrawal latencies among rats that were isolated in a group from their mother or allowed to nurse, and noted that rat pups that nursed showed a greatly increased latency to withdraw from the thermal stimulus compared to the isolated litter (Blass, Shide, Zaw-Mon, & Sorrentino, 1995). A similar effect of non-nutritive suckling increasing paw withdrawal thresholds and latencies has also been demonstrated in rats that experienced CFA-induced inflammation (Anseloni, Ren, Dubner, & Ennis, 2004). Thus, neonate rats experiencing pain might increase their overall time spent nursing as a form of analgesia to mitigate the effects of the pain. It’s possible that the changes in maternal behavior can mitigate against or exacerbate the effects of the neonatal pain which manifest as altered behavior later in life. For example, several studies demonstrated that changes in levels of licking/grooming and arched-back nursing as pups can alter subsequent fear behaviors in adults (Caldji et al., 1998; Francis & Meaney, 1999), and thus maybe a modulating factor for subsequent fear conditioning.

The most similar study to ours might be Anseloni et al., (2005), which observed maternal behavior on pups that received carrageenan injections. In that study, it was found that pup licking/grooming *increased* in conjunction with paw edema, in contrast to the *decrease* we observed, and that dams also increased in nursing which persisted longer than the visible edema, consistent with our observations. Importantly, their study differed from ours on the timing of the observations. Anseloni et al., (2005) engaged in fairly continuous monitoring following pain initiation. Thus, although they saw an initial increase in licking and grooming, by 24 hr post-injection (when we began our behavioral recording) differences in licking and grooming were negligible from controls. Nursing behavior remained elevated up to 48 hrs post-injection. Thus, these apparent discrepancies may be resolved if neonatal pain affects maternal behavior in a non-monotonic fashion. Given that the timing of observations may be critical, it should also be noted that there is a wide variation among studies that observe maternal behavior with regard to daily observation time. For instance Mooney-Leber et al., (2018) observed maternal behavior for a total of 50 mins per session (only 30 mins after pups and mother were reunited) immediately following neonatal injury; Anseloni et al., (2005) observed maternal behavior for 60 mins daily immediately following carrageenan injections; Walker et al., (2003) recorded maternal behavior for 90 mins on the sixth and seventh day of testing, immediately following pain/stress manipulations.

With regard to the effects of inflammatory pain on subsequent fear and somatosensory function, we observed that pups receiving carrageenan injections into the hindpaw showed an attenuation to conditioned fear response later in life, as evident by a reduction in freezing during re-presentation of the conditioned stimulus. Like our previous study (Davis et al., 2018), this fear conditioning effect appears to be transient in that the effect was most pronounced in younger rats (PND 24) and tends to dissipate by PND 45 and 66. This may be due to a variation of expression of the fear/anxiety phenotype in younger rats. Similar findings were reported by Anseloni et al., (2005), who demonstrated a later-life attenuated anxiety response as measured by EPM open-arm entries in PND 50-55 rats that were treated with carrageenan neonatally. In contrast, there is one study which showed inflammatory pain produced a reduction in fear-based extinction, which would produce an increase in freezing, opposite to the reduction in fear we observe (Doenni, Song, Hill, & Pittman, 2017).

However, this study differed from the current work in multiple facets. One, they used a different inflammatory agent (LPS vs. carrageenan). Second, their fear conditioning model differed in both the intensity of the UCS (4 tone/shock pairings at 1 mA vs 10 tone/shock pairings at 0.3 mA) and overall design with dedicated extinction periods for both auditory and contextual modes.

We did not observe any group differences between treatment groups on tactile or thermal sensitivity despite previous work suggesting a hyposensitive response in inflammatory pain-treated rats (Bhutta et al., 2001; LaPrairie & Murphy, 2007; Wang et al., 2004). This is somewhat surprising considering that 24 hours prior to sensory testing rats received an injection of formalin into the hindpaw in an effort to reduce thresholds and latencies in the current studies. However, latencies and thresholds for these rats were still relatively high despite the two inflammatory manipulations. Thus, formalin injections into the hindpaw prior to somatosensory testing did not succeed in reducing threshold/latencies, and we are likely continuing to observe a ceiling effect on these measures, as we previously reported.

There were several limitations in the current study. For one, pain litters were briefly handled daily to obtain paw measurements while undisturbed rats were not. This may have had a small effect on its own. For example, studies have shown that the adverse effects of neonatal separation can be reversed by tactile stimulation (Imanaka et al., 2008). Additionally, brief maternal separation versus prolonged separation has been shown to have protective effects on adverse emotional responses in mice (Bailoo, Jordan, Garza, & Tyler, 2014). Therefore, because rat pups in the inflammatory pain condition experienced brief daily separations, this may have counteracted the pain-induced stress by increasing certain aspects of maternal behavior, thereby eliminating some of the expected effects, particularly the somatosensory responses. In future studies, we could incorporate the same inflammatory pain methodology and subject the pups to a period of maternal separation in order to elucidate how inflammatory pain alters later-life behavior. A final limitation may be that our fear conditioning protocol may have still been too strong. This is apparent by the rat's ability to resist extinction to the auditory cues (see Figure 4). In particular the undisturbed and vehicle treated rats appeared to show an "anti-extinction" by having elevated freezing throughout the 10 tone presentations. We could potentially reduce the number of tone/shock pairings and/or reduce the intensity of the shock to eliminate this effect thereby group differences may become more apparent.

