Investigating the sensitivity of juvenile social exploration at detecting the affective consequences accompanying chronic neuropathic pain

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Abstract

Chronic neuropathic pain debilitates millions of lives globally and exerts substantial emotional hardship, particularly in the form of major depressive disorder, on its sufferers. Despite extensive research, the biological basis of interaction between pain and its affective consequences remains largely elusive. A growing body of literature suggests chronic neuropathic pain and major depressive disorder may have similar underlying causes rooted in neuroinflammation. Establishing a paradigm to study the comorbidity of pain and its affective implications would be invaluable in the process of developing holistic treatment options. Here, we investigate the sensitivity of an anxiety-based behavioral test, juvenile social exploration (JSE), at detecting the affective repercussions of chronic neuropathic pain, simulated by chronic constriction injury, in male Sprague Dawley rats over the course of 28 days. Although statistically significant differences in interaction times were observed between the experimental groups on day 7 post-surgery, these differences were not observed on the following days post-surgery. A morphine time course was employed to exacerbate the effects of inflammation with the hope of creating greater separation between the groups but this manipulation produced contradicting behavioral data. A ceiling effect was suspected so a 3-minute pre-exposure trial was conducted; but again, this modification resulted in unremarkable data. Despite the negative results of JSE in this exploratory study, other behavioral assays might prove to be more sensitive at detecting the affective implications of chronic neuropathic pain. Further research into developing a reliable model of chronic neuropathic pain and its affective complications is desperately warranted.

Introduction

Chronic neuropathic pain impacts the lives of 25 million adults in the United States alone (Nahin et al, 2015). The economic toll of pain based on missed workdays, decrease in work hours, and decreased wages is estimated to be over 500 billion dollars (Gaskin et al., 2011).

Depression is one of the largest causes of disability in the world (Murray et al., 1997; Gelenberg et al., 2010; Kessler et al., 2013) and has been closely associated with chronic neuropathic pain (Bair et al., 2003). Experts have acknowledged the interaction between depression and pain symptoms as the depression-pain syndrome, or depression-pain dyad (Lindsay et al., 1981). Pain and depression have been shown to respond similarly to treatment, exacerbate each other, and share common biological etiologies. (Gallagher et al., 1999; Blier et al., 2001; Nekovarova et al., 2014)

Available treatment options address the physical and affective symptoms separately (Nalamachu et al., 2013). Morphine and other opioids are commonly used to treat cases of acute pain but chronic users are subject to adverse effecting including tolerance, addiction, and abuse (Glare et al., 2006; Baldini et al., 2012). Selective Serotonin Reuptake Inhibitors (SSRI's) are commonly used to treat depression but they have been criticized for fostering dependency and demonstrating lack of benefit in placebo-controlled studies (Kirsch et al., 2008).

Chronic pain and depression display a high comorbidity (Nicholson et al., 2005; Means-Christensen et al., 2008; Alibrandi et al., 2014) and have been shown to have roots in neuroinflammatory processes mediated by glial cells, particularly microglia and astrocytes (Dantzer et al., 2008; Loram et al., 2009; Miller et al., 2009). Chronic neuropathic pain induced using a Chronic Constriction Injury (CCI) model in rats has been linked to increased glial cell activation and production of pro-inflammatory cytokines including interleukin-1β (IL-1β) and

tumor necrosis factor (TNF) (Watkins et al., 2007; Loram et al., 2009). Glial activation may be reversed and neuropathic pain alleviated using an adenosine 2A receptor (A2AR) agonist, ATL-313, that promotes the production of an anti-inflammatory cytokine, IL-10 (Milligan et al., 2006; Loram et al, 2009).

