Long-term Effects of Perinatal Affective Pain on c-Fos and N2RA: N2RB expression in the rACC of adult rats

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May 14, 2022
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Acknowledgments

I would like to thank my advisor Professor Patrese Robinson-Drummer for her tremendous help and expertise, for her willingness to help us troubleshoot and problem-solve, and her cheerfulness and optimism every Monday at 8 am. I would also like to thank my fellow thesis group members Seabrook Jeffcoat and Taylor Siminiski as well as Naomi Kassahun and Arushi Ishwar for their help in the Lab, running, and brainstorming of the immunofluorescence protocol. Thank you to Kira Barnes and Jose Rodriguez for their support in Lab. I would like to acknowledge Dr. Gordon Barr and Dr. Carly Gomes for starting this project and donating the slides needed to make this work possible. In addition, I am grateful for all of Professor Laura Been’s help, sharing her tips and vast knowledge on our protocols, as well as always being an encouraging presence in Lab. Lastly, I would like to thank Luke Troyon for his help with the microscope and the entire Haverford Psychology Department. Without their support, both academic and otherwise, this project would have not been possible.
Abstract

Preterm infants in intensive care are exposed to higher levels of inflammatory injuries and infections than babies born full-term, during a time where noxious stimulations are normally absent. Research by Gomes and Barr (2020) suggests that early life exposures to injury and infection concurrently, as well as injury alone, have long-term effects on the nociceptive and affective components of pain. Following aversive conditioning, regions critical for pain processing (like the anterior cingulate cortex) show increased c-Fos expression and changes in glutamatergic subunit ratios for NR2A and NR2B. Using tissues acquired from Gomes and Barr (2020), the goal of this study was to measure activity in the rostral anterior cingulate cortex (rACC), through c-Fos expression and the semi-quantify the N2RA: N2RB ratio, in adult rats following a formalin-induced conditioned place aversion (F-CPA) task. We compared c-Fos positive cells in the ACC in rats exposed to either infection and inflammatory injury (E. coli and carrageenan), only infection (E. coli and saline), only inflammatory injury (PBS and carrageenan), or a control (PBS and saline). Results showed no significant difference in c-Fos expression in the ACC between any of the four groups. Due to the small sample size, rACC slices were grouped with the rest of the ACC, but the sample size still did not allow statistically powered results. Lastly, we were unable to semi-quantify the N2RA: N2RB ratio as we did not have full brains to run immunofluorescent staining. Contrary to our hypotheses, these findings revealed adult ACC activity did not reflect developmental treatment-related exposures as proposed by Gomes and Barr (2020) as well as previous research studying affective pain and the ACC.

Keywords: Perinatal, Affective Pain, Carrageenan, E. coli, Formalin-Induced Conditioned Place Aversion, ACC, c-Fos, N2RA, N2RB, rat
Long-term Effects of Perinatal Affective Pain on c-Fos and N2RA: N2RB expression in the rACC of adult rats

Preterm babies are all infants born before 37 weeks of gestation have occurred (World Health Organization, 2018). Preterm infants spend an average of 17 days in the newborn intensive care unit (NICU), ranging from 30 days to around a week for infants born at 32 and 37 weeks of gestation respectively (Ann & Robert H. Lurie Children's Hospital of Chicago, 2020). During their time in the NICU, neonates are exposed to an average of 12 painful medical procedures a day (Carbajal, 2008). Although around 70% of medical procedures are considered painful, analgesia is only used in 20.8% of painful procedures (Carbajal, 2008). The fear of adverse effects of analgesia and the underestimation of pain leads doctors to inadequately manage pain in neonates (Williams & Lascelles, 2020). Painful medical procedures expose preterm babies to unknown levels of pain during a time when noxious stimulation is normally absent. The period neonates spend in the NICU coincides with a very sensitive period of brain growth and development (Andescavage et al., 2017). In their report, Fitzgerald et al. (2005) demonstrate a variety of differences in developing nociceptive systems relative to older animals. The authors note that in the neonatal state, nociceptive neuronal circuits are limited, underdeveloped, and much more excitable than in older individuals. Furthermore, during these preterm and neonatal periods, noxious stimulation results in exaggerated cutaneous reflexes, prolonged excitation of the dorsal horn, and large receptive fields of the dorsal horn cells. These systems become more specific and tuned as development occurs (Fitzgerald, 2005). When noxious stimulation during this early stage persists, the period of stimulation is outlasted by the effects which can result in long-lasting changes in the nociceptive pathway (Slater et al., 2010). Exposure to persistent injuries and pain around this stage affects the development and tuning of
the nociceptive neuronal circuit, resulting in hyperexcitability of the nervous system and hyperalgesia (Ren et al., 2004; Ruda et al., 2000). Repetitive pain is associated with lower pain thresholds during development (Anand et al., 1999). In the long-term, exposure to pain, in premature infants, is associated with behavioral and cognitive problems, anatomical and hormonal changes, and acute responses to pain (Brummelte et al., 2015; Ranger et al., 2015; Taddio et al., 1995).

