Acute and Long-term Effects of Early Life Inflammatory Pain on Brain Morphometry and Adult Behaviour

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November, 2010

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Psychiatry

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Abstract

Premature infants receive numerous painful procedures while in the neonatal intensive care unit (NICU). Pain may lead to long-term negative consequences that include cognitive, social, and learning deficits and a higher incidence of depression. The mechanisms by which pain can exert these effects are currently unclear. To examine more precisely the effect of neonatal pain on brain morphology and adult behaviour, a rat model of acute neonatal inflammatory pain was used to mimic pain experienced by preterm infants in the NICU. Male rat pups were injected with either a low formalin dose (0.75%-1.5%), saline (0.9%), or remained uninjected during the first 2 weeks of life. First, we performed magnetic resonance imaging (MRI) on postnatal days (PND) 15 and 35 to identify potential changes in brain volume in response to pain. In a second study, we examined cellular proliferation (using Ki67, an endogenous marker of cell proliferation) and neurogenesis (using survival of BrdU-labelled cells) in response to pain in regions identified from our MRI analysis. Neurons were also counted using NeuN (a marker of mature neurons). Our final study investigated changes in anxiety and fear related behaviours in adult offspring subjected to pain as neonates. We consistently found that neonatal pain causes moderate hypertrophy in the basolateral amygdala (BLA) in PND 15 pups. These volumetric changes were not observed on PND 35. Similarly, neuronal counts (NeuN) showed a moderate increase in the BLA of PND 15 pain-exposed pups compared to controls ($p=0.062$), however, this effect was not seen on PND 35. However, there was no significant treatment effect of pain on Ki67-positive cells on PND 15, suggesting that neuronal proliferation induced by early pain occurred prior to PND 15. In addition, pain had no effect on the number of NeuN

and BrdU positive cells on PND 35, suggesting that pain has transient effects. In adulthood, rats that were exposed to pain as neonates did not differ from controls (either saline injected or uninjected) in behavioural tests measuring anxiety (social interaction and elevated plus maze) and fear (fear conditioning). Overall, these results show that repeated exposure to mild inflammatory pain during the neonatal period produces transient changes in BLA morphology, the consequences of which do not affect adult behaviours mediated by this structure. The early transient hypertrophic response to pain in the BLA may trigger plastic changes sufficient to protect the animal from any potentially negative functional consequences.

Keywords: pain, neonate, amygdala, anxiety, fear, proliferation, survival, neuron

Résumé

Lors de leur séjour dans l'unité des soins intensifs, les bébés prématurés sont soumis a de nombreuses procédures douloureuses qui ne sont pas toujours bien contrôlées et qui ont des conséquences a long terme sur les fonctions cognitives, l'apprentissage et les interactions sociales. Les mécanismes qui sont impliqués sont encore peu connus. Au cours de ce travail, nous avons utilisé un modèle animal de douleur inflammatoire répétée au cours de la période néonatale chez le rat pour comprendre les effets à long terme sur la morphologie du cerveau et le comportement relié à l'anxiété à l'âge adulte. Nous avons injecté les pattes arrières des ratons entre le jour 3 et 14 de la vie postnatale avec soit de la saline (0.9%) ou de la formaline (0.75-1.5%) une fois par jour. Un groupe contrôle n'a pas été injecté. Dans une première étude, nous avons mesure les changements de volume du cerveau induits par la douleur au jour 15 et 35 de vie postnatale. À l'aide de la résonance magnétique (scanner de 7T), nous avons pu démontrer une augmentation presque significative du volume de l'amygdale basolatérale (BLA) chez les ratons de 15 jours soumis à la douleur compares aux contrôles injectes à la saline. Par contre, cette hypertrophie amygdalienne n'était que transitoire car les différences de volume avaient disparu au jour 35. En quantifiant le nombre de neurones matures (en immunohistochimie avec NeuN) dans la BLA à ces mêmes âges, nous avons pu confirmer que le l'exposition à la douleur durant le 2 premières semaines de vie produit une hypertrophie neuronale dans la BLA au jour 15 mais que celle-ci n'est plus observée après le sevrage au jour 35. Nous avons également déterminé que l'hypertrophie au jour 15 était probablement due a une prolifération cellulaire qui avait eu lieu avant cette période car le nombre de cellules

avec un marquage positif pour le Ki-67 (un marqueur endogène de prolifération) était semblable entre les groupes douleur et contrôles au jour 15. De même, le nombre de cellules ayant incorpore le BrdU au jour 15 n'était pas différent au jour 35 selon les traitements. Ceci suggère également que l'effet de la douleur sur la morphologie de la BLA se limite à la période pendant laquelle les ratons sont soumis à la douleur. Finalement, à l'âge adulte, nous n'avons pas observé de différence significative entre les groupes contrôles et douleur en ce qui a trait à l'anxiété (par le test de interaction sociale et « elevated plus maze ») ou par rapport au conditionnement à l'anxiété (par le test de « fear conditioning »). Ces résultats nous permettent de conclure qu'une douleur de type inflammatoire modeste au cours des deux premières semaines de vie chez les ratons produit une hypertrophie neuronale transitoire de la BLA, une structure impliquée dans les émotions et les comportements relatifs à l'anxiété. Nous n'avons pas détecté de conséquences à long terme sur le morphologie neuronale ou les comportements d'anxiété suite a cette expérience néonatale.

Acknowledgements

Firstly, I would like to thank my supervisors, Drs. C.-D. Walker and B.J. Bedell for their guidance, support, optimism and wisdom throughout the past two years. I would also like to thank Drs. Celeste Johnston and Joe Rochford for agreeing to be on my thesis committee, for taking the time to answer my questions and allowing me to gain invaluable knowledge that only their unique specialties can provide. Without these people, this thesis would never have come to fruition.

I was very privileged to have access to some of the best facilities to conduct my research, but most importantly the wonderful people who work in them. From the Douglas Mental Health University Institute, I would especially like to acknowledge and thank Hong Long, Zhifang “Susan” Xu, and Anne Haldane for the invaluable help with the immunostaining and counting. I would also like to express my appreciation to various members of the Small Animal Imaging Lab. Without the talents of Jason Cakiroglu and Jonathan Yu, I would never have been able to conduct the MRI portion of my thesis. Thank you both for your dedication, working late nights that turned into early mornings, all so that this work could be accomplished. I would like to thank Rozica-Jenica Bolovan for her help with processing tissue and sharing with me all her sectioning secrets. Ming-Kai Ho was instrumental in the scanning of slides and providing much needed sugar boosts.

Last, but certainly not least, I would like to thank my family for their never ending love and support even though they don’t always understand what I do. My friends, confidants and occasional roommates, I will forever be indebted to you.

Acute and Long-term Effects of Early Life Inflammatory Pain on Brain Morphometry and Adult Behaviour

I. Premature Infants in Neonatal Intensive Care Units

Infants born less than 37 weeks gestation are classified as preterm infants. Due to the premature state of these infants, they are raised in incubators in the neonatal intensive care unit (NICU). During their stay in the NICU, these infants have numerous painful experiences that occur during routine procedures, including heel lances and intravenous line insertions, required for treatment and monitoring. Standard care involving painful procedures, such as blood draws, injections, circumcision (Howard, Howard, Garfunkel, de Blieck, & Weitzman, 1998) and immunization (Sparks, 2001), are performed with little or no anesthesia or analgesia. In fact, more than 65% of neonates (mean gestational age = 32.5 weeks) did not receive adequate analgesia during various painful procedures (Simons et al., 2003).

Unfortunately, preterm infants experience pain during a period of rapid brain development, a time when infants are normally protected in the mother's womb. In humans, by the third trimester of gestation, ascending pain systems are able to receive and process pain signals (Loizzo, Loizzo, & Capasso, 2009). However, descending modulatory pain systems are not equally mature. Therefore, the infant does not have the appropriate mechanisms required to regulate, buffer and terminate the pain response (Fitzgerald & Beggs, 2001; Fitzgerald & Koltzenburg, 1986). Long-term negative effects have been found in preterm neonates and these negative effects appear to be associated with the frequency of painful procedures experienced while in

the NICU (Johnston & Stevens, 1996). For instance, premature infants, with a mean gestational age of 29 weeks, have poorer cognitive and motor abilities at 8 and 18 months (Grunau et al., 2009). This result is associated with the number of skin-breaking procedures experienced while in the NICU.

Other studies have looked specifically at premature birth and its relation to negative long-term effects. School age children, born gestational age 32-35 weeks, have abnormal levels of hyperactivity (19%) and poor learning abilities, in skills such as writing (32%), arithmetic (29%), and reading (21%) as reported by parents and teachers (Huddy, Johnson, & Hope, 2001). Very premature infants (gestational age less than 33 weeks) are found to have lower verbal and performance IQ scores in adolescence (mean age 15.3 years) and young adulthood (mean age 19.5 years) (Allin, Walshe & Fern, 2008). When parents of teenagers (12-16 years old) who were born prematurely (mean gestational age 27 weeks) were asked to complete the Ontario Child Health Study-Revised questionnaire regarding their teen, they reported greater scores on subscales of attention deficit hyperactivity disorder and depression compared to parents of at term teens (Saigal, Pinelli, Hoult, Kim & Boyle, 2003). Since these children were born prematurely, they most likely experienced painful procedures that could be implicated in these negative long-term effects. Therefore, understanding how pain impacts the developing brain is vital for improving pain treatment and prevention in preterm and term infants, which could in turn reduce some of these negative long-term consequences.

II. The Pain System

There is a clear distinction between nociception and pain. Nociception is the brain's response to perceived or actual tissue damage. Pain, as defined by the International Association for the Study of Pain (IASP), is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." This definition identifies pain as being a combination of a physical, sensory component and an affective component. Thus, when studying pain, it is important to understand how these two distinct components are processed.

The main ascending pain pathway is the spinothalamic tract (STT), which is predominantly involved in the processing of the sensory aspects of pain (for a review, refer to Price, 2002). This pathway contains mostly wide dynamic range (WDR) neurons (75%), which respond to both innocuous and noxious stimuli. The receptive fields and activity levels of WDR neurons increase with noxious stimulation (Iwata et al., 2001) providing evidence for the alteration of pain processing of WDR with experience. The other neurons present in this pathway are nociceptive specific (NS) neurons, which only respond to noxious stimuli. NS neurons have small receptive fields, making them able to accurately localize pain (Coghill, Mayer, & Price, 1993). Nociceptive afferent neurons send nociceptive signals to Lamina I in the spinal cord, which then projects to the thalamus and subsequently to the primary and secondary somatosensory cortices. As this pathway is sensitive to changes in the quality of stimuli (Price, 2002), it is considered to be very important for the sensory processing of pain.

Two other key ascending pathways for the perception of pain are the spino-parabrachio-hypothalamic pathway and parabrachio-amygdaloid pathway (Bernard & Besson, 1990; Bernard, Bester, & Besson, 1996; Bernard, Peschanski, & Besson, 1989). In contrast with the STT, these pathways contain more NS neurons than WDR neurons. Since there are more NS neurons, these pathways are able to accurately detect stimulus location and intensity. The regions targeted by these ascending pathways are important for autonomic processing (hypothalamus) (Luquet & Magnan, 2009) as well as anxiety and fear (amygdala) (Krug & Carter, 2010; LeDoux, 2007), thus making these pathways important for the affective modulation of pain.

There is an alternative pathway, the spinothalamocortical pathway, which has been proposed to integrate somatosensory information with other sensory modalities, as well as learning and memory. This pathway sends signals from the somatosensory cortices to the insular cortex (IC), which then projects to several brain areas including the anterior cingulate cortex (ACC), hippocampus and amygdala (AMY) (Price, 2002). As the target brain regions of this pathway include the IC and AMY, it is important for the affective processing of pain. Attention and motivation are processed by the ACC, which projects to the prefrontal cortex (PFC) (executive functioning) and supplementary motor areas (response selection). Imaging studies in humans (Atlas, Bolger, Lindquist, & Wager, 2010; Berna et al., 2010; Villemure & Bushnell, 2009) and animals (Malisza et al., 2003; Seminowicz et al., 2009) have revealed that the ACC is the brain region most consistently activated during pain (Hsieh et al., 1996; Lamm, Decety, & Singer, 2010; Price 2002). Within the AMY, the basolateral amygdala (BLA) is important for the consolidation of emotional memory (Hubbard, Nakashima, Lee, & Takahashi, 2007) and

storage of fear memory (Ponnusamy, Poulos, & Fanselow, 2007), whereas the central amygdala (CeA), regulates fear, emotional memory and responses to threatening stimuli (Wilensky, Schafe, Kristensen, & LeDoux, 2006).

Descending inputs from the ACC and AMY modulate the pain signal by projecting to the periaqueductal grey (PAG), a relay center that sends afferents to the medulla. In the medulla, three types of neurons can be activated during nociception. These neurons are called “on-cells”, “off-cells”, and “neutral cells” according to the direction of the effect they produce on the pain signal. These three types of neurons project to the dorsal horn, each providing a unique direction for the pain signal. On-cells increase whereas off-cells decrease transmission, thereby allowing for bidirectional control of the descending system. The specific role of neutral cells in nociception is still unclear, but one theory suggests that these cells have the potential to become on- or off-cells during chronic pain states (Miki et al., 2002).

III. Animal Models of Pain During Development

Animal models allow for the study of pain with greater control over variables, such as frequency, timing and intensity of pain distribution, than is permissible with human subjects. Rats are suitable subjects because their growth pattern can be paralleled to that of humans. It is hypothesized that the developmental trajectory of the rat central nervous system (CNS) is comparable to that of a human infant such that the neonatal rat is relatively equivalent to a 24 week old fetus, a seven day old rat is equal to a full-term baby and by three weeks, the CNS of the rat is similar to that of a toddler (Dobbing, 1981; Marsh, Hatch, & Fitzgerald, 1997). Numerous developmental animal

models of pain have been developed to mimic different acute and chronic clinical pain experiences of preterm infants (for a review refer to Johnston, Walker, & Boyer, 2002). For instance, inflammatory pain conditions can be induced by injecting various inflammatory agents, including complete Freund's adjuvant (CFA), carrageenan, and formalin, into the hindpaw of the animal. Other models use either superficial or more profound peripheral nerve damage that causes neuropathic pain.

In this series of studies, we were interested in mimicking the type of repeated, mild pain that preterm infants experience in the NICU when receiving multiple heel lances over periods of 24 hours. While this procedure is less traumatic than other more invasive procedures encountered by preterm infants, it nevertheless causes pain (Johnston et al., 2008; Johnston, Stevens, Craig, & Grunau, 1993) and some degree of inflammation, even if the nursing staff takes precautions. In setting up our animal model, we needed to determine which inflammatory agent would best mimic the pain experience of preterm infants in the NICU. We took into consideration the duration of the solution's effect and the pain intensity produced by the stimulant. CFA has been shown to result in long-term activation of the immune system, resulting in a response that could be comparable to autoimmune disorders. A single injection of carrageenan can produce inflammation, which persists for up to two weeks (Alvares, Torsney, Beland, Reynolds, & Fitzgerald, 2000), but at a low dose (0.25%) causes short lasting (less than 2 days) inflammation (Lidow, Song, & Ren, 2001). The effects of an acute formalin injection have been found to last for 1-2 hours in adults (2.5%) (Guy & Abbott, 1992). We selected daily formalin injections for our pain procedure because of the

relative short recovery time, which means that injections could occur daily over relatively long periods of time without causing severe inflammation.

Formalin has frequently been used to test the inflammatory pain response of rat pups (Guy & Abbott, 1992; Mikhailenko, Butkevich, Vershinina, & Semenov, 2010; Rovnaghi, Garg, Hall, Bhutta, & Anand, 2008). The benefit of using formalin as an inflammatory agent in pups is that the concentration can be modified to complement the age of the animal, reflecting changes in behavioural responses across development (Guy & Abbott, 1992; Teng & Abbott, 1998). We varied the formalin concentration as a function of age to correspond to approximately 50% of the total pain score previously found at different ages (Teng & Abbott, 1998). This approach is an important consideration, since we injected our animals during the first two weeks of life, a time when motor and pain systems of the animal are developing rapidly. Our interest in using an animal model of early life inflammatory pain was to determine whether pain could induce long lasting changes in brain morphology and function. An earlier study by Anand et al. (2007) demonstrated that a high formalin concentration (4% single injection) resulted in greater cell death bilaterally in the AMY on postnatal day (PND) 1 and unilaterally on PND 7. Furthermore, in a pilot study where we performed magnetic resonance imaging (MRI) scans on young rats, we found hypertrophy in the AMY of pups that experienced pain in the neonatal period. This hypertrophy was consistently found to be nearly significant during the neonatal (PND 15) period. Since the AMY is implicated in the emotional processing of pain, we designed our studies to determine if pain would influence brain morphology, cellular development, and behaviours specific to the AMY.