The neuroinflammtory view of depression suggests that microbiota leak out during the proliferation of the endothelial lining of intestines during periods of stress (Dantzer et al., 2008). The presence of bacteria, or more specifically lipopolysaccharide (LPS) in their cell walls, in the periphery activates toll-like receptors (TLR's), particularly TLR-4, on immune cells that initiate the production of pro-inflammatory signals. TLR-4, IL-1 β, plasma LPS, and lipopolysaccharide binding protein (LBP) levels have been demonstrated to be up-regulated using the Chronic Mild Stress (CMS) paradigm in rats (Garate et al., 2011). These pro-inflammatory signals can then propagated to the brain and modulated by the vagus nerve among other pathways (Goehler et al., 2000; Borovikova et al., 2000). In the central nervous system, a cycle of neuroinflammation is perpetuated by the interaction of microglia and astrocytes thus resulting in deregulation of glutamate signaling, excitotoxic neuronal cell death, apoptosis of oligodendrocytes, demyelination, decreased levels of brain derived neurotropic factor (BDNF), and decrease in serotonin levels (Miller et al., 2009).

Developing a paradigm to study chronic pain and its affective implications (Leite-Almeida et al., 2015) is critical to developing holistic treatment options targeted towards attenuating the underlying neuroinflammation rather than other downstream consequences. Pervious studies have demonstrated affective behavioral accompany neuropathic pain models (Wang et al., 2011; Dellarole et al., 2015). In this pilot study, we investigate the sensitivity of an anxiety-based behavioral test, juvenile social exploration (JSE) (File et al., 2003), in detecting

the affective implications of CCI. We specifically choose to use JSE and CCI because these models most closely represent clinical expression of chronic neuropathic pain and depression.

Materials and Methods

Subjects

Sixteen adult male Sprague Dawley rats (325-350 g at time of arrival; Harlan Laboratories) were housed in pairs with standard rat chow and water *ad libitum.* Environment was temperature controlled (23 ± 2°C) with a 12-hour light/dark cycle (lights on at 7:00 AM). Behavioral testing occurred during the light phase. Rats were allowed 7 days of habituation and 3 separate 2 minute handling sessions prior to any experimentation. All procedures were conducted in accordance with the University of Colorado Boulder Institutional Animal Care and Use Committee's regulations.

Experimental Design

Subjects were randomly assigned to two experimental groups: CCI (n=8) or Sham (n=8). On day 0, CCI animals received CCI surgery and Sham animals received an identical surgery with the exception that sutures were not tied around the sciatic nerve. Von Frey (VF) and juvenile social exploration (JSE) testing was conducted prior to surgeries to establish baseline scores and subsequently on a weekly basis until day 28. VF testing was omitted on day 28. Beginning on day 16, a 5-day morphine time course was initiated and groups were subdivided into CCI+Morp. (n=3), CCI+Saline (n=4), Sham+Morp. (n=4), and Sham+Saline (n=4). On day 28, a 3-minute pre-exposure JSE trial was conducted in an attempt to mitigate a suspected ceiling effect. All behavioral tests were conduced by observers blind to experimental conditions.

Chronic Constriction Injury (CCI)

Four 4-0 chromic gut sutures were lightly tied along the left sciatic nerves of the CCI animals at mid-thigh level just prior to the bifurcation of sciatic nerves (Bennett and Xie, 1988). Sham animals underwent an identical surgery with the exception that sutures were not tied around the sciatic nerves. Surgeries lasted approximately 15 minutes and were conducted under isoflurane anesthesia (1.5-2.0% vol. in oxygen) in a sterile environment with appropriate techniques and precautions to avoid infections. Perma-silk 3.0 was used to close the incisions in thigh muscle produced as a result of surgery. Surgical staples were used to mend the site of entry on the skin and were removed 14 days later. Animals were allowed 7 days to recover from surgery before any behavioral tests were conducted.

Von Frey (VF) Testing

Von Frey (VF) testing was used to assess mechanical allodynia (Loram et al., 2009). Animals were habituated to the testing room 3 separate times for one hour each prior to assessing baseline scores. On days of testing, Animals were acclimated to the testing apparatus for one hour. The plantar surface of animals' hind paws was stimulated using a logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (Stoelting) sequentially (from low- to high-intensity threshold) to the left and right hind paws in random order, each for 8 seconds at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by log10(milligrams 10). The range of monofilaments used in these experiments (0.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity [expressed as

log10(milligrams 10)] (Chaplan et al., 1994). The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function (Harvey, 1986).