The International Association for the Study of Pain (2021) describes pain as, “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (para. 1). This definition differentiates two aspects of pain, the nociceptive and the affective components of pain. Nociceptive pain is caused by physical damage to tissue whereas affective pain is the feeling of unpleasantness and aversiveness (Price, 2000). The affective component of pain encompasses the feelings of distress and fear, as well as the desire to escape or end the painful stimuli (Price, 2000). The “pain-sensitive” window is considered the period when infants are especially susceptible to long-term neurodevelopmental changes caused by pain experiences. The sensitive period where short-term injuries and inflammation induce long-term changes was between birth and day 5 (Ren et al., 2004). Pain and stress during this sensitive period can impair cognitive and social development, and result in an increased vulnerability to stress and anxiety disorders in adulthood (Williams & Lascelles, 2020). Poor health outcomes such as diabetes, hypertension, obesity, and cardiovascular diseases are much more common in adults who experienced early-life painful experiences (Williams & Lascelles, 2020). Research conducted on rodent models shows that localized painful stimulation in neonates, from Freund’s adjuvant or carrageenan, alters responses to sensory stimulation and is associated with adult hyperalgesia (Ren et al., 2004; Ruda et al., 2000). In rats, the sensitive
period which results in adult hyperalgesia is their first week of life (Ren et al., 2004). The developmental stage of the central nervous system of rats during this first week corresponds to that in premature human neonates (Semple et al., 2013). Although much is known about the effects of early pain exposure on the long-term changes in the nociceptive pain response, this is not the case for the affective pain response.

Most of the daily painful experiences endured during premature infants’ time in the NICU are caused by medical procedures. The most frequently performed procedure is the heel stick used to retrieve blood (Carbajal, 2008; Courtois et al., 2016). Open wounds, caused by medical procedures, make preterm infants more susceptible to breakthrough infections, leaving them exposed to both infections and physical injuries (Wang et al., 2019). Infections are a common cause of pain for neonates during their time spent in the NICU. Nosocomial infections, infections that occur after the first 72hrs of life, develop in 6.2 to 33% of neonates in the NICU. The incidence of infection is higher in preterm neonates due to their immature immune systems, 20-30% of preterm neonates experience two or more nosocomial infections during their time in intensive care settings. (Clark et al., 2004). The effects of inflammation caused by neonatal bacterial infections such as E. coli (or experimentally induced via lipopolysaccharide (LPS) exposure) have long-lasting effects in adulthood. A study by Boissé et al. (2005) determined that exposure to LPS during the neonatal period resulted in nociceptive disorders. Rats in the LPS group showed decreased nociceptive thresholds and increased responses to painful stimuli in adulthood when compared to the control group. These findings suggest that similarly to neonatal injuries, neonatal bacterial exposures alter nociceptive perception and sensitivity in adulthood (Boissé et al., 2005). Although newborns in intensive care settings are commonly exposed to infections and physical injuries simultaneously, few reports have examined the impact of these
two factors in combination on adult pain processing. It is essential to understand the relationship between infection and injuries as many preterm newborns undergo multiple painful procedures, during a time when infections are the most common (Polin et al., 2012).