IV. The Amygdala and its Relationship to Pain and Stress

The AMY is a limbic structure located in the medial temporal lobe that is important for processing and regulating responses to emotional stimuli. It is composed of several nuclei, which can be classified into three main subgroups: lateral (LA), basolateral (BLA), and central (CeA). Each nucleus has distinct inputs and outputs relating to their functional roles. As well, there are intra-amygdaloid connections, which are important for further processing of information.

The LA is referred to as the gatekeeper (LeDoux, 2007) since it is the nucleus, which receives and integrates inputs from all sensory systems (auditory, visual, somatosensory, gustatory and olfactory) and the thalamus (Turner & Herkenham, 1991). Consolidation of emotional memory (Hubbard et al., 2007) and storage of fear memory (Ponnusamy et al., 2007) occurs in the BLA. Projections from the PFC (Cressman et al., 2010), hippocampus (Kishi, Tsumori, Yokota, & Yasui, 2006), and thalamus (Turner & Herkenham, 1991) are received by the BLA. The BLA also receives intra-amygdaloid connections from the LA, which is important for attaching emotional significance to sensory information, in particular anxiety and fear (Hubbard et al., 2007; Neugebauer, Galhardo, Maione, & Mackey, 2009). The CeA is the main output nucleus of the AMY. It receives afferent fibers from the PFC, brainstem and viscero-sensory cortex (LeDoux, 2007). Nociceptive information can go directly from the parabrachial nucleus in the brainstem, bypassing the thalamus, to the CeA (Bernard & Besson, 1990). In terms of intra-amygdaloid connections, the CeA receives inputs from both the LA (von Bohlen und Halbach & Albrecht, 1998) and BLA (Savander, Go, LeDoux, & Pitkänen, 1995).

Intra-amygdaloid connections, in association with afferent and efferent projections with other brain regions, are extremely important for emotional processing and memory, in particular anxiety and fear. First, the LA receives sensory information from various sensory brain regions and the thalamus. Efferent projections from the LA are received by the BLA, which also has reciprocal connections with the hippocampus (Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000) and PFC (Gabbott, Warner, Jays, Salway, & Busby, 2005; Ishikawa & Nakamura, 2003). Thus, the BLA receives information regarding sensations (LA), context and memory (hippocampus) and executive functioning like attention and cognition (PFC). The BLA gathers all this information and associates an emotion, particularly anxiety and fear, to the stimuli. Therefore, the BLA is important for emotional processing, in particular consolidation of emotional memory (Hubbard et al., 2007) and storage of fear memory (Ponnusamy et al., 2007). This emotionally associated information is projected from the BLA to the CeA, the main output source of the AMY (Hopkins & Holstege, 1978; Rizvi, Ennis, Behbehani, & Shipley, 1991). The CeA is also known as the “nociceptive amygdala” (Ikeda, Takahashi, Inoue, & Kato, 2007; Palazzo, Fu, Ji, Maione, & Neugebauer, 2008) since it is the target of the parabrachio-amygdaloid pain pathway and is high in nociceptive neurons (Bernard, Huang, & Besson, 1992). In particular, the CeA regulates pain responses via its projections to pain pathway centers, including the PAG (Neugebauer, 2007; Oka, Tsumori, Yokota, & Yasui, 2008; Ortiz, Heinricher, & Selden, 2007; Pedersen, Scheel-Krüger, & Blackburn-Munro, 2007). In addition to receiving inputs from the BLA, the CeA also receives direct inputs from the LA (Jolkkonen &

Pitkänen, 1998). Once the AMY processes the information, it is projected to relevant brain regions for further processing (For a review, refer to Neugebauer et al., 2009).

Interestingly, several conditions, including stress, pain, and mental disorders, such as major depressive disorder and anxiety, have been associated with different AMY volumes in both humans and rodents, results, however, are inconsistent. In humans, AMY volumes across the entire spectrum, including large, small, and no difference, have been implicated in behavioural dysfunctions. A large AMY volume has been observed in adult patients with major depressive disorder, specifically in the left hemisphere (Lorenzetti, Allen, Whittle, & Yücel, 2010). In children, those who stayed longer in orphanages (range 0-28 months) and, therefore, were exposed to an unstable care giving regimen, had larger AMY volumes and increased anxiety, but no difference in hippocampal volume at 8 years of age compared to controls (Tottenham et al., 2010). Adopted children from severely deprived institutions also had larger left AMY volumes, such that children who lived longer in an institution had a larger AMY volume (Mehta et al., 2009).

On the other hand, a smaller AMY volume has been associated with other disorders involving fear, anxiety, and depression. From a group of adults who experienced the same traumatic event, namely the Tokyo subway sarin attack, those who developed post-traumatic stress disorder (PTSD) had a smaller bilateral AMY volume and PTSD severity was greater in individuals with a smaller AMY (Rogers et al., 2009). Reduced AMY volumes have been correlated with symptoms of dysphoric disorder, including emotional instability, aggression and irritability (van Elst, Groffmann, Ebert, & Schulze-Bonhage, 2009). Interestingly, the AMY regulates these symptoms.

One study documented that adolescents (mean age = 16) who suffer from bipolar disorder have smaller left AMY volumes (Chen et al., 2004). These studies demonstrate that different AMY volumes are found amongst individuals who suffer from disorders with AMY-regulated symptoms.

Van Elst et al. (2009) hypothesized that symptoms of affective disorders can be divided into categories depending on the size of the AMY. Mainly, symptoms related to depression such as depressed mood, loss of drive, and phobic anxiety are associated with a larger AMY volume, whereas emotionally unstable, psychotic symptoms like irritability, impulsiveness, and paranoid anxiety, manifest in individuals with a smaller AMY.

Most reports of a smaller AMY volume have been based on adults who experienced or suffered from a trauma in adulthood. The experience of trauma or chronic stress early in development might lead to a different outcome in adulthood. Healthy adults (males and females, mean age = 40) who experienced early life stress, did not have differences in AMY volume, but did have smaller ACC and caudate volumes (Cohen et al., 2006). No differences in AMY volume were found in adult females (mean age = 20) who were sexually abused as children, but smaller volumes were found in the hippocampus and corpus callosum (Andersen et al., 2008). Similar results, including no altered AMY volume and decreased hippocampal volume, were found in another study that looked at the effects of both childhood sexual and physical abuse on brain volume in adulthood (mean age = 41) (Bremner et al., 1997). This lack of altered AMY volume in response to early life traumatic events may be explained by the natural development of the AMY. By the age of four, the AMY reaches its full adult

size in human females (Andersen et al., 2008). Therefore, the sensitive period in development for the AMY may have occurred prior to the stressful events, thus having little impact on the structure of the AMY. The rat CNS at three weeks is equivalent to a human toddler (Dobbing, 1981; Marsh et al., 1997). Therefore, we could use rats to detect if stress earlier in life has an impact on AMY development.

In animals, much like in humans, studies exploring the effects of stress and pain on AMY volume have conflicting results, particularly between the different nuclei. Chronic immobilization stress in adult rats results in atrophy in the hippocampus, hypertrophy in the BLA, and increased anxiety on the elevated plus maze. Twenty-one days after the termination of the stressor, hypertrophy in the BLA and increased anxiety still occur, however, there is no further atrophy in the hippocampus (Vyas, Pillai, & Chattarji, 2004). Adult rats with chronic pain, in the form of spinal nerve injury (SNI), show increased volume and cell numbers in the CeA and BLA, but not in the LA compared to controls. This increase in volume was mediated at least in part by increased cell numbers, specifically neurons (BrdU+NeuN and BrdU+calbindin), but not glial cells (GFAP) or dendritic length (Gonçalves et al., 2008). However, a MRI study on SNI rats found decreased volume in pain-associated areas including the ACC and PFC, but failed to find any volumetric differences in the AMY compared to controls (Seminowicz et al., 2009). In adult male rats that were prenatally stressed, the LA volume was approximately 30% larger and contained more neurons and glial cells compared to non-prenatally stressed rats (Salm et al., 2004). Although research has demonstrated how stress and pain in adulthood affects the volume and number of

neurons in the AMY, as well as AMY-regulated behaviours, how early life pain impacts AMY development and function requires further investigation.

V. The Amygdala and Development

Since preterm infants are found to have social and anxiety problems later in life (Charkaluk, Truffert, Fily, Ancel, & Pierrat, 2010; Saigal et al., 2003), and knowing the relative sensitivity of the AMY to stress-related stimuli, we hypothesized that early pain exposure might lead to significant changes in AMY structure and function. Indeed, our pilot MRI data revealed that formalin-induced pain during the first two weeks of life induced significant hypertrophy in the BLA in neonates and near significant hypertrophy in post-weaning rats. Therefore, identifying how pain alters normal development of the AMY can help us understand the implications these changes have on behaviour. A study exploring the natural development of the BLA in the rat found that the volume of this structure increased from PND 20 to PND 35 and that the number of neurons and glial cells decreased from adolescence (PND 35) to adulthood (PND 90) (Rubinow & Juraska, 2009). This observation suggests that the BLA has a period of rapid growth early in development, which could potentially be sensitive to environmental stressors. In fact, one study found that the BLA and CeA of prenatally stressed rats had smaller volumes, fewer neurons, and fewer glial cells compared to controls on PND 25 (Kraszpulski, Dickerson, & Salm, 2006). However, in this same study, the LA, which also had a smaller volume on PND 25, had fewer neurons and glial cells on PND 7. Another study found that prenatal stress caused an increase in volume, number of neurons, and glial cells, in the LA, but not the BLA or CeA of adult male rats (Salm et

al., 2004). These results demonstrate that each nucleus in the AMY is differentially sensitive to early life stressors during development.

VI. Stress-induced Neuronal and Glial Proliferation: Use of Molecular Markers

In order to identify if repeated inflammatory pain during the neonatal period could induce differences in cellular development in the BLA, we selected three molecular markers (Ki67, NeuN and BrdU) for their ability to provide specific information about the timing of proliferative events. Ki67 is a protein implicated in cell proliferation as it regulates the progression of cell division. It is present in the nucleus during all active phases of the cell cycle and mitosis, but is not present in resting cells. The advantage of Ki67 is that it is an endogenous protein, therefore it does not need to be introduced into the animal's system. Identification of Ki67-positive cells is typically used to provide information about acute proliferation in tumor cells (Gimotty et al., 2005; Montebugnoli et al., 2009; Weigel & Dowsett, 2010), but since it is present in neurons, it can also be used to assess acute proliferative stages at the time of tissue collection. In contrast, neuronal nuclei (NeuN) is an antigen that is only present in the nuclei of mature neurons. Along the development of a neuron, it is first present when the neuron leaves the cell cycle and/or the cell differentiates into a neuron (Mullen, Buck, & Smith, 1992). Counting NeuN immunoreactive (-IR) cells is a well-accepted method for counting mature neurons in the brain (Clark et al., 2008; He, Overstreet, & Crews, 2009; Kempermann, Jessberger, Steiner & Kronenberg, 2004; Stratmann et al., 2010).

The other marker we chose to use, 5-bromo-2-deoxyuridine (BrdU), is a synthetic thymidine analog, which is incorporated into the DNA of a cell during the S-phase of the

cell cycle (Kee, Sivalingam, Boonstra, & Wojtowicz, 2002; Taupin, 2007; Wojtowicz & Kee, 2006). BrdU has frequently been used to determine the number of newly proliferated cells as well as the survival of these cells. BrdU, like Ki67, can provide information regarding cellular proliferation when tissue is collected within 24 hours of BrdU administration. The fate of newly proliferating cells can be obtained 2-4 weeks after BrdU is injected because cells that incorporate BrdU will retain it until cell death. In addition, BrdU can be double labeled with other molecular markers to determine cell type, i.e. NeuN for neurons or glial fibrillary acidic protein (GFAP) for astrocytes. A number of studies have investigated neurogenesis in adult animals with chronic pain. In particular, adult rats with SNI have more proliferating neurons that are double-labeled with BrdU+NeuN in the BLA and CeA than controls (Gonçalves et al., 2008). This result demonstrates that there is an increase in the number of neurons in the AMY following pain.

VII. Behavioural Tests Involving the Basolateral Amygdala

We attempted to determine if early life pain affects AMY morphology and whether this change results in altered functionality as reflected by modified AMY-regulated behaviours. In particular, we decided to select behavioural tests that involve activation of the BLA. Furthermore, we wanted to select tests that would measure similar behaviours that are altered in children that were premature infants in the NICU, such as social behaviour and anxiety. Thus, we selected social interaction and memory, elevated plus maze, and cue/context fear conditioning tests to determine if early life pain could alter anxiety and fear in adulthood.

i. Social interaction.

The social interaction test has been validated as an animal model of anxiety (File & Seth, 2003). Since anxiety and social interaction involve contradictory behaviours, it is assumed that animals displaying decreased social interaction are anxious (Lapiz-Bluhm et al., 2008). Both stress and stress hormones have been found to alter social interaction behaviour and brain morphology, specifically in the AMY. CRF is a neuropeptide that is released in response to stress. The role of the BLA has been emphasized in studies using CRF infusions, which increase anxiety resulting in decreased social interactions (Sajdyk, Schober, Gehlert, & Shekhar, 1999). Alternatively, administration of a CRF antagonist into the BLA decreased anxiety during social interaction testing following a stressor in rats (Gehlert et al., 2005). Acute stress was found to reduce whereas chronic stress was found to increase aggressive behaviour in rats (Wood, Young, Reagan, & McEwen, 2003). Since the BLA is strongly implicated in the regulation of social interaction, we decided to use this paradigm to determine if neonatal rats that experience pain would behave differently in a social interaction test in adulthood.

ii. Elevated plus maze.

The elevated plus maze is a valid and frequently used test for anxiety. The design of the elevated plus maze creates an ethological approach-avoidance conflict for the rat. Rats are naturally curious and want to explore their environment, as well they want to avoid dangerous situations, i.e. bright light, heights. The four arms of the maze provide a novel environment for the rat to explore. However, the two elevated open arms create an environment that the rat naturally fears, i.e. open and elevated. Anxious

rats are less inclined to explore the open arms of the maze. For example, adult male rats that received an acute dose of corticosterone, a stress hormone, had hypertrophy in the BLA and spent less time exploring the open arms of the elevated plus maze, but displayed no differences in fear conditioning 12 days after administration (Mitra & Sapolsky, 2008). This finding demonstrates that an acute dose of a stress hormone is sufficient to alter the morphology of the AMY and some AMY-regulated behaviours, such as anxiety, but not fear. Prenatal stress has been found to increase anxiety behaviour in male offspring in adulthood on the elevated plus maze, demonstrating that early life stress can permanently modify behaviour in this test (Brunton & Russell, 2010).

iii. Fear conditioning.

Fear conditioning consists of the classical conditioning paradigm where a conditioned stimulus, such as a light or tone, is paired with an unconditioned stimulus, such as a shock. If conditioning is successful, the animal will learn to fear the conditioned stimulus even in the absence of the unconditioned stimulus. Both context (Gresack et al., 2010; Hubbard et al, 2007) and cue (Mountney, Anisman, & Merali, 2008; Orman & Stewart, 2007; Walker & Davis, 2008) can be used as the conditioned stimulus in fear conditioning.