Morphine Injections

Animals were divided into groups (CCI+Morp. (n=3), CCI+Saline (n=4), Sham+Morp. (n=4), and Sham+Saline (n=4)) as discussed previously. Morphine was administered to the assigned animals subcutaneously at 5 mg/kg, twice a day. Injections were separated by at least six hours. Saline injections were also administered to the appropriate animals in an identical fashion to serve as a control. Morphine time course lasted 5 days (day 16 to day 20). VF and JSE testing was conducted on day 21.

Juvenile Social Exploration (JSE)

JSE testing was conducted as described previously (Christianson et al., 2008, 2010). In a separate testing room, animals were placed individually in plastic tub cages with shaved wood bedding and wire lids. After one hour of acclimation, a 2-3 week-old juvenile rat was introduced to the cage for 3 min and two observers, blinded to treatment, timed exploratory behaviors (sniffing, pinning, and allogrooming) initiated by the adult animals. Total interaction time was calculated using the average of observers' scores. A novel juvenile was presented to each animal during every JSE trial. The wire lids of cages were replaced with an elevated lid and a mirror was placed behind the testing cage to allow observers to better visualize behavior. JSE trials were recorded using a 2005 Sony HDR camcorder. On day 28, a 3-minute pre-exposure trial was

conducted. In this modified version, juveniles and adult rats interacted for 6 minutes but only the last 3 minutes of interaction was scored. All other conditions remained constant.

Statistical analysis

VF and JSE interaction time data was plotted in Microsoft Excel and statistically analyzed using a using StatPlus (AnalystSoft). VF data from baseline to day 21 were analyzed using a simple compare means T-test. Interaction time values from baseline, day 7, day 14, and day 28 were analyzed using a simple compare means T-test. JSE data from day 21 (morphing time course) was analyzed using a one-way ANOVA. All data is reported as mean ± standard error.

Results

CCI produces sustained mechanical allodynia

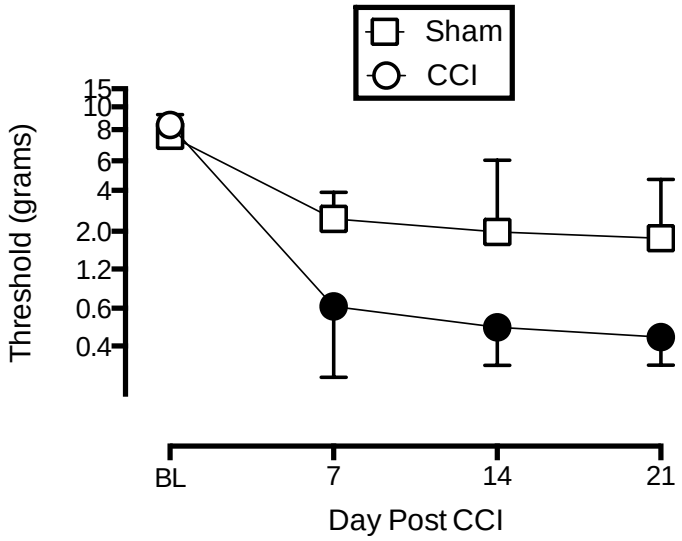

Figure 1. CCI produces sustained mechanical allodynia. CCI animals (4.96 ± 0.03 g) and Sham animals (4.89 ± 0.05 g) display no significant differences (p=0.24) in mechanical allodynia on day 0 during baseline testing prior to surgery. CCI animals exhibited significantly increased nociceptive behavior in comparison to Sham animals on days 7 (CCI: 3.85 ± 0.15 g; Sham: 4.39 ± 0.06 g; p=0.006), 14 (CCI: 3.72 ± 0.08 g; Sham: 4.31 ± 0.15 g; p=0.005), and 21 (CCI: 3.66 ± 0.06 g; Sham: 4.27 ± 0.13 g; p=0.0007) post-surgery. Filled circles represent significant differences.