As the previously mentioned reports suggest, infection and physical injury in neonates independently alter the affective and the nociceptive pain response in adulthood, they decrease the nociceptive threshold and enhance the perception of pain (Boissé et al., 2005; Ren et al., 2004; Ruda et al., 2000). Although the effects of infection and injury on adulthood pain responses are known, less certainty exists on the neural pathways the early exposures alter. Current reports suggest that different neural pathways process each pain component. Research suggests that noxious stimulus follows a projection from the spinal dorsal horn through the lateral thalamus to the primary somatosensory cortex and the insular cortex; in contrast, affective information is believed to be processed through a pathway starting in the dorsal horn, through the medial/intralaminar thalamic nuclei to the anterior cingulate cortex (ACC) (Minami, 2009; Treede et al., 1999). Additional evidence suggests that the anterior cingulate cortex plays an important role in the processing of emotions, memories, and pain (Lamm et al., 2011). The ACC, located in the medial wall of each cerebral hemisphere, above and next to the corpus callosum, is a part of the cingulate cortex (Paxinos and Watson, 2013). The ACC’s connections to the prefrontal cortex and the limbic system make it an important player in the integration of affect regulation (Stevens et al., 2011). Early research implicating this brain region in the affective component of pain was the result of surgical ablations of the ACC. Lesions to the ACC resulted in a decrease in the unpleasantness caused by pain without affecting the ability to localize or feel the pain (Hurt & Ballantine, 1974).
In more recent years, studies have observed the role of the ACC in pain processing with the help of neuroimaging (Casey, 1999) or by using Immediate Early Genes (IEGs) as indirect markers for neuronal activity (Gallo et al., 2018). IEGs are the first genes, following stimulation, to undergo regulation of expression (Tischmeyer and Grimm, 1999). A specific IEG called c-Fos is a proto-oncogene expressed within neurons following action potentials and is commonly used as an indirect marker for neuronal activity (Bullitt, 1990). c-Fos expression is the highest when the presented stimulus is novel and learning is required, once the experimental procedure is familiar, c-Fos induction is attenuated (Tischmeyer and Grimm, 1999). Assessing c-Fos expression facilitates the quantification of activated neurons during nociception, allowing neuronal activity to be measured by immunohistochemical techniques 20 to 90 minutes following stimulus exposure (Bullitt, 1990; Harris et al., 1998). Importantly, c-Fos expression is correlated with learning and memory and its expression has been observed in regions such as the ACC, the amygdala, and the paraventricular nucleus of the hypothalamus (Barr, 2011; Cao et al., 2009; Gallo et al., 2018; Lei et al., 2004a).

High c-Fos expression in brain regions such as the ACC and the amygdala has been observed following conditioned place aversion (CPA) tasks as the rats successfully learn to avoid the area or the object associated with the aversive stimuli (Barr, 2011; Jarrin et al., 2020; Lei et al., 2004). In a CPA task, the animal learns an association between an environment with contextual cues and a negative stimulus, this allows for the assessment of the affective component of pain avoidance (Gomes & Barr, 2020; Minani, 1997). During the conditioning trials, the negative reinforcer, often a formalin or carrageenan injection or a shock, is introduced and the animal is placed into a compartment with a distinctive cue. The animal, once conditioned, is placed in an apparatus with multiple compartments, one of them being the same
compartment with the distinctive cue. Time spent in each compartment is measured to determine if a learned association occurred between the aversive stimulus and the environment; if learned, animals will avoid the compartment paired with the negative reinforcer (Tappe-Theodor and Morgan, 2019).

Formalin-induced conditioned place aversion tasks (F-CPA), the combination of the hind-paw formalin model and the place-conditioning paradigm, allows us to measure behaviors that reflect both the nociceptive and the affective components of pain separately (Gao et al., 2004; Tzschentke, 2014). The place avoidance induced by the pairing of the CPA and the formalin injection exclusively reflects the affective component of pain; in contrast, the acute behaviors such as paw licking, biting and flinching resulting from the injection correspond to the nociceptive component (Johansen et al., 2001). Animal behavioral studies show that formalin-induced CPA is mediated by the ACC (Gao et al., 2004; Johansen et al., 2001; Johansen & Fields, 2004). The ACC is activated by noxious stimuli and is specifically involved in affective pain processing (Minami, 2009; Li et al., 2009; Price 2000). Experiments lesioning the ACC before F-CPA tasks resulted in a reduction in formalin-induced conditioned place avoidance while leaving nociceptive behaviors intact. Attenuation of F-CPA was observed when lesions originated exclusively from the rostral, not the caudal, ACC. This suggests that an intact rACC is necessary to produce aversion to painful stimuli; and lesions to this region exclusively attenuate the affective quality of pain (Johansen et al., 2001). This evidence supports the idea of separate neural pathways for the distinct components of pain, implicating the ACC critically in the processing of the affective component.

Glutamatergic receptor activity is implicated in pain processing in the ACC, their expression increases with the presence of painful stimuli. Glutamatergic receptors are highly
expressed in the rostral ACC, play an important role in pain processing, and are required for pain aversion learning (Jarrin et al., 2020; Johansen et al., 2004). N-methyl D-aspartate (NMDA) receptors are ionotropic glutamate (an excitatory neurotransmitter) receptors (Chen et al., 2021). NMDA receptors play an essential role in synaptic transmission and plasticity of the central nervous system. They induce the activation of long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD are the two major forms of synaptic plasticity (Massey et al., 2004). LTP enhances synaptic function while LTD attenuates its function (Zhuo, 2009).