The AMY has been found to have a key role in the learning and subsequent behaviour displayed in response to fear. Traditionally, it was believed that the hippocampus was critical for contextual fear conditioning and that the BLA was important for cued fear conditioning. However, Maren, Aharonov, and Fanselow (1997) proposed the competition hypothesis, which states that under healthy conditions, the

hippocampus and AMY compete for processing of contextual information and under normal circumstances the hippocampus wins (Biedenkapp & Rudy, 2009). The AMY is able to compensate for the hippocampus if it is damaged prior to conditioning. Thus, the BLA receives cue information from appropriate sensory regions and context information via the hippocampus. However, if the hippocampus is damaged, the BLA receives context information directly. Cue (Huff & Rudy, 2004), context (Mountney et al., 2008), and aversive stimuli (i.e. shock) (Shi & Davis, 1999) all converge on the BLA, which assigns an emotion to the sensations prior to sending the information to the CeA. The CeA, the major output center of the AMY, then exerts its effects on the descending pain pathway via its efferent connections (Bernard et al., 1992; Neugebauer, Li, Bird, & Han, 2004), such as those to the PAG.

Some studies have explored the relationship between fear and AMY volume. PTSD is a failure to extinguish fear. One study found that the severity of PTSD in adults was negatively correlated with the size of the left AMY (Rogers et al., 2009). Another study that looked at the correlation between the AMY and fear found that the volume of the right AMY was positively correlated with fear, but only in females (van der Plas, Boes, Wemmie, Tranel, & Nopoulos, 2010). However, this study was conducted on healthy children and teenagers (age range 7-17) and the measure of fear was the parent's rating of their child's behaviour using the Pediatric Behavioural Scale. These results demonstrate that differences in AMY volume are associated with how different individuals respond to fear. Since we expected hypertrophy in the AMY of animals exposed to early life inflammatory pain, we hypothesized that these animals would

exhibit a greater fear response (i.e. more freezing) because of the AMY's role in regulating fear.

We measured fear by scoring the amount of time the animal spent freezing as this variable is a widely used and accepted indicator of fear in rats (Roche et al., 2010; Takeda, Tamano, Imano, & Oku, 2010). We used two different contexts, with different wall colours and floor textures, in order to distinguish the strength of the association animals made directly with the context and cue (tone) during training.

Objectives of the Study

Pain in development may have long-term consequences, although we do not know by which mechanisms early pain can affect later behaviour. Based on human and animal studies, both hypertrophy and atrophy have been found in different brain areas involved in processing pain. These morphological changes have been associated with behavioural dysfunctions that are specifically related to the affected brain region. Some of these deficits are seen into adulthood. In particular, we were interested in the BLA because of its central role in the interplay between the processing of nociception and emotion. Studies in adult rats have shown that pain can modify both the morphological structure and function of the BLA, an effect possibly mediated by neuropeptides released in response to stress, such as CRF (Fu & Neugebauer, 2008; Hubbard et al., 2007) and neurotrophic factors like BDNF (Ou, Yeh, & Gean, 2010). Little is known about the effects of pain experienced early in life on the developing brain and whether the structure and function of the BLA is permanently altered. This study was designed to answer the following questions:

i. What brain areas display volumetric changes following exposure to early life inflammatory pain? Are these changes persistent in adulthood?

Using MRI, we examined the brains of PND 15 and PND 35 rats to determine whether repeated formalin injections during the first two weeks of life would lead to either hypertrophy or atrophy of structures specifically involved in pain circuitry. Specifically, we targeted regions such as the somatosensory cortices (involved in the sensory processing of pain) (Price, 2002), thalamus (role in pain sensation and unpleasantness) (Price, 2002), ACC, which is the most consistent brain area found to be activated during pain imaging studies (involved in motivation and the integration of sensory, attention, memory and motor responses) (Price, Verne, & Schwartz, 2006), PAG (emotional behaviour) (Gauriau & Bernard, 2002; Kong, Tu, Zyloney, & Su, 2010), IC (implicated in identifying negative emotions in self and others) (Kurth, Zilles, Fox, Laird, & Eickhoff, 2010), hippocampus (use of context for appraising stimuli) (Ziv, Tomer, Defrin, & Hendler, 2010), PFC [cognition (Apkarian, Bushnell, Treede, & Zubieta, 2005), motor response planning (Baron, Baron, Disbrow, & Roberts, 1999), and modulating pain (Seifert et al., 2009)] and AMY (implicated in emotions, fear and anxiety) (Bernard & Besson, 1990; Phelps et al., 2001; Ziv et al., 2010) (Table 1).

ii. Does exposure to early life inflammatory pain induce proliferation and alter cell survival in the basolateral amygdala?

In this set of experiments, we focused on the BLA to examine whether early life inflammatory pain a) induced cellular proliferation and b) if new cells that were generated in response to pain integrated and survived in the brain. To measure the

amount of cellular proliferation, we immunostained for Ki67, which labels newly proliferated cells. In order to analyze the number of newly generated cells that survived three weeks after the pain treatment, we selected BrdU, which incorporates into the DNA of a cell during the cell cycle (Kee et al., 2002; Taupin, 2007; Wojtowicz & Kee, 2006) and remains present in cells that proliferated at the time of injection. Finally, we used NeuN, a marker for mature neurons, to quantify the number of neurons in the BLA.

iii. Are there long-term effects of early life inflammatory pain on behaviours that involve activation of the basolateral amygdala?

In this set of experiments, we tested adult male offspring using well-characterized behavioural tests that reflect regulation by the BLA. In particular, we used tests of social interaction, anxiety, and fear. The choice of these tests was based primarily on findings in human preterm infants showing that pain during neonatal life is associated mostly with social problems and increased anxiety.

Methods

General Methods

i. Animals.

Female Sprague-Dawley rats (Charles River, St. Constant, Quebec) arrived on approximately gestational day 16-18 and were housed individually. The day animals gave birth was designated as PND 0. On PND 1, all litters were culled to 10-12 pups per dam. Six males per litter were randomly assigned to either the formalin, saline, or uninjected (where applicable) group. In order to identify to which group each pup belonged, the front paws were injected with India ink (formalin – right paw, saline – left

paw). Animals were maintained in the Animal Facility at the Douglas Hospital Research Center with regulated conditions of light (lights on at 08:00 h and off at 20:00 h), temperature (18–25^{o}C) and humidity (25–40%) and had *ad libitum* access to food and water. All procedures were approved by the Animal Care Committee of McGill University and followed ethical guidelines from the Canadian Council on Animal Care.

ii. Pain paradigm.

We selected an acute repetitive inflammatory pain paradigm as we felt that this approach best mimicked the pain experienced by infants in the NICU. The inflammatory agent used was formalin since its effects are relatively mild compared to other inflammatory stimulants used to induce pain. The formalin concentration used was altered as a function of age to correspond to approximately 50% of the total pain score previously found at different ages (Teng & Abbott, 1998). Pups were removed from the nest between 8:30am-10:00am to administer injections from PND 3-14. The injected paw alternated between left and right each day. Pups were randomly assigned to either the formalin, saline or uninjected group. Animals in the formalin group received formalin injections (10µl) of 0.75% from PND 3-8 and 1.5% from PND 9-14. Saline pups received saline (10µl; 0.9% NaCl) injections. Uninjected pups were also removed from the nest, but did not receive an injection. All pups were placed back in the nest with the dam following injections. The pain paradigm used was consistent across all studies.

iii. Behavioral observations: Scoring of pain and inflammation.

During each injection day, body weights were recorded. In addition, we observed the pups every minute for 15 minutes after injections to monitor their motor movements and behavioural response to pain. The motor and pain scoring systems were based on

Teng and Abbott (1998) with a few additional behaviours based on a study previously conducted in our lab (de Medeiros, Fleming, Johnston & Walker, 2009). Motor behaviours were scored on a scale from 0-3: lying still (0), normal movement (1), pacing, repeated movements (2), and rolling around (3). Pain scoring was based on a scale from 0-4: normal (0), favor (1), lift (2), lick/shake/flinch (3), and paw in mouth (4). We also recorded paw inflammation to ensure that the inflammatory agent did not cause necrosis. The paw inflammation scoring system ranged from 0-2: normal (0), swollen/red (1), and inflamed (2).

Specific Methods

Prior to Study 1, we conducted a pilot study where animals were scanned at three different ages: PND 15, 35 and 70. This study was necessary to set the most appropriate parameters for volumetric determination at the young age of PND 15. Few studies have reported volumetric determinations with high sensitivity at this age. We, therefore, used this pilot study to test our scanning and analysis parameters and obtain preliminary evidence for the effect of early life pain on brain structures. Pups received injections from PND 3-14 of either formalin (10μl, 0.2% from PND 3-8, 0.4% from PND 9-14) or saline (10μl, NaCl 0.9%) twice daily (morning and evening). Animals were scanned for volumetric determination at three ages: PND 15, 35 and 70. Results of this pilot study provided evidence for significant hypertrophy in the basolateral amygdala (BLA) of formalin compared to saline injected pups on PND 15 and a near significant effect on PND 35. No significant differences were found in PND 70 adult offspring. The following study (Study 1) was performed in order to determine if the differences found in the BLA could be replicated and if any other brain areas would also be affected by early

life inflammatory pain. Based on our pilot results, we decided to scan animals at two ages, namely PND 15 and PND 35. In an attempt to increase the group differences caused by the pain experience, we decided to increase the formalin dose injected. Based on results of a dose dependent pilot study, we decided to administer 0.75% (PND 3-8) and 1.5% (PND 9 -14) formalin, but with a single injection in the morning instead of two daily injections.

Study 1: Volumetric Assessment in the Brain of Pups Exposed to Early Life Pain

i. Animals.

As discussed above, two sets of animals were scanned, the first set (Set A) was scanned only on PND 15/16 and the second set (Set B) of pups was scanned on PND 15/16 and PND 36/37. Three pregnant Sprague–Dawley mothers were used for each set of animals. All litters were culled to 10 pups per dam on PND 1 and of these, 6 male pups were randomly assigned to either the formalin or saline group (3 males/group). For each set, there were 9 formalin and 9 saline animals. On PND 9-10, dams and litters were transferred to the Genome animal facility (McGill University). On PND 15, both sets of animals received their first scan. Animals in Set A were then perfused on PND 17. After the first scan on PND 15, animals in Set B were left undisturbed except for cage cleaning and body weight recording. Set B animals were then weaned and pair-housed with one saline and one formalin animal from the same litter per cage on PND 22. All procedures were approved by the Animal Care Committee of McGill University and followed ethical guidelines from the Canadian Council on Animal Care.

ii. MRI scanning and deformation-based morphometry.

Animals were scanned for deformation-based morphometry (DBM) analysis on PND 15/16 (Set A and Set B) and PND 36/37 (Set B). Animals were anesthetized and maintained on isoflurane (1-2%) for the duration of the scan. During the scans, core temperature and heart rate were monitored and recorded continuously using SA Instruments MRI-compatible physiological monitoring system. Core temperature was maintained using a circulating warm air feedback system synchronized with the monitoring system. Animals were scanned with a 7 T Bruker Pharmascan MRI system with a 3D inversion recovery rapid acquisition with relaxation enhancement (RARE) sequence with the following parameters: TR = 3000 ms, TE = 9.7 ms, inversion time = 600 ms, field-of-view (FOV) = 3.2 cm, voxel resolution = 0.0125 cm x 0.0125 cm x 0.0250 cm, image matrix = 256 x 256 x 128 and a total scan time of 77 min. Immediately following the scan, animals were given 0.5 ml (PND 15/16) or 1 ml (PND 36/37) of 0.9% physiological saline subcutaneously to compensate for any possible fluid loss during the time that they were separated from the dam. Once animals recovered from anesthesia they were placed back in their home cage.

Automated DBM analysis was used to determine local volumetric differences using the procedure described in Lau et al (2008). DBM was performed using methods similar to those described by Lau *et al.* (2008) in order to examine potential neuroanatomical differences between groups. The scaled, log-transformed Jacobian determinant was computed at every voxel, for each scan, in order to examine the local volume change.

iii. Perfusions.

Perfusions were performed after scanning was completed on PND 17 (Set A) and PND 41/42 (Set B). Animals were deeply anesthetized with an intraperitoneal injection of a ketamine:xylazine cocktail (0.16 ml/100 g body weight). Animals were then perfused with 0.9% saline, 3 min for Set A and 5 min for Set B, followed by cold (4^{o}C) 4% paraformaldehyde (PFA) in 0.1M PBS, 20 min for Set A and 25 min for Set B. Brains were then removed and cut using a brain matrix (1 mm slots) in slices comprising 1) 10 mm to 6.2 mm (anterior commisure, anterior hippocampus, and caudate putamen), 2) 6.2 mm to 2.0 mm (AMY, medial preoptic area, hypothalamus, thalamus, and hippocampus), and 3) 2.0 mm to 0.9 mm (dentate gyrus, hippocampus, thalamus, and substantia nigra) for Set A (21 days; Sherwood & Timiras, 1970). Slices for Set B were comprised of 1) Bregma -12.84 mm to -4.56 mm (hippocampus, posterior thalamus, and motor cortex), 2) Bregma -4.56 mm to 0.00 mm (AMY, thalamus, hippocampus, hypothalamus, and primary somatosensory cortex), and 3) Bregma 0.00 mm to 5.64 mm (motor cortex, nucleus accumbens, cingulate cortex, and frontal cortex) (Paxinos & Watson, 2007). The three slices were placed in cassettes in 4% PFA for 24 hours. Tissue was then placed in 70% ethanol for 24 hours. The cassettes were then transferred to an automated tissue processor and underwent a series of 11 dehydration/ infiltration steps, each lasting 4 hr with the following solutions: 70% ethanol, 95% ethanol (x 2), 100% ethanol (x 3), xylene (x 3), and paraffin (x 2). Following the dehydration process, brains were embedded in paraffin in preparation for sectioning. Brains were sectioned at 5 μm on the rostro-caudal extent of the AMY (Set A: A 2.9 mm to A 4.4 mm; Set B: Bregma -4.08 mm to -1.56 mm). Six serial sections were collected for analysis followed by 10 discarded sections. Tissue sections were prepared for

quantification of NeuN-IR cells in the BLA.

iv. Immunohistochemistry – NeuN.

The brains from Study 1 – Set A and Set B were immunostained for NeuN. Slides were de-waxed in an oven overnight at 60^{o}C. Rehydration was then performed by placing the slides in each of the following solutions for 3 min each: xylene (x 2), xylene:ethanol (1:1), 100% ethanol (x 2), 95% ethanol, 70% ethanol, and 50% ethanol. Slides were then placed in cold water for 5 min then rinsed with 1X TBST until antigen retrieval. A pressure cooker was used to bring 10mM sodium citrate buffer to a boil for antigen retrieval. Antigen retrieval was performed by placing the slides in the boiling sodium citrate buffer for 20 min. Slides were then removed and placed in water followed by 1X TBST for approximately 1 hr. Another rinse in 1X TBST for 5 min was followed by treatment with 3% H_2O_2 (Sigma, St. Louis, MO) for 5 min. Slides were then rinsed again with 1X TBST prior to the block of normal goat serum (2%), 10% Triton X-100 in 1X TBS (0.2%), and 1X TBST for 5 min. Incubation with the primary antibody (mouse-anti-NeuN, 1:1000, Chemicon International, Temecula, CA) in Ultra Ab Diluent (Fisher Scientific, New Lawn, New Jersey) occurred for 1 hr. After the incubation period, slides were rinsed with 1X TBST then the secondary antibody was applied (M.O.M. biotinylated anti-mouse IgG reagent, 1:250, Vector Laboratories, Inc., Burlingame, CA) in Ultra Ab Diluent (Fisher Scientific, New Lawn, NJ) for 30 min. Another rinse with 1X TBST was performed prior to applying ABC solution (Vectastain Standard, Vector Laboratories, Inc., Burlingame, CA) for 35 min. Tissue was then rinsed with 1X TBST and incubated with AEC (Thermo Scientific, Lab Vision Corp, Fremont, CA) for 30 min. Another rinse in 1X TBST occurred followed by a 15 min rinse

in double distilled water. Tissue was then counterstained with Hematoxylin (SelecTech, Surgipath, Richmond, IL) then immediately rinsed under running water for 15 min. Slides were coverslipped with Aquatex.

v. Quantification and statistical analysis.