CCI significantly decreases JSE interaction time on day 7 but not day 14 post-surgery

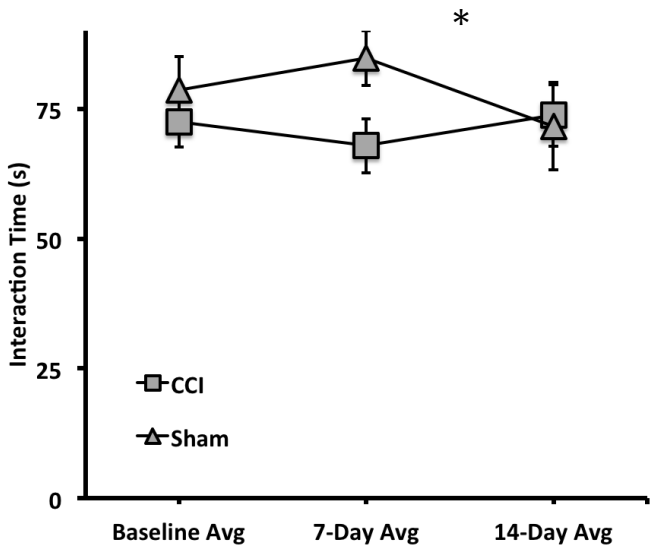

Figure 2. CCI significantly decreases JSE interaction time on day 7 but not day 14 post-surgery. CCI (72.41 ± 4.76 s) and Sham (78.55 ± 6.47 s) showed no significant difference (p=0.46) in baseline testing. CCI animals (76.85 ± 5.20 s) showed significantly decreased JSE interaction times (p=0.03) in comparison to Sham animals (84.75 ± 5.32 s) on day 7 post-surgery. CCI animals (73.72 ± 5.95 s) and Sham animals (71.64 ± 8.47 s) showed no significant difference (p=0.84) on day 14 post-surgery.

5-day chronic morphine administration produces no significant differences in JSE interaction time on day 21 post-surgery

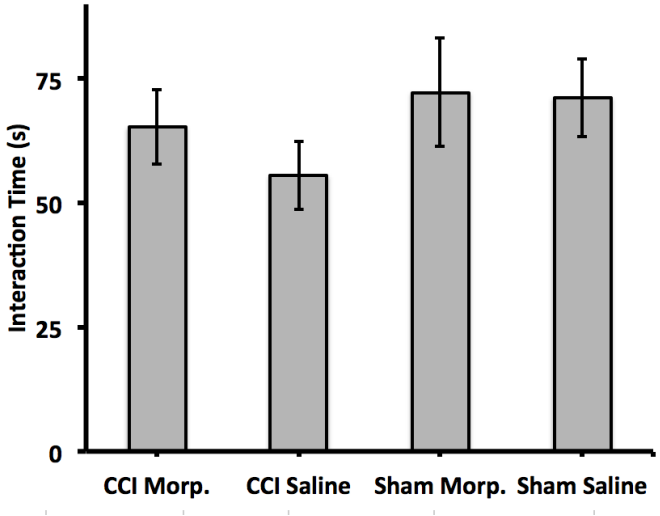

Figure 3. 5-day chronic morphine administration produces no significant differences in JSE interaction time on day 21 post-surgery. CCI Morp. group (65.21 ± 7.49 s), CCI Saline group (55.48 ± 6.14 s), Sham Morp. group (72.21 ± 10.49 s), and Sham Saline group (71.20 ± 7.83 s) showed no significant difference ($p=0.50$, $F_{(3,11)}=0.84$) on day 21 post-surgery.

3-minute pre-exposure produces no significant differences in JSE interaction time on day 28 post-surgery

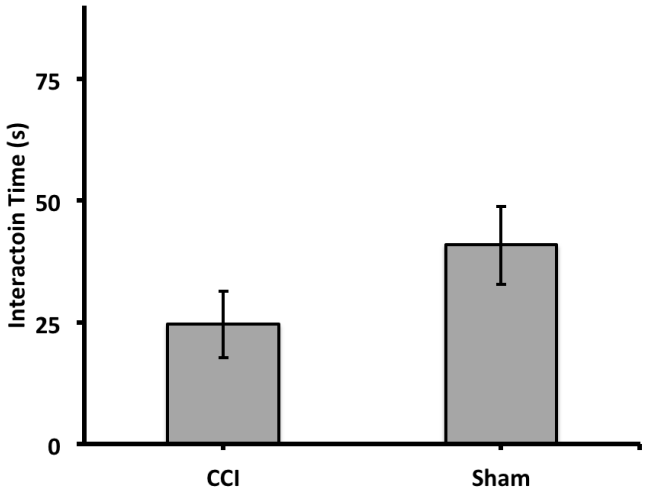

Figure 4. 3-minute pre-exposure produces no significant differences in JSE interaction time on day 28 post-surgery. CCI animals (24.54 ± 6.86) and Sham animals (40.85 ± 7.99) exhibited similar levels of interaction (p=0.15) on day 28 post-surgery.