Current research links NMDA receptor expression in the rACC to pain processing and aversion learning (Jarrin et al., 2020; Johansen & Fields, 2004). Experiments demonstrate that the introduction of an NMDA antagonist, such as intra-ACC injections of AP5 or an antagonist of glycine sites of the NMDA receptors in the rACC, during conditioning blocks learning (Lei et al., 2004b; Ren et al., 2016). The inhibition of NMDA receptors, through an antagonist, is sufficient to reduce the F-CPA conditioning score and c-Fos expression in the ACC (Lei et al., 2004b). rACC glutamatergic transmission through NMDA receptors is essential for the acquisition of formalin-induced conditioned avoidance and consequently plays a major role in the affective component of pain (Johansen & Fields, 2004).

NMDA receptors contain combinations of the NR1 subunit plus one or more NR2A-NR2D subunits, GluN2A (or NR2A) and GluN2B (NR2B) subunits predominate in the forebrain (Monyer et al., 1994). NMDA receptor subtype NR2B is specifically required in LTD while NR2A is involved in LTP but it is not essential for its induction (Massey et al., 2004). The composition of NMDA receptors changes throughout development. According to Sheng et al. (1994), at birth, forebrain NMDA receptors contain exclusively NR1 and NR2B subunits with high levels of N2RB remaining constant throughout adulthood. In contrast, NR2A is not
expressed at birth but instead appears progressively for the first three weeks of life until it plateaus, remaining the same throughout adulthood (Sheng et al., 1994). By the third or fourth week of postnatal development, once NR2A has plateaued, the NR2A: NR2B ratio has decreased and remains steady throughout life (Sheng et al., 1994). Li et al. (2009) investigated the contributions of NMDA subunit type in the ACC to CPA activity and reported an upregulation of both NR2A and NR2B subunit expression in the rACC following neonatal formalin injection (i.e., the presence of a painful stimulus). The authors also found that the attenuation of either subunit with an antagonist result in the inhibition of F-CPA acquisition and formalin nociceptive conditioning induced c-Fos expression. In the same report, it was noted that overexpression of NR2A and NR2B leads to heightened learned pain response and their attenuation inhibits a learned response. Additional research conducted in transgenic mice, with overexpressed NMDA receptor subunit NR2B in the forebrain, suggests that NR2B overexpression causes an enhanced response to inflammatory stimuli (Wei et al., 2001). In the prefrontal cortex, the N2RB subunit is critically involved in the formation of contextual fear memories and LTP, blocking this subunit results in their impairment (Zhao et al., 2005). Similarly, NR2B is a key player in the processing and long-term changes of visceral pain in rats (Zhou et al., 2013). Unlike localized inflammatory pain, visceral pain is perceived diffusely throughout the body and produces strong affective responses (Sikandar & Dickenson 2012). Despite the overexpression of both NR2A and NR2B subunits, most of the research suggests NR2B expression specifically plays a role in increased pain responses. All research on the changes of NMDA receptor subunit expression is in the context of inflammatory injury or infection, there is currently no research looking at the interaction of perinatal injury and inflammatory. Nonetheless, previous literature would suggest
that individuals exposed to pain and infection would have a smaller NR2A: NR2B ratio in their ACC than individuals not exposed to these conditions.

Gomes and Barr (2020) began to examine the behavioral components of the relationship between perinatal infection and inflammatory injury on future nociceptive and affective responses to painful stimuli in rat pups. For their study, rats were infected with E. coli or PBS at postnatal day 2 (PN2) and injected with carrageenan or saline at PN3. At PN65, they underwent formalin-induced conditioned place aversion tasks to study the differences in learned pain aversion between rats in the control, the infected, the injured, and the combined group. The study found that early inflammatory injury and early E. coli infection independently increased pain scores in P8, but these effects continued into >PN65 group only for the carrageenan group. The combination of E. coli and carrageenan had the greatest effect on pain aversiveness in adulthood (>P65) with animals exposed to both showing the strongest response and aversion to pain (Figure 1). These findings show an age-dependent interaction between injury, infection, and long-term effects on responses to painful stimuli.

**Figure 1.**

*Effects of E. coli Infection and Carrageenan Injury on Learned Aversion to Formalin Stimuli*
two pup groups E. coli or saline, while the circles indicate the carrageenan group and squares specify the saline control group. This graph shows a significant learned aversion to painful stimuli following exposure to carrageenan \((F(1,17) = 5.631, p = 0.030)\). As well as a significantly higher aversion to painful stimuli following combined exposure to injury with carrageenan and E. coli infection \((F(1,17) = 5.728, p = 0.028)\) relative to E. coli alone.