Experimenters were blind to treatment groups when counting. In order to identify the number of NeuN-IR cells in the BLA, three (Set A) and six (Set B) sections per animal were counted. Slides were scanned using a MIRAX scanner (Carl Zeiss, Göttingen, Germany) and NeuN-IR cells were counted manually at 40X magnification with the MIRAX viewer software. The BLA perimeter was determined according to the rat brain atlas by Sherwood and Timiras (21 days, 1970) for Set A and Paxinos and Watson (2007) for Set B. The regions used to count the BLA for Set A included ABL and ABM, whereas Set B included BLA, BLP, and BLV. The average number of NeuN-IR cells in the left and right BLA was calculated separately. Statistical analysis was conducted using a two-way ANOVA with laterality (left vs. right BLA) and treatment (formalin vs. saline) as factors to determine any differences in the number of NeuN-IR cells. The number of animals analyzed per treatment group were: Set A: formalin ($n = 5$) and saline ($n = 4$) and Set B: formalin ($n = 3$) and saline ($n = 4$). For any significant differences found ($p < .05$), Bonferroni post hoc tests were conducted.

Study 2: Neuronal Proliferation and Neuronal Survival in the Basolateral Amygdala After Early Life Pain

We required separate sets of animals in order to investigate both cell proliferation (Study 2A) and cell survival (Study 2B). In addition, we were interested in looking at the number of neurons present in both the neonatal and post-weaning periods. For Study

2A, we used Ki67 as a marker for cell proliferation and NeuN to detect the number of neurons. For survival (Study 2B), we selected BrdU to identify newly proliferated cells that survived and integrated, and NeuN to quantify the number of neurons. Pups from the same litters were divided and used in either Study 2A (Ki67) or Study 2B (BrdU). Animals from Study 1 were used for immunostaining NeuN, in particular, Set A was used to correspond with the neonatal period (proliferation) and Set B for the post-weaning period (survival). For details on NeuN, please refer to Study 1.

Study 2A: Proliferation – Ki67 (PND 15)

i. Animals.

Eight pregnant Sprague–Dawley female rats (Charles River, St. Constant, Quebec) were received on gestational day 16–18 and housed individually. Animals were maintained in the Animal Facility at the Douglas Hospital Research Center with the same controlled lighting, temperature, and humidity as stated in *General Methods 1. Animals*. The day of birth was designated as PND 0. On PND 1, litters were culled to 12 pups per dam (7 males and 5 females). Males from each litter were randomly assigned to one of three injection groups: formalin, saline or uninjected. From PND 3-14 animals received daily formalin or saline injections as specified in the *General Methods 2. Pain Paradigm*. All procedures were approved by the Animal Care Committee of McGill University and followed ethical guidelines from the Canadian Council on Animal Care.

ii. Perfusions.

Perfusions were performed on PND 15 as described in *Study 1 iii. Perfusions*.

Briefly, animals were anesthetized and perfused with saline (0.9% NaCl) followed by 4% PFA in 0.1M PBS. However, unlike in Study 1, brains remained whole. Following perfusion, intact whole brains were extracted and placed in 4% PFA for 4-5 hr, then transferred to 30% sucrose in 1M phosphate buffer for 24-48 hr. Tissue was frozen and stored in -80^{o}C until sectioning. Brains were sectioned at 50 μm and transferred to well plates filled with a cryoprotectant solution and maintained at -20^{o}C. Just prior to mounting, tissue was removed from the freezer and allowed to reach room temperature.

iii. Immunohistochemistry – Ki67.

Tissue sections were rinsed in PBS 3 x 5 min then mounted onto Superfrost Plus slides (Fisher Scientific, New Lawn, New Jersey). After drying for 1 hr, slides were placed in the oven (70^{o}C) for approximately 21 hr. Slides were then placed in distilled water (dH_2O) for 5 min. Antigen retrieval followed by placing slides for 15 min in a solution of 10mM sodium citrate buffer that was brought to a boil in a steamer. Slides were then removed from the steamer, but remained in the sodium citrate buffer for 30 min to reach room temperature. The tissue was then rinsed in dH_2O three times followed by a wash in 1X PBS. Sections were incubated with 3% H_2O_2/PBS (Sigma, St. Louis, MO) for 20 min at room temperature then rinsed 3 x 5 min with 1X PBS. A blocking solution consisting of 2% normal horse serum in 1X PBS with 0.2% Triton X-100 was applied for 30 min. The block was removed and the primary antibody, mouse anti-Ki67 (BD Pharmingen, San Diego, CA; 1:1000 in 2% normal horse serum in 1X PBS with 0.2% Triton X-100) was applied and left to incubate overnight at 4^{o}C in a humidified chamber. The following day, sections were rinsed 3 x 5 min with 1X PBS. The secondary antibody, biotinylated horse anti-mouse, rat adsorbed (Vector

Laboratories, Inc., Burlingame, CA; 1:200 in 2% normal horse serum in 1X PBS with 0.2% Triton X-100) was applied for 2 hr in a humidified chamber. Tissue was rinsed 3 x 5 min with 1X PBS prior to incubation with ABC solution (Vectastain Standard, Vector Laboratories, Inc., Burlingame, CA) for 30 min in a humidified chamber. Tissue was washed 3 x 5 min in 1X PBS. Tissue was developed for 10-30 min with SG (Vector Laboratories, Inc., Burlingame, CA) prepared in 1X PBS. To stop the reaction, slides were placed in dH_2O for 5 min then washed 4 x 5 min in PBS. Tissue was then processed through a series of dehydrating steps each lasting 15 s (dH2O, 70% ethanol, 95% ethanol, 2 x 100% ethanol, 2 x xylene) then cover slipped using Permount (Fisher Scientific, New Lawn, New Jersey).

iv. Quantification and statistical analysis – Ki67.

Experimenters counting Ki67-IR cells were blind to the treatment groups. In order to identify the number of Ki67-IR cells in the BLA, the left and right BLA were counted from 4-5 sections per animal in a rostro-caudal distribution then averaged. Tissue sections were examined at 10X magnification and counted with the aid of ImageJ. The BLA perimeter was defined according to Sherwood and Timiras (21 days, 1970) using the following coordinates, A 2.9 mm – A 4.4 mm and landmarks: optic tract (OPT), hypothalamus (HPV and HVM), corpus callosum (CCR), and central amygdala (ACE). Statistical analysis was calculated using five animals per treatment group. One-way analysis of variance (ANOVA) was conducted to compare the number of Ki67-IR cells between the formalin, saline and uninjected animals.

Study 2B: Neuronal Survival – BrdU (PND 35)

i. Animals.

The same eight pregnant Sprague–Dawley females from Study 2A were used. Briefly, animals were maintained in the Animal Facility at the Douglas Hospital Research Center with the same controlled lighting, temperature, and humidity as stated in General Methods 1. Animals. The males in each litter were randomly assigned to one of three injection groups: formalin, saline or uninjected. From PND 3-14 animals received daily formalin or saline injections as specified in the General Methods 2. Pain Paradigm. In addition, on PND 13 (20h00) and 14 (08h00), pups were injected with BrdU (final concentration = 150 mg/kg, i.p., Sigma, St. Louis, MO). On PND 22, animals were weaned and pair housed with an animal from a different treatment group. All procedures were approved by the Animal Care Committee of McGill University and followed ethical guidelines from the Canadian Council on Animal Care.

ii. Perfusions.

Perfusions were performed on PND 35 as described in Study 2A ii. Perfusions. Briefly, animals were anesthetized and perfused with saline (0.9% NaCl) followed by 4% PFA in 0.1M PBS. Following perfusion, brains were extracted and placed in 4% PFA for 4-5 hr, then transferred to 30% sucrose in 1M phosphate buffer for 24-48 hr. Tissue was frozen and stored in -80^{o}C until sectioning. Brains were sectioned at 50 μm and transferred to well plates filled with a cryoprotectant solution and maintained at -20^{o}C.

iii. Immunohistochemistry – BrdU.

Sections were removed from the freezer to allow the tissue to reach room temperature. All steps were performed at room temperature unless otherwise specified.

Tissue was rinsed in 1X TBS (0.1M, pH = 7.4, tris = 121.1 g/mol) 3 x 5 min. 0.02% H_2O_2 was then applied for 30 min followed by 3 x 5 min washes with 1X TBS. Denaturation in formamide (50% formamide/2X SCC) at 65^{o}C occurred for 2 hr. Sections were then rinsed for 5 min in saline sodium citrate (2XSSC) buffer. Tissue was further denatured via incubation in 2N HCl at 37^{o}C for 1hr. Neutralization followed using 0.1M borate buffer (pH = 8.5) for 10 min. Tissue was then rinsed with 1X TBS (4 x 5 min) followed by incubation in the blocking solution (3% normal goat serum in 0.3% Triton X-100 in 1X TBS with 0.03% NaN_3) for 1 hr. The primary antibody, anti-BrdU rat (Accurate, NY; 1:1000 in blocking solution) was applied for 60 hr at 4^{o}C. Tissue was rinsed with 1X TBS (3 x 5 min) prior to incubation with the secondary antibody, biotinylated goat-anti-rat (Vector Laboratories, Inc., Burlingame, CA; 1:200 in blocking solution) for 2 hr. Sections were rinsed with 1X TBS (3 x 5 min) then incubated with ABC solution (Vector Laboratories Inc., Burlingame, CA) for 30-60 min. Sections were washed again in 1X TBS (3 x 5 min). DAB was made according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA) then applied to the tissue. Tissue reacted with DAB for approximately 1-2 min then rinsed with 1X TBS (4 x 5 min) and stored in 1X TBS until mounting. Tissue sections were mounted on Superfrost Plus slides and dried using desiccators with a vacuum for 5 min then left overnight at 4^{o}C to finish drying. The following day, sections were hydrated in descending concentrations of ethanol: 100% ethanol (5 min), 90% ethanol (2 min), 70% ethanol (<1 min), dH_2O (<1 min) prior to staining with cresyl violet (Sigma, St. Louis, MO; 0.5% in dH_2O) for 2 min. Sections were then dehydrated in a series of ascending concentrations of ethanol: dH2O (30 s), 70% ethanol + 2 drops of acetic acid (glacial) (1 min), 90% ethanol (1

min), 100% ethanol (2 x 1 min), and xylene (10 min) and coverslipped with Permount (Fisher Scientific, New Lawn, New Jersey).

iv. Quantification and statistical analysis - BrdU.

Experimenters were blind to treatment groups when counting. In order to identify the number of BrdU-IR cells in the BLA, which included the BLA, BLP and BLV (Bregma: -4.08 mm to -1.56 mm; Paxinos & Watson, 2007), the right and left BLA of six sections per animal were counted. Tissue sections were examined at 40X magnification. The average number of BrdU-IR cells in both the left and right BLA were calculated per brain. Statistical analysis was calculated using six animals per treatment group. One-way analysis of variance (ANOVA) was conducted to compare the number of BrdU-IR cells between the formalin, saline and uninjected animals.

Study 3: Long-term Effects of Early Life Pain on Amygdala Related Behaviours in Adult Offspring

Based on our pilot data showing hypertrophy in the BLA, we selected behavioural tests known to be regulated by the BLA, specifically social interaction, elevated plus maze and fear conditioning. These tests are used to assess behaviours associated with anxiety (social interaction and elevated plus maze) and fear (fear conditioning). Decreased social interaction has been established as an indicator of anxiety, (File & Hyde, 1978; File & Seth, 2003; Khoshbouei, Cecchi, & Morilak, 2002). The elevated plus maze is another validated and frequently used test for anxiety (Lapiz-Bluhm et al., 2008). Based on an ethologic approach-avoidance conflict, it uses the rat's natural curiosity to explore a novel environment, while at the same time possessing features it

fears (i.e. open spaces). Anxious animals spend less time exploring the open arms of the maze. We were interested in determining if pain experienced early in life would make animals more anxious. Fear conditioning uses classical conditioning to pair a conditioned stimulus (i.e. tone, light) with an unconditioned stimulus (i.e. shock) to assess the strength of the association made with the context and the cue. Animals that learn to associate the context or cue with the unconditioned stimulus will freeze when presented with the context in which the association was learned and/or the cue.

All animals were tested in each paradigm in the same order as depicted in Figure 1. Briefly, animals were tested for social interaction on approximately PND 60, then tested for social memory approximately 24 hr later. Following a 3-4 day rest period, animals were tested on the elevated plus maze. After another 3-4 day rest period, animals were habituated to the fear conditioning chambers. The next day animals underwent training where they received five tone + shock pairings. Twenty-four hours later, animals were exposed to a different context than that in which training occurred with ten tone alone presentations. The next day, animals were placed in the same context as in training, but with no presentation of tone or shock.

iii. Animals.

The same eight pregnant Sprague–Dawley dams from Studies 2A and 2B were used. An additional four dams (gestational day 16-18) were ordered to complete the number of pups required per treatment for adequate statistical analysis. Animals were treated as previously described in *Study 2A i. Animals*. When pups were weaned on PND 22, they were pair housed with a conspecific from a different treatment group.

Animals remained undisturbed until testing in adulthood on approximately PND 60.

In addition, a separate set of adult males (350g) was ordered (Charles River, St. Constant, Quebec) to serve as stimulus rats for social interaction and memory testing. Stimulus rats were pair housed and maintained using the same lighting, temperature and humidity conditions as experimental animals.

iv. Social interaction & social recognition.

On approximately PND 60, all experimental animals were separated and housed individually for one week in preparation for social interaction testing. Research shows that animals are more social when housed individually for one week prior to testing (File & Seth, 2003; Niesink & Van Ree, 1982). During each of the three days prior to the social interaction test, both experimental and stimulus rats were handled (5 min) and habituated (5 min) to the test arena. The testing arena (99.1 cm x 99.1 cm x 45.7 cm) was made out of wood with the floor painted grey and the walls black. Habituation and testing were conducted under standard lighting.

Social interaction testing occurred between 09h00-11h45. Experimental animals were habituated to the testing arena individually. Stimulus rats were habituated to the testing chamber with a different stimulus rat each day to ensure that they acclimated to the novel environment and were not anxious around novel animals. On the first test day, social interaction testing occurred over a 5 min period. The stimulus rat was placed in a corner of the arena followed by the experimental rat, which was placed in the opposite corner of the stimulus rat. The testing session began after the experimenter left the room. Animal interactions were recorded with a camera mounted

above the arena allowing for undisturbed behavioural observations. To distinguish the stimulus rat from the experimental rat, stimulus rats were marked on the back with a Sharpie marker. At the end of the test, animals were placed back in their respective home cages. The arena was cleaned with Peroxyguard and rinsed with water between each test. Behaviours were coded at a later time. The behaviours of interest were divided into 2 categories: social and anxiety. Social behaviours included: i) sniff (the experimental animal had its snout close to the stimulus rat and sniffed any part of the stimulus rat's body), ii) approach (the experimental animal moved towards or followed the stimulus rat), iii) communal grooming (the experimental rat engaged in grooming/licking the stimulus rat), and iv) wrestling (the experimental rat went on top or underneath the stimulus rat). Anxiety behaviours included: i) freezing (total absence movement except for that associated with breathing), ii) self-grooming (the experimental rat groomed itself), and iii) aggression (the experimental rat attacked [upright and boxing] or bit the stimulus rat). The amount of time spent performing each of these behaviours was recorded.

Approximately 24 hr after the social interaction test, social recognition testing was performed. Social recognition testing was performed in two sessions, one in the morning and the other in the afternoon. Each session was separated by 4 hr. Social recognition was examined by presenting the experimental animal with a familiar stimulus rat (the same stimulus rat that was presented during the social interaction test) and a novel stimulus rat (a stimulus rat that the experimental rat had never encountered) during 5 min sessions for each encounter. The presentation order of the familiar and novel rat was counterbalanced across morning and evening sessions

amongst the treatment groups. Testing routine and behaviours of interest were identical to those performed during the social interaction test as stated above. The day following social recognition testing, experimental animals were re-paired with the same conspecific they were paired with prior to individual housing. Animals remained pair housed for the remainder of the study.

a. *Statistical analysis.*

Two-way ANOVAs were conducted on the average duration (seconds) of all individual social and anxiety behaviours performed on each test day (social interaction compared to social recognition of a familiar rat and social recognition of a novel rat) and neonatal treatment (formalin [n=15], saline [n=14], and uninjected [n=13]) as factors. Subsequently, the combined amount of time each animal spent performing either social behaviours or anxiety behaviours was calculated for each test day to determine if there was an overall difference in the amount of time spent being social or anxious. For total time spent exhibiting either social or anxiety behaviours, two-way ANOVAs with test day (social interaction, social recognition of a familiar rat, and social recognition of a novel rat) and neonatal treatment (formalin [n=15], saline [n=14], and uninjected [n=13]) as factors were conducted. Bonferroni post hoc tests were performed on statistically significant ($p < .05$) behaviours.

v. Elevated plus maze (EPM).