In conclusion, this study demonstrated that neonatal inflammatory pain attenuated conditioned fear responses in comparison to undisturbed controls. Inflammatory pain also influenced maternal behavior with litters that experienced pain showing a reduction in licking and grooming and an increase in nursing during the later days. Finally, there was no observed group difference in thermal or tactile hypersensitivity.

Acknowledgements:

This work was funded by NIGMS P20GM103643 (Meng PI) and NICHD/NIGMS 1R15HD091841 (Burman PI), as well as the generous support of the UNE summer undergraduate research experience (SURE) program, and the College of Arts and Sciences. The authors report no conflicts of interest. We'd like to thank Jared Zuke for his dedicated assistance with data collection as well as reviewing drafts of this manuscript. Additionally, we'd like to

thank Brandon Buck, Jacob Rudlong, Victoria Eaton, Ben Sasso, Josh Schultz, and Taylor Paquin for their help with data collection for this project.

Data Availability Statement:

The data that support the findings of this study are available from the corresponding author, Michael Burman, upon reasonable request.

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A. Experimental Timeline **Figure 1 –.**

Experimental timeline (A) depicting the four-day fear and somatosensory testing protocol along with the number of litters used from each breeding protocol. Daily neonatal manipulations and observation points (B) indicating when *i.p.l.* carrageenan injections (red arrows) occurred relative to paw thickness measurements (green arrows) and maternal observation recording (blue arrows).

Figure 2 -

Paw thickness measurements (in mm) for vehicle- and carrageenan-treated rats over the seven-day neonatal manipulation period. Carrageenan-treated rats showed greater paw edema than vehicle-treated rats following injection on PNDs 2-6. * indicates $p < 0.05$.

Figure 3 -

Percent of observations in which each of the 5 maternal behavior measures occurred: Licking/Grooming (A), Nursing (B), Nest Building (C), Self-grooming (D), and Maternal Contact (E) among undisturbed (white symbols; $N = 6$) and experimental (black symbols; $N = 6$) litters. Licking and grooming was significantly decreased, while nursing increased in vehicle/carrageenan-treated rats relative to undisturbed controls. * indicates a statistically significant main effect ($p < 0.05$) when collapsed across day.

Figure 4 -

Percent freezing for rats on the three contextual freezing variables (habituation, context, novel context) and two conditioned freezing variables (average tone, tone difference). Top two panels represent postweaning (PND 24) rats with males (A) and females (B) in the undisturbed ($N = 6M, 5F$), vehicle ($N = 5M, 5F$), and carrageenan ($N = 7M, 7F$) groups. Adolescent (PND 45) rats are represented in the middle two panels with males (C) and females (D) in the undisturbed ($N = 7M, 6F$), vehicle ($N = 8M, 7F$), and carrageenan ($N = 9M, 5F$) groups. Adult (PND 66) rats are depicted on the bottom two panels with males (E)

and females (F) in the undisturbed ($N = 5\text{M}, 8\text{F}$), vehicle ($N = 7\text{M}, 8\text{F}$), and carrageenan ($N = 8\text{M}, 8\text{F}$) groups. When collapsed across age and sex, carrageenan treated rats showed a reduction of freezing in the AVG Tone variable when compared to undisturbed controls.

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Figure 5 -

Average percent freezing for rats during the tone presentations collapsed across sex and age. Rats treated with carrageenan ($N = 42$) showed an attenuation in tone freezing compared to undisturbed rats ($N = 35$) but not vehicle-treated ($N = 40$) rats. * indicates a statistically significant effect ($p < 0.05$).

Figure 6 –.

Percent freezing for rats after each of the 10 CS tone presentations in the novel environment. Top two panels represent postweaning (PND 26) rats in the undisturbed ($N = 6M, 5F$), vehicle ($N = 5M, 5F$), and carrageenan ($N = 7M, 7F$) groups. Adolescent (PND 47) rats are represented in the middle two panels in the undisturbed ($N = 7M, 6F$), vehicle ($N = 8M, 7F$), and carrageenan ($N = 9M, 5F$) groups. Adult (PND 68) rats are depicted on the bottom two panels in the undisturbed ($N = 5M, 8F$), vehicle ($N = 7M, 8F$), and carrageenan ($N = 8M,$

8F) groups. There was a main effect of condition, in which carrageenan treated pups demonstrated less freezing. There was also an effect of tone.

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Figure 7 –.

Paw withdrawal thresholds on the von Frey mechanical allodynia measure for males (A) and females (B) for postweaning (PND 27) rats in the undisturbed ($N = 6M, 5F$), vehicle ($N = 5M, 5F$), and carrageenan ($N = 7M, 7F$) groups. Paw withdrawal thresholds on the von Frey mechanical allodynia measure for adolescent males (C) and females (D) for adolescent (PND 48) rats in the undisturbed ($N = 7M, 6F$), vehicle ($N = 8M, 7F$), and carrageenan ($N = 9M, 5F$) groups. Paw withdrawal thresholds on the von Frey mechanical allodynia measure for adult males (E) and females (F) for adolescent (PND 69) rats in the undisturbed ($N =$

5M, 8F), vehicle ($N=7$ M, 8F), and carrageenan ($N=8$ M, 8F) groups. We didn't observe any effect of neonatal pain condition on subsequent sensory function.

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