Research on the interaction between infection and inflammatory injury on long-term changes in brain activity is limited. By using the Gomes and Barr (2020) study we attempted to understand the long-term effects of this inflammatory injury and infection interaction on c-Fos expression and semi-quantify the N2RA: N2RB ratio of NMDA receptors, in the rostral anterior cingulate cortex. The rostral ACC excitatory neurotransmission is essential for the acquisition of formalin-induced conditioned avoidance but not for its expression and c-Fos expression is the highest when the stimulus is novel and learning is required (Johansen & Fields, 2004; Tischmeyer and Grimm, 1999). Thus, we expected to see c-Fos expression in the rACC in the rats with heightened pain aversion following the F-CPA task. Additionally, we expected to see an upregulated expression of NMDA receptors, more specifically a decrease in the NR2A: NR2B ratio in the rACC, caused by an upregulation of expression of the NR2B subunit.

The original goal of this study was to compare c-Fos expression and semi-quantify the N2RA: N2RB ratio in the rACC between the E. coli infection and inflammatory injury interaction group and the PBS and saline control group following adult F-CPA. Due to a lack of whole brains, we were unable to complete the immunofluorescence staining protocol and thus did not study the prevalence of NMDA receptor subunits N2RA and N2RB in the rACC. Additionally, the small sample size of adult rats in the E. coli-carrageenan group resulted in a
comparison c-Fos expression in the ACC as a whole, in all four condition groups (E. coli-carrageenan, E. coli-saline, PBS-carrageenan, PBS-saline). Only a few of the animals had rACC slides so the study grouped the rACC with the rest of the anterior cingulate cortex. Nonetheless, our hypothesis remained, and we expected the interaction group to have the highest c-Fos positive cells in the ACC and the control group to have the lowest c-Fos positive count. This hypothesis is supported by previous studies showing that early injury in pup rats results in increased learned aversion, and consequently a higher count of c-Fos positive cells than their control (Barr, 2011).

Methods

Animals and behavioral testing

Brains originated from Long-Evans Hooded rats used by Gomes & Barr (2020). Rat pups were separated into four groups, PBS-saline, E. coli-saline, PBS-carrageenan, and an E. coli-carrageenan group. Two days after birth (PN2) rat pups were inoculated with either 0.1 mL of Phosphate-buffered saline (PBS) or 0.1x 10⁶ CFU E. coli suspended in 0.1mL of PBS. The following day, PN3, pups were either injected with 0.25% carrageenan (1μl/g) in saline or saline alone, on the plantar surface of the left hind paw.

Conditioned place aversion tests, formalin, and thermal tests for inflammatory and acute pain respectively were used at PN8, PN15, and >PN65 to assess nociceptive and affective pain response. Formalin testing was used to measure nociceptive susceptibility. Rats received hind paw injections of formalin before being placed in a compartment with a lemon odor. Behavior was observed 30-45 minutes after injection (Figure 2). The conditioned place aversion test was conducted to assess the affective component of pain and quantify the learned aversion to the painful stimulus. The testing site consisted of a chamber divided into three zones, a neutral area,
a chamber with an unscented cotton ball, and a chamber with a lemon-scented cotton ball. Rats were placed in the neutral chamber and the time present in each chamber was measured to determine learned aversion. Plantar thermal withdrawal tests, to record latency of withdrawal time, were used to measure baseline responses to pain and changes in nociceptive responses to acute pain. The thermal withdrawal test consisted of placing the rats in a plexiglass chamber where both hind paws were exposed to a focused heat lamp; the recorded latency was the time it took for rats to lift each paw from the glass surface.

Rats used in the Gomes and Barr (2020) study were sacrificed and their brains were removed, flash-frozen, and stored at (-20°C). Brains from all four experimental groups were used for the quantification of c-Fos expression. The sample consisted of 15 pups in either of the four groups: E. coli-carrageenan group (n = 1), E. coli-saline (n = 2), PBS-carrageenan (n = 5), and PBS-saline (n = 7).

Figure 2.

Timeline for treatments for the ≥PN65 group run by Gomes and Barr (2020)

Note. At PN2 pups were inoculated with E. coli or saline. At PN3 pups were injected with either PBS or carrageenan. At ≥PN65 pups underwent thermal, formalin, and conditioned place aversion tests (figure adapted from Gomes & Barr, 2020).