Three to four days after social recognition testing, animals were tested using the EPM. EPM was selected to test for anxiety-like behaviour based on the approach-avoidance conflict. The maze was made of Plexiglas with two white open arms (50.8

cm x 10.2 cm) and two closed arms (50.8 cm x 10.2 cm x 33 cm) with white floors and black walls in the shape of a cross. The center of the maze where all arms intersected was 10.2 cm x 10.2 cm. The entire maze was elevated 53.3 cm above the floor.

Animals were brought to the testing room to acclimate for 15-20 min. Each animal was removed from its home cage and placed in the center of the EPM. The experimenter left the room and the animal's behaviour was recorded for 5 min with a camera mounted above the maze allowing for undisturbed behavioural observations. At the end of the test, the animal was returned to its home cage. The maze was cleaned with Peroxyguard and rinsed with water between each test. Behaviours were coded at a later time by an experimenter who was blind to the experimental treatment of each animal.

The behaviours of interest included: i) time spent on open arms, ii) time spent on closed arms, iii) number of open arm entries (defined as all four paws placed in the open arm), iv) number of closed arm entries (defined as all four paws placed in the closed arm), and v) stretch/attend (the rat extended the front half of its body into another division of the maze while its rear paws remained in the previously occupied section).

a. *Statistical Analysis.*

One-way ANOVAs were conducted on all behavioural parameters to analyze differences between the different neonatal pain treatments (formalin [n=15], saline [n=14], and uninjected [n=13]). Time spent in each set of arms is represented as time (seconds +SEM) and number of entries is represented as the mean (+SEM).

vi. Fear conditioning.

Three to four days after EPM testing, animals underwent fear conditioning. Fear conditioning occurred across four consecutive days (habituation, training, cue-test and context-test). Three testing chambers were used simultaneously allowing three animals to be tested at the same time. Two different chamber contexts were used. In Context A, the chamber was made of Plexiglas with three black walls and one clear panel at the front to permit viewing and video recording (43.8 cm x 22.2 cm x 19.7 cm). The floor of the chamber was stainless steel rods (1.9 cm apart) connected to a shock scrambler, which was set to 0.5 mA. In Context B, the chamber was made of Plexiglas with three white walls and one clear panel at the front to allow viewing and video recording (42.5 cm x 20.3 cm x 20.3 cm). The floor was a smooth black board also made of Plexiglas. Training, cue conditioning and context conditioning sessions were recorded with video cameras on tripods located directly across each chamber.

On day 1, experimental animals were habituated to both chamber contexts to ensure that the animals' responses were not influenced by the novelty of the chamber and procedure. Animals were placed in each chamber for 20 min per context during which time neither tone nor shock were presented. The chambers were cleaned with Peroxyguard between each animal. The order of habituation was counterbalanced amongst animals in each treatment group.

Approximately 24 hr after habituation, animals underwent training (day 2). Training occurred in Context A. Animals being tested were brought into the testing room and each rat was placed in a testing chamber. The testing program (Motor Monitor, Kinder Scientific) was initiated simultaneously for all chambers once all animals were placed inside. Animals acclimated to the chamber for 2 min prior to the first of five

tone+shock pairings. The tone (80 db, 4 kHz) was presented for 10 s. The shock (0.5 mA) was administered for 1 s and co-terminated with the tone. The inter-trial interval between tone+shock pairings was 2 min. After the fifth and final tone+shock pairing, animals remained in the chamber for 5 min. Total duration of training was 13 min. Chambers were cleaned with Peroxyguard between each rat.

The following day, animals were tested for cue conditioning (day 3). Cue conditioning took place in Context B. Animals were brought into the testing room and placed in their own chambers. Once all animals were in the chambers, the testing program (Motor Monitor, Kinder Scientific) was initiated. Tone presentation and behavioural recording was simultaneous for all chambers. The testing session began with a 5 min acclimation period during which no tone or shock was presented. After acclimation, animals were presented with 10 tone alone (80 dB, 4 kHz) trials, each lasting 10 s, similar to the training session. An inter-trial interval was set to 2 min. Once all 10 tone alone sessions were presented, animals remained in the chamber for 5 min. The cue condition session was 28 min total. Chambers were cleaned with Peroxyguard between rats.

Twenty-four hours after cue-conditioning, animals were tested for context conditioning (day 4). Context conditioning took place in Context A. Animals were placed in the testing chamber and left undisturbed (no tone or shock) for 28 min. Chambers were cleaned with Peroxyguard between rats.

Freezing behaviour during training, cue conditioning and context conditioning was manually scored every 5 s from videos. Experimenters scoring behaviour were blind to

the experimental treatment of the animals. During cue conditioning and context conditioning, the entire 28 min test was scored for a total possible freezing score of 336 (28 min x 60 s/5 s intervals = 336) per test. Percent of time spent freezing was calculated by dividing the total freezing score for each animal per test by the total possible test score (336) then multiplying by 100. One-way ANOVAs were performed with neonatal pain treatment as a factor (formalin: n=15 [cue test] and n=14 [context test], saline: n=14, uninjected: n=13).

Results

Study 1: Volumetric Assessment in the Brain of Pups Exposed to Early Life Pain

i. MRI scanning and deformation-based morphometry.

Statistical analysis was performed using a two-tailed t-test. Results from our pilot data found hypertrophy in the BLA (10 days; Sherwood & Timiras, 1970) of formalin animals compared to saline injected animals on PND 15 (green: $p = .009$) (Figure 2A). On PND 35, near significant differences were found in the BLA (Paxinos & Watson, 2007; pink: $p = .061$) (Figure 2B). However, analysis of Study 1 failed to replicate significant differences in the BLA or any other region.

ii. NeuN.

NeuN was used to assess the number of mature neurons present in the BLA of animals that received mild inflammatory pain during the neonatal period at both neonatal and post-weaning ages. The brains of animals from Study 1, Set A (PND 17) (formalin: n = 5; saline: n = 4) were processed immediately after the pain procedure.

For Set A, the BLA, consisting of the ABL and ABM (Sherwood & Timiras, 1970) from three sections per animal was counted and analyzed. Separate NeuN counts were made for the right and left BLA. A two-way ANOVA with laterality (right vs left) and treatment (formalin vs saline) as factors was conducted. Analysis revealed no significant effect of laterality [$F(1, 7) = 1.662$, $p = .2383$], but a near significant treatment effect was found [$F(1, 7) = 4.905$, $p = .0624$] (Figure 3A) such that animals injected with formalin had more NeuN-IR cells compared to saline animals. There was no interaction [$F(1, 7) = 2.817$, $p = .1372$]. When neuronal cell counts were performed on Set B brains (PND 41/42) (formalin: $n = 3$; saline: $n = 4$) three weeks after the pain procedure, this difference disappeared. For Set B, the BLA, consisting of the BLA, BLV, and BLP (Paxinos & Watson, 2007), of six sections per animal was analyzed. A two-way ANOVA with laterality (right vs left) and treatment (formalin vs saline) as factors was conducted. Analysis revealed no significant effect of laterality [$F(1, 5) = 0.5748$, $p = .4825$] (Figure 4A) or treatment [$F(1, 5) = 1.600$, $p = .2617$]. As well, there was no interaction between laterality and treatment [$F(1, 5) = 0.6465$, $p = .4579$].

Overall, these data suggest that early exposure to pain produces transient hypertrophy in the BLA that is evident immediately at the end of the pain treatment. This BLA hypertrophy is not observed after weaning. These data are consistent with our pilot imaging data suggesting that repeated mild inflammatory pain in neonates results in BLA hypertrophy in young animals, but these effects disappear towards weaning age.

Study 2: Neuronal Proliferation and Neuronal Survival in the Basolateral Amygdala After Early Life Pain

We first determined if cellular proliferation occurred at the termination of the pain procedure in PND 15 neonates using Ki67 expression as an index of acute proliferative events. Next, we examined whether new cells labeled with BrdU at the end of the pain procedure (i.e. PND 13-14) would be maintained three weeks later in post-weaning animals (PND 35).

Study 2A: Proliferation – Ki67 (PND 15)

Brains (PND 15) were processed for Ki67 to determine differences in the number of proliferating cells in the BLA immediately following the pain procedure. The number of cells from the left and right BLA were combined and averaged per brain. A one-way ANOVA revealed no significant differences in the number of Ki67-IR cells in the BLA between the treatment groups (n = 5 animals/treatment; 4-5 sections/animal) [$F(2,14) = 0.6269$, $p = .5509$; Figure 5A].

Study 2B: Neuronal Survival – BrdU (PND 35)

BrdU was injected at the end of the neonatal pain procedure allowing for incorporation into newly proliferating cells at that time. Three weeks after the BrdU injections, animals were sacrificed to determine if any newly developed cells that incorporated BrdU survived and integrated into the BLA. BrdU-IR cells were counted in both the right and left BLA and averaged. A one-way ANOVA was performed on the number of BrdU-IR cells in the BLA (formalin: n = 6, saline: n = 6, uninjected: n = 6). No significant effect of treatment was found in the number of BrdU-IR cells [$F(2,17) = 0.0432$, $p = .9579$] (Figure 6A).

Overall these data demonstrate that early life pain does not alter the number of

newly proliferating cells. As well, there is no difference in the number of newly proliferating cells at the end of the pain procedure that survive based on early life pain experiences. However, if we double label these markers of cell proliferation (Ki67) and cell survival (BrdU) with markers for cell phenotype (i.e. NeuN for neurons or GFAP for glial cells), perhaps we could complement the NeuN data from the neonatal animals.

Study 3: Long-term Effects of Early Life Pain on Amygdala Related Behaviours in Adult Offspring

This set of studies was designed to determine whether exposure to early life inflammatory pain could have a significant impact on behavioural responses involving the AMY. We focused our attention on tests of social interaction, anxiety, and fear conditioning since behavioural responses to these tests are known to be dependent upon a functional AMY (for reviews refer to LeDoux, 2007; Schumann, Bauman, & Amaral, 2010).

i. Social interaction & social recognition.

As indicated in the Methods section, social interaction test behaviours were divided into two categories: social and anxiety behaviours. Two-way ANOVAs (test day x treatment [formalin: n = 15, saline: n = 14, uninjected: n = 13]) were conducted on all individual social and anxiety behaviours, but no significant effects or interactions were found. Time spent performing individual social (Figure 7A) and anxiety (Figure 7B) behaviours displayed during the social interaction test were compared to those displayed during social recognition tests when animals were presented with a novel and a familiar rat. All social behaviours were combined, as were all anxiety behaviours, for

analysis of overall time spent being social and anxious. A two-way ANOVA (test day x treatment [formalin: n = 15, saline: n = 14, uninjected: n = 13]) was conducted on the total time animals spent exhibiting all social behaviours. No significant treatment effect on total social behaviour was found [$F(2,78) = 1.531$, $p = .2291$], but a significant effect of test day was revealed [$F(2,78) = 5.042$, $p = .0087$] (Figure 7A). Bonferroni post-hoc tests revealed that animals in the uninjected group performed more social behaviours during the social interaction test compared to when they were presented with a familiar rat during the social recognition test. However, no interactions or other significant test day effects were found for total social behaviours. Total anxiety behaviours were also analyzed using a two-way ANOVA with the same test day x treatment factors. No significant effect of test day [$F(2,78) = 1.043$, $p = .3573$] or treatment [$F(2,78) = 0.9499$, $p = .3956$] was found for total anxiety behaviours.

These results suggest that early life inflammatory pain does not cause differences in adult social or anxiety behaviours in social interaction and social recognition tests. Animals did not interact differently with novel or familiar rats, suggesting no differences in anxiety levels.

ii. Elevated plus maze.

A one-way ANOVA was performed to determine if early life inflammatory pain altered the amount of time animals spent in the open and closed arms of the EPM. Results show no significant effect of neonatal inflammatory pain treatment on the amount of time spent in the open arms [$F(2,41) = 0.2108$, $p = .8109$] (Figure 8A) and closed arms [$F(2,41) = 0.7408$, $p = .4833$] (Figure 8B). Analysis of the number of

entries into open arms [F(2,41) = 0.9423, p = .3984] (Figure 8C) and closed arms [F(2,41) = 1.293, p = .2860] (Figure 8D) also failed to find any significant treatment effects. Exploratory behaviour analysis, specifically stretch/attend, revealed no significant differences between treatment groups [F(2,41) = 0.3212, p = .7272].

iii. Fear conditioning.

The total freezing score during each test day was converted into percent freezing score for each 28 min test (cue conditioning and context conditioning). One-way ANOVAs were performed to examine if any significant treatment effects were found on percent freezing during cue conditioning and context conditioning tests in adulthood. Analysis of percent freezing score during cue conditioning [F(2,41) = 0.1786, p = .8371] (Figure 9A) and context conditioning [F(2,40) = 1.802, p = .1788] (Figure 9B) failed to produce significant differences between treatment groups.

Discussion

In this set of studies, we used an animal model of early life inflammatory pain to mimic the pain experience of preterm infants in the NICU. Early life inflammatory pain did not alter physiological or behavioural measures, specifically those related to the BLA. MRI analysis failed to reveal volumetric differences between animals that received pain during the neonatal period from those that did not in brain areas associated with pain circuitry. Early life inflammatory pain did not alter cell proliferation (Ki67) or cell survival (BrdU) in the BLA. Animals injected with formalin had more mature neurons (NeuN) in the BLA compared to saline injected animals on PND 17. However, when animals were sacrificed three weeks after the pain procedure on PND

42, the difference in neuronal number between the two treatment groups disappeared. To determine if early life inflammatory pain would alter behaviour in adulthood, we selected behavioural tests known to be regulated by the BLA. In particular, we chose the social interaction test and elevated plus maze to assess behaviours associated with anxiety and fear conditioning to assess fear. No significant differences were found between the different treatment groups on any of the behavioural tests. Our findings suggest that inflammatory pain produces transitory effects that disappear shortly after the pain subsides. Our interest in investigating the long-term effects of early life pain is based on the human literature documenting mostly negative cognitive, emotional, and behavioural effects in children who were born premature and subjected to painful procedures, such as heel lances and intravenous line insertions, during their stay in the NICU (Grunau et al., 2009; Huddy et al., 2001). In fact, the frequency of painful procedures received while in the NICU is associated with behavioural immaturity of pain responses (Johnston & Stevens, 1996). Preterm infants at 8 and 18 months have poorer cognitive and motor abilities, which has been associated with the number of skin breaking procedures (Grunau et al., 2009).

Studies investigating the negative effects of pain on preterm infants are limited because of ethical considerations involved in investigating this extremely sensitive population. Only observations can be made, making it difficult to control factors that could be important for understanding the effects of pain, such as the actual age of the infant, as well as the timing, magnitude and frequency of the pain. Without the ability to control these elements, it is difficult to establish the underlying mechanism causing these effects.

In order to have greater control over the numerous variables implicated in studying pain in preterm infants, several animal models of early life pain have been designed (for a review refer to Johnston et al., 2002). Some of these models include inflammatory agents such as carrageenan, complete Freund's adjuvant (CFA) and formalin. Animal studies that used inflammatory pain during the neonatal period have found long lasting effects in adult offspring such as hypersensitivity to formalin (Walker, Xu, Rochford, & Johnston, 2008), decreased pain thresholds (hot-plate test) (Anand, Coskun, Thrivikraman, Nemeroff, & Plotsky, 1999), and greater cell death in several brain regions (Anand et al., 2007). Behaviourally, most studies have looked at the impact of early life inflammatory pain on later pain sensation, whereas few have investigated its effects on social tasks, anxiety and fear (Anand et al., 1999; Anseloni et al., 2005).