VF was used to verify mechanical allodynia produced as a result of CCI. CCI animals displayed significant nociceptive behavior throughout the course of the experiment (Figure 1). Although VF testing was not conducted on day 28, it may be reasonably assumed animals continued their trend as CCI has been shown to be consistent for nearly 10 weeks (Loram et al., 2009). Sham animals showed no signs of mechanical allodynia or nociceptive behavior in VF (Figure 1). JSE was used to assess the level of anxiogenic behavior, measured a function of interaction time, expressed by animals in chronic neuropathic pain simulated by CCI. We expected CCI animals to exhibit lower levels of interaction time in JSE in comparison to Sham animals; however, this was only observed on day 7 (Figure 2). CCI and Sham animals showed no significant differences on day 14 (Figure 2). One day after a 5-day morphine time course, we expected animals treated with morphine to exhibit lower interaction times because morphine has been shown to

exacerbate the effects of CCI (Grace et al., in press); however, on day 21, CCI+Morp., CCI+Saline, Sham+Morp., and Sham+Saline showed no significant differences (Figure 3). A ceiling effect was suspected so a 3-minute pre-exposure trial was conducted on day 28; but again, no significant differences were observed between the CCI and Sham animals (Figure 4). At the day 28 time point, we do not expect the morphine time course conducted earlier to have an effect so the morphine and saline sub-groups were collapsed back into CCI and Sham.

Discussion

Although a significant difference in JSE interaction time was observed on day 7 post-surgery, this was not replicated on day 14 post-surgery (Figure 1). This suggests that the difference observed on day 7 might have been due to chance as the p-value of 0.03 was only slightly below the standard alpha value of 0.05 (Figure 1). Furthermore, the morphine time course that has been shown to exacerbate the effects of 1-suture CCI surgery and prolong the duration of mechanical allodynia produced by 4-suture CCI (Grace et al., in press), did not produced a change in JSE interaction time day 21 post-surgery (Figure 3). This suggests that the molecular mechanism and neural circuitry involved in the JSE behavior is resilient to increased stressors. Rats are known to be extremely social animals so we suspected that the lack in difference between groups might be due to a ceiling effect. We employed a 3-minute pre-exposure trial on day 28 to negate the initial novelty effect of juvenile rats; however, this manipulation did not result in a significant difference between the groups. Rather, a 3-minute pre-exposure seems to suggest almost a floor effect. Taken together, this might suggest there is a window of time between 0 to 6 minutes where a difference between experimental groups may be detected. This might also suggest that

animals' interaction behavior decreases gradually overtime, ultimately reaching a floor-level of expression, regardless of experimental condition.

Given the results of this pilot study, JSE does not seem to be sensitive to the affective implications of chronic neuropathic pain as induced by CCI given the experimental conditions. One possible explanation is that CCI does not induce enough pain to result in affective behaviors; however, this is unlikely because (1) Dellarole et al. (2015) has shown that CCI is effective at inducing affective behaviors in TNF receptor 1 (TNFR1) KO mice detected using sucrose preference test (SPT) (a measure of anhedonia) and open field test (OFT) (a measure of anxiety); and (2) Wang et al. (2011) has shown that the spared nerve injury (SNI) model of chronic neuropathic pain is sufficient to induce affective behaviors in rats as detected by the SPT and forced swim test (FST) (a measure of behavioral despair). Although SNI has been shown to be effective at inducing affective behavior in rats, it involves severing a portion of animals' sciatic nerves (Wang et al., 2011). This results in neuronal cell necrosis and other related cellular processes that do not mimic clinical expression of chronic neuropathic pain as closely or for as long as CCI (Dowdall et al., 2005). It may be reasonable to test if increasing the severity of CCI by tying sutures to both of rats' hind paws results in significant difference in interaction time between CCI and Sham animals.