Cryosectioning

The cryostat was used to cut 20µm thick coronal sections. The coronal sections were then thaw-mounted onto microscope slides for immunofluorescence protocol.
c-Fos Immunostaining

Although we did not have access to the exact protocol, Gomes and Barr (2020) performed immunohistochemistry on the ACC tissue. Slices were incubated for 46hrs in was a rabbit anti-c-Fos primary antibody. The second antibody used was a goat anti-rabbit, sample incubated for 1hr. A 10 min H₂O₂ and DAB incubation was used to cause a precipitate at the site of c-Fos positive cells. c-Fos positive cells were identified by their brown, round staining (Figure 4).

Figure 4.

Photomicrograph of c-Fos Positive Cells in the cACC

Note. The image shows c-Fos staining of the cACC at a lower and higher magnification. The black arrow in the right image indicates a c-Fos positive cell.

Immunofluorescence

Day 1, sections were washed for 5 min 8 times with 0.1M PBS at room temperature before incubating for 15 min with hydrogen peroxide (H₂O₂). Sections were then washed 5 min 5 times in PBS to suppress endogenous peroxide. 200ml of 1:5000 dilution of c-Fos (9F6) Rabbit monoclonal antibody and a 1º master mix, 0.4% Triton-X in PBS (4:1000). Slides were
incubated for 46 hours at room temperature. Day 3, sections were washed before being covered in 2º master mix with Jackson, Alexa Fluor488 Conjugated AffiniPure Goat anti-rabbit IgG (H+L), slides incubated for 1hr in the dark. Slides were washed 5 times for 5 minutes. EverBrite TrueBlack® Hardset medium was applied before coverslipping.

**Imaging and Cell counting**

Tissue sections used for cell counting were imaged and examined at 2.5-3.2x, using a dissection microscope. Two slices were counted per animal (except where noted, see results). The regions of interest used were the rACC (plate 8-13), the ACC (plate 14-22), and the cACC (plate 23-36) (Figure 3). Plate numbers were based on Watson and Paxinos’s *The Rat Brain in Stereotaxic Coordinates* (2013). Immunoreactive nuclei were identified and counted using ImageJ/Fiji (Schindelin et al., 2012). Scoring was conducted blindly, with no knowledge of the conditions of the animals. A minimum threshold for particle selection was manually set by the scorer in a 250x250pixel area within the region of interest. The automated particle selection threshold chosen by the scorer was adjusted per image to 5% of the manual count before being applied to the entire region. The left and right sides of the ACC were counted separately, and the final c-Fos count per animal was the average of both sides from both slices (Figure 3,4).

**Figure 3.**

*Sections of rACC, ACC, and cACC Captured and Counted*
Note. The sections outlined in red correspond to the areas captured and counted for the rACC, the ACC, and the cACC respectively.

Statistics

A 2-way ANOVA was used to study the variability of c-Fos expression between the PN2 treatment (carrageenan and PBS) and PN3 treatment groups (formalin and saline). c-Fos positive cells were counted in all slides containing ACC, including rostral through caudal. For statistical analysis we used the average of all total c-Fos counts per animal. The alpha level was 0.05.

Results

The immunofluorescence protocol was successful in practice rats exposed to Novel Object Recognition tests. These tests do not require ACC activation, so we did not observe any c-Fos expression in the ACC in the practice rats. Nonetheless, the protocol was considered successful as staining of c-Fos positive cells and cell bodies were observed in the thalamus (Figure 5).

Figure 5.

_GFP and DAPI Results from Immunofluorescence Protocol in the Thalamus and the ACC_
Note. A, photomicrograph showing c-Fos expression in the thalamus. The yellow arrows indicate c-Fos positive cells. B, represents cell bodies in the thalamus. C and D, are GFP and DAPI photomicrographs of the left rACC, slide 12 (Paxinos and Watson, 2013).

A 2-way analysis of variance was used to study differences in c-Fos expression between the PN2 treatment (E. coli, PBS) and the PN3 treatment (carrageenan, saline). Results show no significant differences in c-Fos expression between the two PN2 treatment groups, E. coli and PBS ($F(1, 3) = 1.593, p = .233$). The was no significant main effect of PN3 treatment ($F(1, 3) = .191, p = .670$), carrageenan and saline groups contained similar levels of c-Fos expression. Lastly, no interaction effect was observed between PN2 and PN3 treatments ($F(1, 3) = .087, p = .774$) (Table 1).

Table 1.

Analysis of the Variance of c-Fos Expression and Treatment Group

<table>
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<td>55768</td>
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Note. PN2 Treatments were E. coli or PBS, while PN3 treatments were either Carrageenan or Saline. Alpha level was 0.05.