In many of these studies, it is obvious that the long-term effects observed in adulthood vary depending on the experimental model of pain and methodology used. For instance, a single injection of carrageenan (2%) administered to rat pups within 24 hours of birth results in inflammation lasting two weeks, but has no effect on inflammatory responses or mechanical and heat thresholds in adulthood (Alvares et al., 2000). Male rats that were injected with CFA during the neonatal period (PND 0, 1, 3, and 14) exhibited lower pain thresholds in response to a noxious thermal stimulus in adulthood 24 hours after being injected with CFA (Ruda, Ling, Hohmann, Peng, & Tachibana, 2000). In particular, results reported on animals that received early life formalin injections have varied depending on the dose of formalin, age of administration and testing paradigm. When a very high dose of formalin (10%) was given from PND 1-

7 in rat pups, these rats had higher thermal pain thresholds on the hot plate test in adulthood (Bhutta et al., 2001). However, when a moderate dose of formalin (0.2%-0.4%) was administered from PND 3-14, adult animals were more sensitive to inflammatory pain caused by formalin, but not thermal pain measured using the Hargreaves test (Walker et al., 2008). The benefit of using formalin is that it has a relatively short recovery time, thus allowing for repeated administration over a prolonged period of time. Our lab previously reported that daily modest formalin injections (0.2%-0.4%) caused inflammation of the injected paw (de Medeiros et al., 2009). The concentration of formalin can also be altered to complement the age of the animal, reflecting changes in behavioural responses across development (Teng & Abbott, 1998). For these reasons, we chose to use repeated daily formalin injections from PND 3-14 to cause mild inflammatory pain in rat pups. We selected doses shown to cause 50% of total pain scores previously found at different ages (Teng & Abbott, 1998). Using this paradigm of early life inflammatory pain exposure, the goal of our studies was to determine whether early life pain would alter i) brain volume of sites involved in the CNS pain circuitry during the neonatal and post-weaning periods, ii) cell proliferation and survival in the BLA, and iii) behaviours regulated by the BLA, specifically anxiety and fear, in adulthood.

Our first objective was to determine if pain experienced during the neonatal period would alter brain volume in areas important for pain processing. Originally, we targeted several brain regions primarily involved in the neurocircuitry subserving pain responses (Table 1). In particular, we focused on the somatosensory cortices, thalamus, ACC, PAG, IC, hippocampus, PFC and AMY. The AMY is of particular

interest because it is implicated in the processing of emotions, especially fear and anxiety, as well as in associations between stress, emotions and memory formation via connections with the hippocampus. In our first set of experiments using MRI scans, we consistently found bilateral hypertrophy in the BLA of PND 15 rat pups that received formalin during the neonatal period. When the same animals were scanned on PND 35, we found that hypertrophy was nearly significant in the right BLA. This laterality in the right AMY in response to pain is not unprecedented. Using an arthritic model of pain in rats, Ji and Neugebauer (2009) found that neurons in the right, but not left, CeA develop increased responsiveness to mechanical stimulation. In contrast, some studies on human chronic pain in adults have reported atrophy, rather than hypertrophy in the AMY (Rodriguez-Raecke, Niemeier, Ihle, Ruether, & May, 2009; Valfrè, Rainero, Bergui, & Pinessi, 2008). Other studies have found changes in the brain volume of adult rats following pain experienced in adulthood (Gonçalves et al., 2008; Seminowicz et al., 2009). A fairly consistent finding both in humans (Obermann et al., 2009; Rodriguez-Raecke et al., 2009) and rodents (Seminowicz et al., 2009) is that of significant atrophy in the ACC and dorsolateral prefrontal cortex (DLPFC).

Interestingly, chronic pain in humans has resulted in reduced grey matter in some regions that is, at least partially, reversible once the pain has subsided. Two regions, which are relatively consistent in their reversible decreased grey matter in response to pain, are the ACC and DLPFC. For example, patients with chronic headaches associated with whiplash had decreased grey matter in the ACC and DLPFC three months after the incident, but by one year, the decrease had reversed (Obermann et al., 2009). Another study investigating chronic pain in patients with hip

osteoarthritis found that the decreased grey matter in the ACC and DLPFC, increased following surgery and the alleviation of pain (Rodriguez-Raecke et al., 2009). These studies suggest that certain brain regions remain plastic into adulthood.

In contrast to these regions that decrease in response to pain and are reversible, other regions in the human brain show volume increases. Migraine patients have a higher density in the PAG compared to controls (Rocca et al., 2006). Increased gray matter has also been found in the thalamus of patients with headaches induced by whiplash injury (Obermann et al., 2009) and chronic back pain (Schmidt-Wilcke et al., 2006). More recently, a study looking at gray matter volumes in patients with myofascial temporomandibular pain found that increased gray matter in the ACC is associated with self-reports of pain severity, whereas longer pain durations are related to increased grey matter in the posterior cingulate, hippocampus, midbrain and cerebellum (Younger, Shen, Goddard, & Mackey, 2010). However, few studies have investigated the potential reversal of these volumetric increases following the cessation of pain. One study did, however, show that gray matter in the brainstem, thalamus, and cerebellum remained enlarged even after the pain abated (Obermann et al., 2009). A distinction can be made between regions that returned to normal volumes compared to those that remained enlarged after cessation of pain in humans. Intriguingly, some of the regions that remained enlarged, such as the PAG, are involved in descending pain modulatory pathways. It has been proposed that the persistent enlargement of these brain regions is important for the better attenuation of pain (Obermann et al., 2009).

Stress has also been associated with volumetric changes in the AMY with most of the reported changes involving hypertrophy. For example, chronic immobilization

stress resulted in hypertrophy of the AMY and increased anxiety persisting even 21 days after cessation of the stressor (Vyas et al., 2004). This hypertrophy has been found in the three main subgroups of the AMY, mainly the LA, BLA and CeA. Volume in the LA increased by 30% and contained more neurons and glial cells in adult rats having received stress prenatally (Salm et al., 2004). Acute and chronic administration of corticosterone, a glucocorticoid released during stress, leads to dendritic hypertrophy in the BLA and increased anxiety in adult male rats (Mitra & Sapolsky, 2008). In the CeA, more neurons containing corticotropin-releasing hormone were found following chronic unpredictable mild stress (Wang, Yan, Hofman, Swaab, & Zhou, 2010). Taken together, these results suggest that plasticity of the AMY is highly sensitive to stress, thereby resulting in hypertrophy.

We demonstrated that early exposure to pain induced by formalin injections results in a moderate increase in the number of mature neurons (NeuN-IR) in the BLA on PND 15, 24 hours after the last formalin injection, which is consistent with our MRI results at the same age. In order to detect whether this trend towards hypertrophy in the BLA was associated with acute cellular proliferation, we stained for Ki-67, an endogenous marker associated with proliferating cells. Surprisingly, we did not find that early life inflammatory pain altered proliferation in the BLA on PND 15. Thus, the increase in neuronal number might be due to proliferative events that occur prior to PND 15 or an inhibition of normal cell death during the first two weeks of life, i.e. during the period of pain exposure.

Alternatively, we could use BrdU instead of Ki67 to investigate cell proliferation. Since BrdU is incorporated into newly proliferating cells, sacrificing animals 24 hours

after injecting BrdU would provide us with another way of measuring the number of cells being produced immediately at the time of the pain experience. The rat brain goes through a period of rapid growth, including synaptogenesis, myelination and proliferation, around PND 10 (Bouza, 2009). Perhaps analyzing brains at a younger age, such as PND 7 or 10, when the brain is undergoing many changes, would result in greater group differences and might allow us to distinguish between the different mechanisms causing these effects. If the difference in neuronal number is a result of altered cell death, we could use Fluoro-Jade, an anionic fluorescein derivative that detects neuronal degeneration, to determine if pain increases or decreases cell death (Schmued & Hopkins, 2000a,b). Fluoro-Jade has been successfully used to detect increased cell death in the brains of neonatal rat pups that were injected with 4% formalin in the hind paw (Anand et al., 2007; Rovnaghi et al., 2008).

We also investigated whether there would be a long lasting, pain-induced difference in the number of newly developed cells that survive and integrate in the brain, specifically in the BLA. BrdU was injected during the last two days of the pain procedure (PND 13 and 14) and animals were sacrificed on PND 35, i.e. three weeks after termination of the pain procedure. This approach allowed us to determine how many of the newly proliferated cells that incorporated BrdU remained and incorporated into the neural circuitry. We found no differences in the number of BrdU-IR cells between formalin and control (saline and uninjected) animals. When we used NeuN, a marker for mature neurons, no group differences in post-weaning animals were found. Despite the moderate effects found in young animals (PND 15), this difference was absent by PND 35. This observation suggests that chronic inflammatory pain early in

life has transient effects on neuronal survival in the BLA. Since rapid brain growth occurs for most brain regions earlier than PND 15 (Bouza, 2009), it is possible that acute proliferative events caused by repeated inflammatory pain occurred within the first week of life. Future experiments might examine this possibility by injecting pups with BrdU within the first postnatal week.

Although we selected BrdU, which is a well-established marker for neurogenesis, other markers could be used to assess plasticity and the potential for neurogenesis in the AMY after neonatal pain exposure. Poly-sialated neural cell adhesion molecule (PSA-NCAM) can be used to detect young, immature neurons since it is located on the cell surface of developing neurons. Whereas NCAM promotes cell adhesion and mediates cell-cell interactions (Bonfanti, 2006; Edelman, 1986), PSA has a high negative charge, functioning to attenuate adhesion forces, negatively regulating cell surface interactions (Rutishauser & Landmesser, 1996). PSA-NCAM synthesis is modulated by cell activation (Kiss et al., 1994) and, thus, may provide an indication of the state of cellular activation during plastic changes and/or proliferative events. The distribution of PSA-NCAM varies throughout development. It is more widespread, but diffuse, during the embryonic stage and early postnatal period (Aaron & Chesselet, 1989; Peretto, Giachino, Aimar, Fasolo, & Bonfanti, 2005) compared to adulthood (Seki & Arai, 1993). Since PSA-NCAM modulates cell migration, axon growth, myelination, synaptic and morphologic plasticity early in development (for a review refer to Bonfanti, 2006), it would be interesting to measure levels of PSA-NCAM during the indicated peak period (PND 5 -10) of neuronal proliferation in response to pain.

In addition to PSA-NCAM, BDNF is also an important factor associated with neuroplasticity. BDNF helps regulate neural development and neuronal survival during development and in adulthood. It is also released in response to pain and injury (Chien et al., 2007; Chung, Bian, Xu, & Sung, 2009), which may be particularly critical during the neonatal period (Fitzgerald, 1995). In view of our data, we propose that early inflammatory pain might lead to specific increases in BDNF expression in the BLA around the first week of postnatal life.

A third factor, synaptophysin, could provide information on synaptogenesis during this period of neuronal proliferation. This protein is expressed in presynaptic vesicles and participates in the secretory process (for a review refer to Valtorta, Pennuto, Bonanomi, & Benfenati, 2004). If pain alters structural plasiticity of neurons, we could use synaptophysin to detect differences in the number of synapses between the treatment groups. Studies investigating changes in synaptogenesis in response to chronic constriction injury have found increased synaptophysin expression, thus an increase in the number of synapses, in the dorsal horn of the spinal cord (Chou et al., 2002; Peng, Lin, Shang, Yang, & Wang, 2010). This increase in synaptophysin expression was correlated with thermal hyperalgesia, but not tactile allodynia (Chou et al., 2002).

The AMY is composed of several nuclei, but we only counted the number of Ki67, BrdU and NeuN immunoreactive cells in the BLA because this region showed the most consistent changes in our volumetric MRI analysis. Differences in the number of cells in the other nuclei may exist. Therefore, it would be beneficial to investigate other nuclei of the AMY, such as the CeA, which plays a key role in modulating pain

experiences (Neugebauer, 2007; Ortiz et al., 2007). We could analyze each nucleus separately and the AMY as a whole to determine overall differences. The AMY is a challenging region to examine because its composite nuclei do not have well-defined boundaries. It is especially difficult in neonatal brains since many brain regions are not yet fully developed.

Thus far, our morphological and imaging results suggest that early exposure to modest formalin-induced inflammatory pain leads to transient BLA hypertrophy that subsides after weaning. We also propose that neuronal proliferation in the BLA occurs prior to the second week of life, most likely during our pain procedure, particularly between PND 5 - 10, which is a vulnerable period of brain growth. Although this set of data fails to provide support for the long-term effects of early life inflammatory pain caused by formalin on brain morphology, it does not preclude subtle alterations in AMY function. As discussed earlier, we cannot rule out modifications in other regions that project to and from the AMY. For this reason, we wanted to test behavioral outcomes of pain-exposed offspring in adulthood. Since our main finding involved changes in AMY morphology, we selected behaviours that are regulated, at least in part, by the AMY. In particular, we selected tests that would measure social interaction, anxiety and fear behaviours.

Social deficits are found in children who are born prematurely. We wanted to test whether animals that experienced pain early in life would also exhibit altered social interaction behaviours later in life. As such, we conducted social interaction and recognition tests in adult rats that were exposed to pain during the neonatal period. The AMY, in particular the BLA, is implicated in the regulation of social interaction. Studies

have shown that infusions of CRF, a neuropeptide released in response to stress, into the BLA results in decreased social interaction, an indicator of anxiety (Sajdyk et al., 1999). On the other hand, when rats were administered a CRF antagonist into the BLA, they were less anxious in a social interaction test following a stressor (Gehlert et al., 2005).

For social interaction testing, we categorized behaviours as either social (including sniffing, approaching, communal grooming, and wrestling) or anxious (including freezing, self-grooming, and aggression). We failed to find any group differences in the amount of time spent displaying either social or anxious behaviours during the social interaction test. Fewer social behaviours during a social interaction test is an indicator of anxiety, thus, our results suggest that mild inflammatory pain during the neonatal period did not have an effect on social anxiety in adulthood. When tested for recognition of a conspecific, animals from the various treatment groups reacted similarly when presented with a novel rat as they did with a familiar one. However, uninjected animals were an exception as they performed more social behaviours during the social interaction test compared to when they were presented with a familiar rat. This finding is expected since rats will normally investigate familiar rats less and novel rats more (Markham & Juraska, 2007; Rudebeck et al., 2007). Thus, our unmanipulated animals are performing according to their natural inclinations. However, the formalin and saline rats treated each stimulus animal the same, regardless if they were familiar or novel. Perhaps the pain experienced by these animals caused social recognition deficits, which is plausible since the AMY and hippocampus share reciprocal connections (Pitkänen et al., 2000).

Modifying the social interaction procedure may reveal group differences. We conducted our interaction studies with weight-matched conspecifics in an arena. Other studies testing social recognition in adults have used juveniles as stimulus rats (Burman & Mendl, 1999; Rudebeck et al., 2007) and perform the test in the experimental animal's home cage (Markham & Juraska, 2007; Rudebeck et al., 2007). Also, since we found a moderate difference in the number of NeuN-IR cells on PND 15, it would be interesting to conduct social interaction and recognition testing at a younger age, i.e. during the juvenile period (Macrì, Laviola, Leussis, & Andersen, 2010; Trezza et al., 2008) to determine if the neuronal differences would be reflected in the behaviour of the animals. Reducing the amount of time between presentation of novel and familiar conspecifics to less than 2 hours during social recognition testing may also improve the animals' memory for the stimulus rats (Markham & Juraska, 2007; Mathiasen & DiCamillo, 2010; Rudebeck et al., 2007).

Similar to the social interaction test, we found no treatment-induced differences in the amount of time spent in the open versus closed arms of the elevated plus maze. This result suggests that early life pain does not affect anxiety in adult offspring. The elevated plus maze is most effective at detecting the impact of drugs on anxiety levels (Kumar, Kalonia, & Kumar, 2010; Zarrindast et al., 2010) and might not be sensitive enough to detect basal levels of state anxiety in our animals.

Finally, using a test of fear conditioning, formalin injected animals did not differ from saline injected and controls in their percent freezing score. We tested animals for their response to both the cue (an AMY-dependent task) and context (a hippocampus-dependent task), yet neither association caused a difference in freezing score. These

results suggest that the transient AMY hypertrophy during exposure to pain in neonatal life did not yield significant consequences in behavioural outcomes in tests that involve the AMY.

Overall, our behavioural results indicate that repeated, mild inflammatory pain early in life does not impact AMY-regulated behaviours in adulthood. However, this failing does not mean that other behavioural outcomes are not affected. Other tests of anxiety could be used that may potentially be more sensitive to our treatment outcomes. Some of these tests include the Geller-Seifter conflict test, light/dark exploration, and defensive burying (File, Lippa, Beer, & Lippa, 2004). Tests that measure other factors, such as mechanical (i.e. von Frey) or thermal sensitivity (i.e. hot plate), could be employed as well. Previous research in our laboratory found that early life inflammatory pain results in greater pain sensitivity to formalin in adulthood (Walker et al., 2008). Longer hot plate latencies, decreased ethanol preference, and reduced locomotor activity were found in adult animals that received acute inflammatory pain during the neonatal period (Bhutta et al., 2001).

Although we took great care in designing these experiments, there are some limitations that must be addressed. We tried to use an animal model of mild inflammatory pain to mimic the pain experienced by preterm infants in the NICU. However, we do not know how accurately our pain paradigm mimics that of the real experience. Pain is a complex process involving many brain regions. Due to time constraints and the evidence gathered from our preliminary data, we decided to focus solely on the AMY, particularly the BLA. It would be beneficial to investigate other nuclei of the AMY as well as other brain regions implicated in pain processing, including

the ACC, PFC, hippocampus, and PAG. In addition, we could study other markers of plasticity, such as PSA-NCAM, BDNF, and synaptophysin, and at younger ages, in order to more thoroughly assess the manner in which pain may alter brain structure throughout development. We only focused on anxiety and fear related behaviours, which means that other behaviours may have been altered by our pain paradigm, but we were unable to detect them. Cognitive or memory tests should be conducted since the PFC and hippocampus are also implicated in pain processing. Since delayed cognitive development (Grunau et al., 2009) is one of the primary negative consequences found amongst preterm infants, it may be particularly important to investigate in our model.

Although numerous studies have reported that the pain experienced by preterm neonates in the NICU causes long-term negative consequences, our results suggest that perhaps it is not pain, or at least not just pain, which produces these outcomes, in particular those related to anxiety and fear. Other stressors are present in the environment of a preterm infant, including the fact that its environment should still be the mother's womb. The infant is separated from its mother while in the NICU, which could play a key role in the developmental course of these children. Lights, noises, and vibrations from the incubator, machinery and other babies' cries are constantly surrounding the infant. Although it is tempting to translate animal data directly to human implications, we believe that caution should be used when interpreting and translating data from rodents to humans. In particular, improving the detection sensitivity of our measures, refining the variables that we choose to measure, and including additional

behavioural tests to critically address the mechanisms underlying the long-term consequences of early life pain would be beneficial.

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Table 1

Brain regions of interest and the integral role they play in processing pain. Columns indicate the brain region of interest, the pain-related function it performs, and the information source.

Brain region	Major function	Reference
Amygdala	Implicated in emotions, fear and anxiety	Bernard & Besson, 1990 Phelps et al., 2001 Ziv, Tomer, Defrin, & Hendler, 2010
Anterior cingulate cortex	Motivation Integration of sensory, attention and motor responses	Price, Verne, & Schwartz, 2006
Hippocampus	Use of context for appraising stimuli	Ziv, Tomer, Defrin, & Hendler, 2010
Insular cortex	Identifying negative emotions in self and others	Kurth, Zilles, Fox, Laird, & Eickhoff, 2010
Periaqceductal grey	Emotional behaviour	Gauriau & Bernard, 2002 Kong, Tu, Zyloney, & Su, 2010
Prefrontal cortex	Cognition Motor response planning Modulating pain	Apkarian, Bushnell, Treede, & Zubieta, 2005 Baron, Baron, Disbrow, & Roberts, 1999 Seifert et al., 2009
Somatosensory cortices	Sensory processing of pain	Price, 2002
Thalamus	Pain sensation and processing	Price, 2002

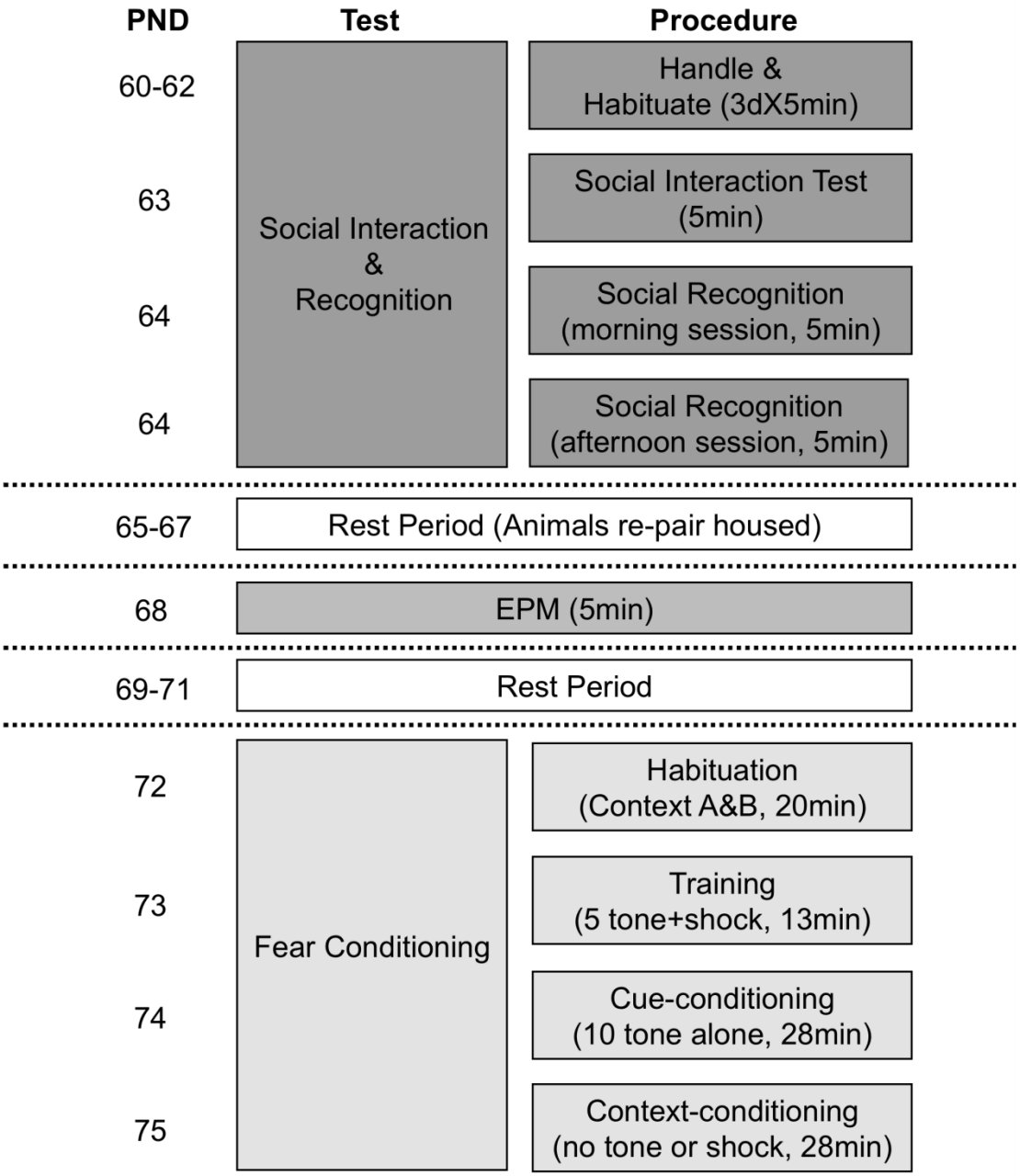

Figure 1. Adult behavioural testing schedule. Behavioural testing began on postnatal day (PND 60). Social interaction and recognition testing took place from PND 60-64 (dark grey boxes). Elevated plus maze testing (EPM) occurred on PND 68 (medium grey box). Fear conditioning occurred between PND 72-75 (light grey boxes). Three day rest periods were provided between each behavioural paradigm (white boxes).

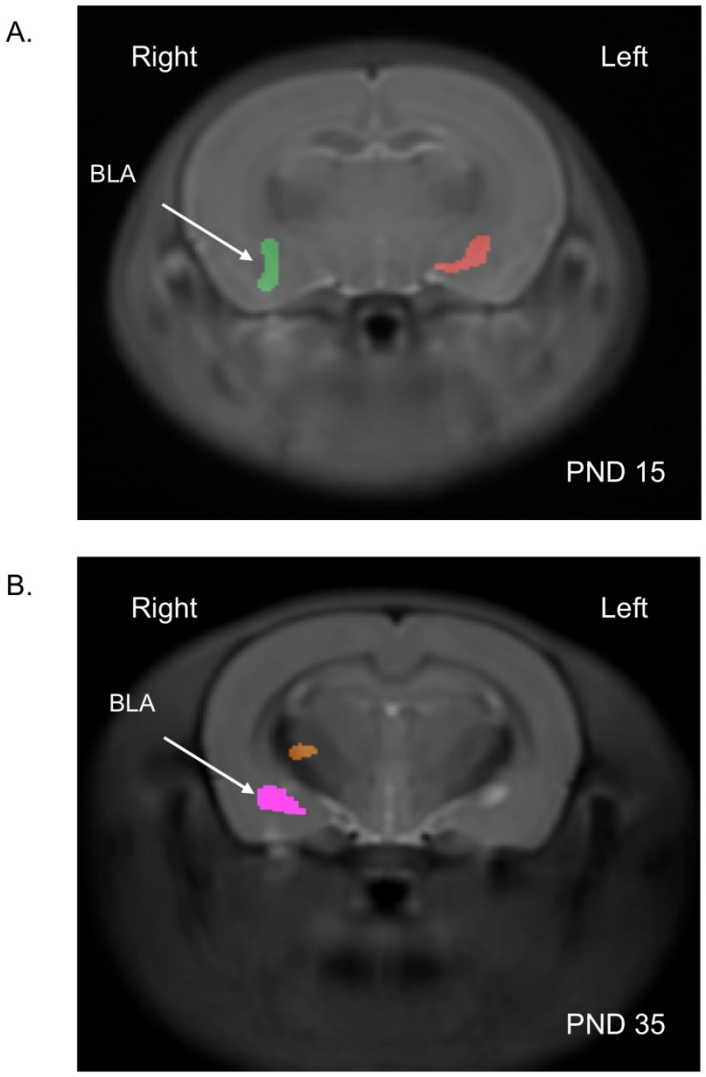

Figure 2. Changes in brain morphometry following early life inflammatory pain. Morphometric results from pilot data on PND 15 (Figure 2A) and PND 35 (Figure 2B). Analysis revealed hypertrophy in the right BLA on PND 15 (Green: $p = .009$) and a near significant effect of hypertrophy in the right BLA on PND 35 (Pink: $p = .061$). The number of animals scanned per treatment were Formalin: n=9 and Saline: n=9.

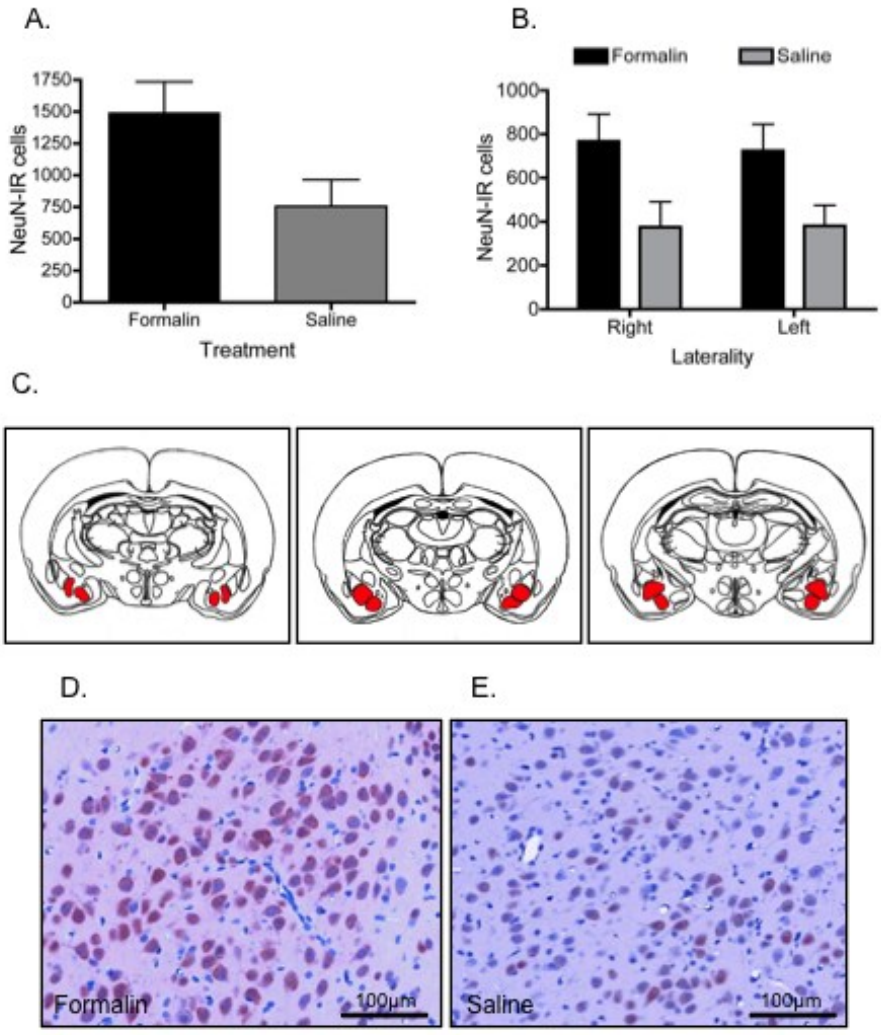

Figure 3. The effect of neonatal pain treatment on NeuN immunoreactive (NeuN-IR) cells in the basolateral amygdala (BLA) during the neonatal period (PND 17). Results are expressed as the number of NeuN-IR cells (Mean +SEM). Formalin: n=5 [black bars] and Saline: n=4 [grey bars] (Figure 3A). Analysis of the right and left BLA reveals a near significant effect of pain treatment ($p = .0624$), but no effect of laterality (Figure 3B). Representative atlas figures used to determine the BLA region (Figure 3C). Photos of NeuN-IR cells (40X magnification) in Formalin (Figure 3D) and Saline (Figure 3E) animals.