The difference in c-Fos expression in the E. coli-Carrageenan ($M = 5$), E. coli-Saline ($M = 168$, $SEM = 40$), PBS-Carrageenan ($M = 300$, $SEM = 94$), and PBS-Saline ($M = 323$, $SEM = 102$), was not statistically significant (Figure 6).
Discussion

Understanding the brain regions and the receptors that are affected by exposure to painful stimuli during the first months of life in rats is important as this research is easily translatable to humans, especially for individuals born prematurely or where in the NICU. In 2020, in the United States, one of every ten children was born prematurely (CDC, 2021). As these exposures cause long-term cognitive defects, behavioral problems, and hypersensitivity to pain it is important to figure out how to effectively treat these patients and prevent these long-term outcomes. The methods used in the Gomes and Barr (2020) study were specifically chosen to mimic the clinical experience of neonates in the NICU. Inoculation with E. coli and carrageenan injections were conducted at P2 and P3 respectively, as rats at this stage have a similar brain development as premature infants (Semple et al., 2013). While E. coli was used as it is
commonly observed in infants in the NICU and causes early-onset sepsis in preterm babies (Barbera et al., 2011). Carrageenan was used to simulate the injuries caused by common skin-breaking procedures used in NICU settings, such as venipunctures and heel sticks.

The results of this study show no relationship between early exposure to inflammatory injury or infection and increased c-Fos expression in the ACC. These findings suggest that early painful exposures do not result in increased ACC activity. Our first hypothesis predicted an increase in c-Fos expression specifically in the rostral anterior cingulate cortex; unfortunately, the sample size used did not allow for an analysis of the comparison of c-Fos expression in the rACC between the interaction and control group. Instead, we used pooled ACC slides to look at c-Fos expression differences between groups in the entirety of the ACC and found no significant differences between any of the four groups. My second hypothesis, regarding a change in the N2RA: N2RB subunit ratio, in the rACC, in pups exposed to neonatal infection and inflammatory injury was not carried out as there were no whole brains available. The brains used in this study, had been previously sliced, stained for c-Fos expression, and mounted on slides, and thus could not be used for the semi-quantification of the N2RA: N2RB ratio.

Although we found no significant differences in ACC c-Fos expression between groups there are numerous limitations to this work that most likely affected our results. Our sample size was small, $N=15$, making the detection of any significant results unlikely especially since our interaction group had a sample size of one. The small sample size was due to small quantity and bad quality of our ACC slides. The small sample size led us to combine all ACC groups, from rostral to caudal. The grouping of the different regions of the ACC might have concealed any significant differences. Especially considering that previous research suggests it is only within the rACC that much of the activity, and thus c-Fos expression is located. Additionally, the slides
used in this study were poorly mounted, there was a lot of debris as well as tears in the slices. The inconsistencies in the slides made counting c-Fos positive cells much harder and likely affected our results. Lastly, not having access to whole brains we were not able to complete the main hypothesis regarding the NMDA subunit ratio. Although part of the protocol appeared successful, and we were able to observe c-Fos positive cells in the brain, we did not have any experimental brains to run the immunofluorescence and consequently did not attempt to study N2RA or N2RB expression.

The next step in this research is to run the protocol suggested originally, using whole brains from the Gomes and Barr study. The use of whole brains would allow us to section them ourselves making sure to collect the entirety of the ACC, including rACC, as well as the ability to control the immunostaining and limit dirt particles and tears on the slides making counting more efficient and more accurate. This would allow us to study the rACC directly, as well as to compare it to the rest of the ACC. It would also allow for the semi-quantification of the N2RA: N2RB ratio. Once we have a much larger sample size it would be possible to run a 2-way ANOVA to compare c-Fos count between all four groups as well as compare the rACC to the rest of the ACC. This analysis could also be done to compare N2RA: N2RB ratio between all four groups. In addition, once we have full brains, we can look at other possibly involved brain regions such as the amygdala, the mPFC, and the hippocampus as suggested by Gomes and Barr (2020). Once c-Fos expression and N2RA: N2RB ratio between groups has been compared in the ACC and other brain regions the next step would be to look at other kinds of injections commonly observed in the NICU as well as increasing the number of injuries. In Gomes and Barr’s study, the pups received one hind-paw injury, and the average NICU baby receives around 12 injuries a day (Carbajal, 2008). An experiment where pups are injected with carrageenan
more than once would likely be more comparable to infants in the NICU. We could also look at the difference in c-Fos and learned aversion when infections and injuries were treated, as infants in the NICU are treated for infections. A combination of all these models would allow for a more accurate and realistic comparison of the clinical model.