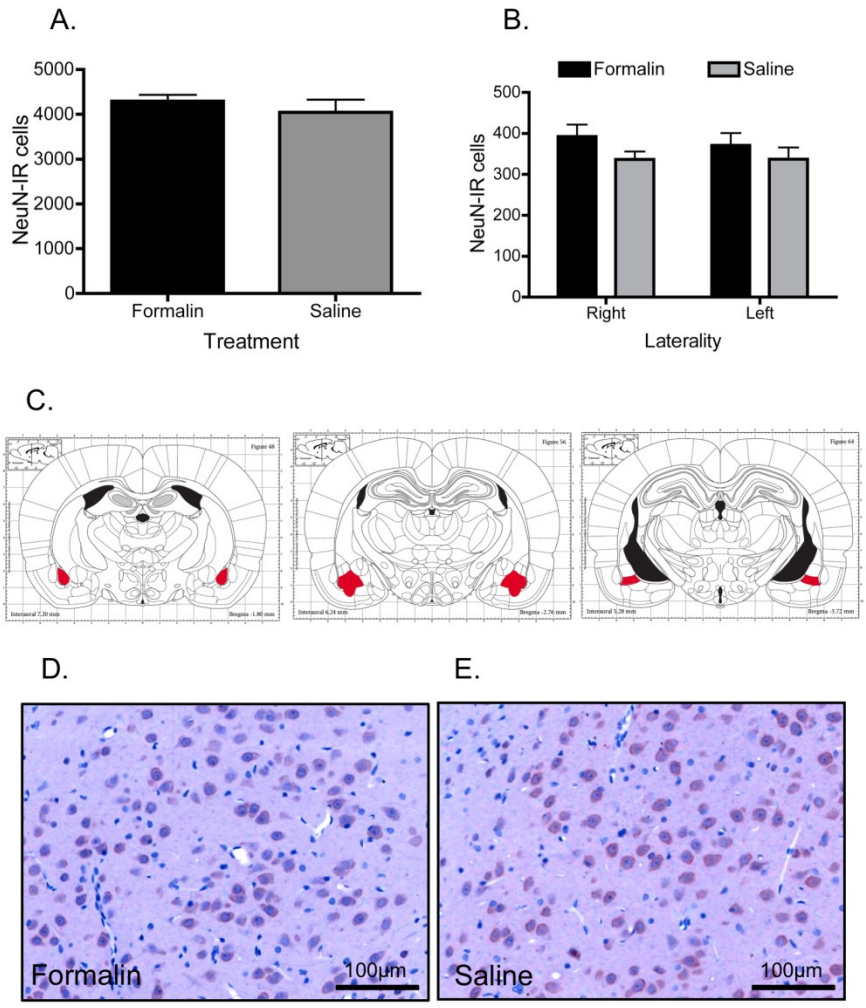

Figure 4. The effect of neonatal pain treatment on NeuN immunoreactive (NeuN-IR) cells in the basolateral amygdala (BLA) during the post-weaning period (PND 42). Results are expressed as the number of NeuN-IR cells (Mean +SEM). Formalin: n=3 [black bars] and Saline: n=4 [grey bars] (Figure 4A). Analysis of the left and right BLA did not find a significant effect of laterality or treatment (Figure 4B). Representative atlas figures used to determine BLA region (Figure 4C). Photos of NeuN-IR cells (40X magnification) in Formalin (Figure 4D) and Saline (Figure 4E) animals.

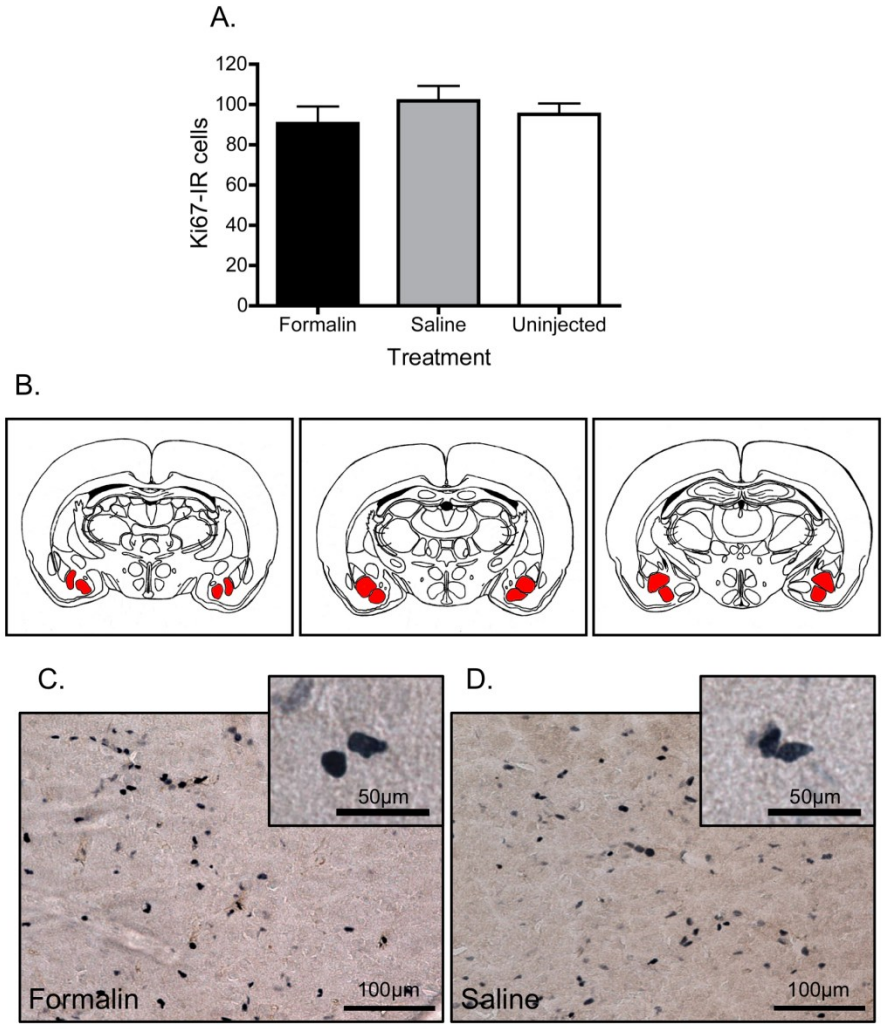

Figure 5. Number of Ki67 immunoreactive (Ki67-IR) cells located in the basolateral amygdala (BLA) of neonatal animals following early life inflammatory pain. The graph represents the combined number of Ki67-IR cells in the right and left BLA (Mean +SEM). Formalin: n=5 [black bars], Saline: n=5 [grey bars], Uninjected: n=5 [white bars] (Figure 5A). Representative atlas figures used to determine the BLA region (Figure 5B). Photos of Ki67-IR cells (20X magnification) in Formalin (Figure 5C) and Saline (Figure 5D) animals with inserts at 40X magnification.

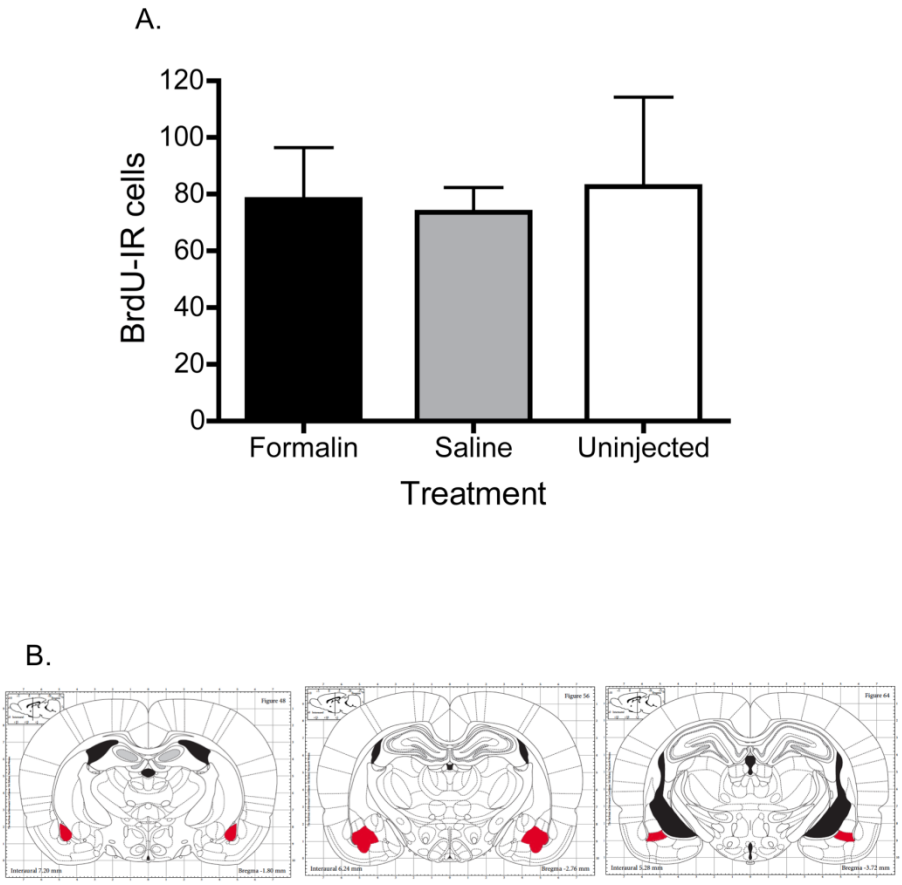

Figure 6. Differences in the number of BrdU immunoreactive (BrdU-IR) cells in the basolateral amygdala (BLA) of post-weaning animals following early life inflammatory pain. Combined number of BrdU-IR cells located in the right and left BLA (Mean + SEM). Formalin: n=6 [black bars], Saline: n=6 [grey bars], Uninjected: n=6 [white bars] (Figure 6A). Atlas figures used to determine the BLA region (Figure 6B).

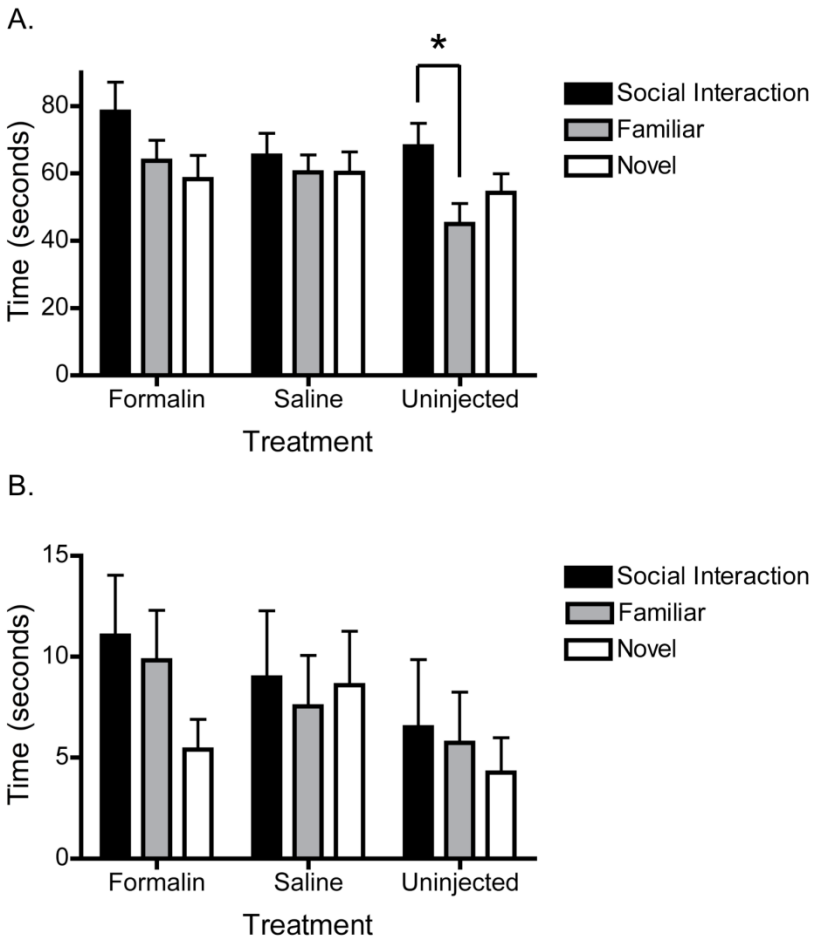

Figure 7. The effect of neonatal pain treatment on adult social interaction and recognition. Results are expressed as the amount of time spent performing either social (Figure 7A) or anxious (Figure 7B) behaviours (seconds + SEM). All animals were tested for social interaction [black bar], social recognition with a familiar rat [grey bar], and social recognition with a novel rat [white bar]. Statistical analysis revealed that uninjected animals performed more social behaviours during the social interaction test compared to the social recognition test with a familiar rat ($p = 0.009$). Formalin: n=15, Saline: n=14, Uninjected: n=13. * indicates a significant difference of $p < .05$.

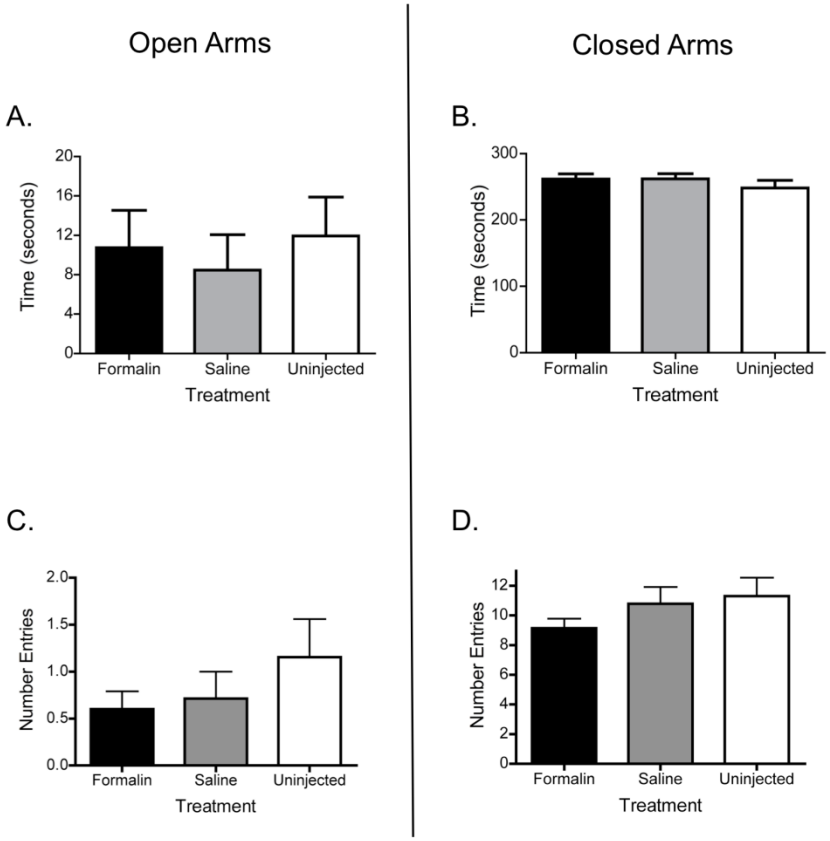

Figure 8. Spatio-temporal measures on the elevated plus maze in adult rats that experienced pain during the neonatal period. Graphs represent the amount of time (seconds + SEM) spent in the open arms (Figure 8A) and closed arms (Figure 8B). Frequency of entries (Mean + SEM) into open (Figure 8C) and closed (Figure 8D) arms. Formalin: n=15 [black bar], Saline: n=14 [grey bar], Uninjected: n=13 [white bar]. No significant treatment effects were found.

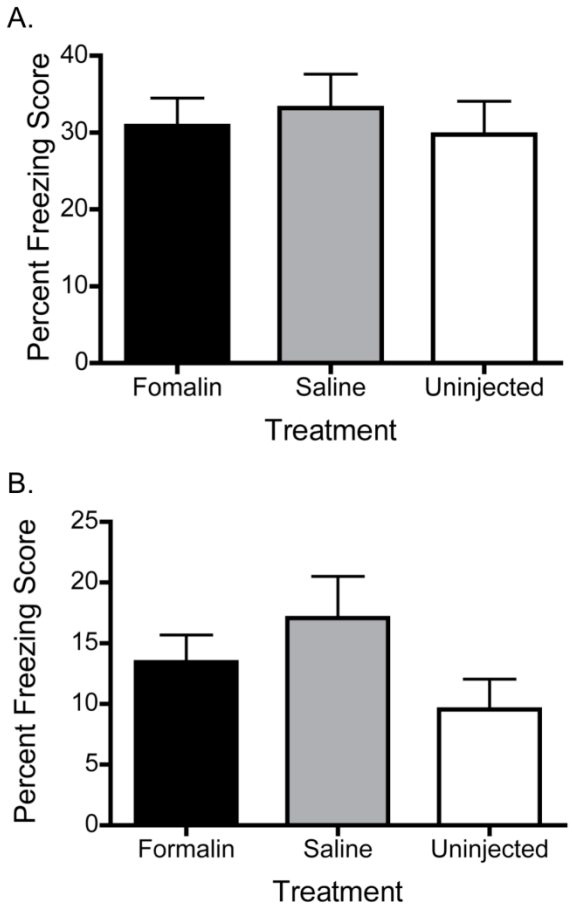

Figure 9. Percent freezing score during fear conditioning in adult animals that received pain during the neonatal period. Results are expressed as percent freezing score (Mean+SEM) during Cue (Figure 9A) and Context (Figure 9B) tests. Formalin: n=15 [black bar; cue test] and n=14 [context test], Saline: n=14 [grey bar], Uninjected: n=13 [white bar]. No significant treatment effects were found on either test day.