Despite this study showing no significant results, it is not representative of previous research in the field and thus needs to be restudied correctly. The research conducted by Gomes and Barr (2020), with the rodents used in this study, showed an increased learned aversion in the interaction group. When pups were exposed to either carrageenan alone or concurrently with E. coli, there was an observed increase in learned aversion during the formalin-induced CPA task. An increase in learned aversion suggests increased susceptibility to painful stimuli. These findings imply that we should have seen an increase in c-Fos expression in cases where learning was more prevalent. This means that the interaction group should have the highest c-Fos count, followed by either carrageenan or E. coli exposure, with the control group having the least expression.

The ACC is one of the regions specifically involved in the affective component of pain (Minami, 2009; Li et al., 2009; Price 2000). It plays a critical role in formalin-induced CPA tasks making it an important region to study and merits further investigation (Gao et al., 2004; Johansen et al., 2001; Johansen & Fields, 2004). Additionally, it is suggested that the rACC specifically is the area involved in the affective pathway, previous research showed that lesions to the rACC result in the reduction of CPA and attenuate the affective component (Gao et al., 2004; Johansen et al., 2001). Lesions to the rACC result in the destruction of the neurons, in the region, and consequently their receptors. N2RA and especially N2RB are the dominant subunits of the NMDA receptors in the forebrain, making this the reason these subunits should be studied
in the rACC (Monyer et al., 1994). Previous studies found that blocking NMDA receptors in the rACC block learning and are sufficient to create a learned aversion (Johansen & Fields, 2004; Lei et al., 2004b; Ren et al., 2016). From this and research showing that overexpression of N2RB causes heightened aversion to painful stimuli, we could predict that the interaction group would have the highest expression of N2RB and thus the largest ratio.

Preterm humans in the NICU are exposed to both infections and injuries at an age when their nociceptive circuitry is most vulnerable to long-term changes (Ren et al., 2004). Understanding the brain regions involved in pain perception and processing is an essential step in the larger understanding of how preterm infants respond to injuries and infection at a time when it is not common. Although our understanding of the effects of these early aversions on the nociceptive component of pain is extensive, knowledge of the effects on the affective component is limited, the goal of this study was to add to our understanding of the effects of combined exposure to inflammatory injury and infection on behavior and development. Although thus far the study of the affective component of pain has been minimal it is as important to understand as the nociceptive component. While physical, nociceptive pain involves reflexes and nociceptors, affective pain activates the frontal region of the cerebral cortex, and thus higher-level processes (Minami, 2009). In many cases, the long-term effects of pain are more commonly associated with the affective component instead of the nociceptive component (Ren et al., 2004; Ruda et al., 2000). Affective pain is thus more likely to have lasting effects on brain activity and functioning. It is important to study the affective component of pain in as much detail as the nociceptive component of pain has been studied in the past. The characterization of the affective component of the pain response would allow a comprehensive understanding of the long-term behavioral and developmental effects of early painful stimulation in the preterm population. The high
prevalence of premature births makes exposure to painful procedures and infections a common occurrence in this neonatal population. A thorough understanding of the pathways, the brain regions activated, and the receptors involved would increase the possibilities for treatment in adult populations. As well as would allow for preventative measures to be taken to avoid heightened pain sensitivity, behavioral problems, and cognitive defects in adulthood.
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References


Clark, R., Powers, R., White, R. et al. Nosocomial Infection in the NICU: A Medical
https://doi.org/10.1038/sj.jp.7211120

Courtois, E., Droutman, S., Magny, J. F., Merchaoui, Z., Durrmeyer, X., Roussel, C., Biran, V.,
Eleni, S., Vottier, G., Renolleau, S., Desfrere, L., Castela, F., Boimond, N., Mellah, D.,
Epidemiology and neonatal pain management of heel sticks in intensive care units:

Neuroscience. 6. 507-20. 10.1038/nrn1701.

ey genes, memory, and psychiatric disorders: Focus on c-Fos, EGR1, and Arc.

cingulate cortex and amygdala to pain- and fear-conditioned place avoidance in rats.

Gomes, Carly & Barr, Gordon. (2020). Local injury and systemic infection in infants alter later
nociception and pain affect during early life and adulthood. Brain, Behavior, & Immunity


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pain-related negative affect? Journal of Neurochemistry, 96(6), 1636–1647.
https://doi.org/10.1111/j.1471-4159.2006.03677.x


https://doi.org/10.1097/SPC.0b013e32834f6ec9


https://doi.org/10.1016/j.pneurobio.2013.04.001