Another possible explanation of why the affective compilations of CCI were not detected could be because of the behavioral assay, JSE, itself. The floor and ceiling effects observed present a unique obstacle of determining the appropriate window of testing to detect a consistent, significant difference between experimental groups. In addition to pre-exposure, other manipulations such as increasing the intensity of light over the testing cage and using older juvenile rats may help detect a greater difference between CCI and Sham animals in JSE. Yalcin

et al. (2011) reports that the affective behaviors accompanying neuropathic pain onset in a time-dependent manner depending on the behavioral tests used. This might suggest that affective implications of chronic neuropathic pain may not be detectable with JSE until many weeks after CCI. For instance, Yalcin et al. (2011) did not report significant differences in FST and TST until 8-9 weeks after the induction of neuropathic pain. Interestingly, Gai et al. (2014) reports that the affective symptoms accompanying CCI in mice were observable 4 weeks after CCI in FST and TST (measuring behavioral despair) but not the light-dark test (measuring anxiety). This might suggest that anxiety-based behavioral tests are less sensitive to detecting affective implications of chronic pain; however, given that OFT has been demonstrated to be effective in mice CCI (Dellarole et al., 2015), it is not completely clear how anxiety-based behaviors manifest themselves following the induction of chronic pain. Despite these difficulties, JSE is a powerful model because in more closely mimics clinical expression of depression by measuring level of anxiety as a function of interaction time in comparison to other behavioral tests including the tail suspension test (TST) (a measure of behavioral despair), FST, SPT, and OFT.

In future studies, we may incorporate other behavioral assays including the TST, FST, SPT, and OFT in addition to JSE as these tests have been demonstrated to be effective at detecting affective implications accompanying chronic neuropathic pain (Wang et al., 2011; Gai et al., 2014; Dellarole et al., 2015). We might also look to use mouse model of CCI as this has been demonstrated to be sensitive to affective behavioral tests after CCI (Gai et al., 2009; Dellarole et al., 2015). We may also include a positive control in the form of an intraperitoneal (IP) LPS injection as this has been shown to consistently produce depressive-like behaviors in rats and mice. A positive LPS control would allow us to compare the differences in affective behavior induced by immune system compromising substances (LPS) vs. chronic neuropathic

pain. In particular, we would investigate why affective implications of neuropathic pain seem to have delayed onset in comparison to LPS-based models. This type of study could help decipher if molecular mechanisms or neural circuits involved in affective behaviors are unique and dependent on their causes.

Once we establish a stable model to investigate the affective implications of chronic neuropathic pain, we can then test the efficacy of various anti-inflammatory drugs – particularly ATL-313 of which a single dose has been shown to attenuate the effect of CCI for 4 weeks in rats (Loram et al., 2009). Others have also explored the efficacy of anti-inflammatory treatments on depression (Andrade et al., 2014; Eyre et al., 2015; Fonseka et al., 2015; Li et al., 2015; Ayorech et al., 2015) but not in conjunction with chronic neuropathic pain. Few have also explored the efficacy of traditional tri-cyclic anti-depressants, cannabinoid agonists, and novel compounds on treating the affective complications accompanying chronic pain with promising results (Hu et al., 2009; Fukuhara et al., 2012; Gai et al., 2014); however, these studies did not explore the role of cytokines or neuroinflammation.

fMRI studies have shown that similar regions of the brain are activated when participants experience pain and social reject (emulating depression) (Kross et al., 2011). This suggests that similar brain regions and circuits may be involved in the regulation of chronic pain in depression. We hope to investigate the contributions of glial cells (particularly microglia and astrocytes) and cytokines (particularly IL-1β, TNF, and IL-10) in the medial pre-frontal cortex (mPFC), amygdala (AMYG), dorsal raphe nucleus (DRN), nucleus accumbens (NAc), ventral tegmental area (VTA), periaqueductal gray (PAG), and hippocampus (HIPP) as all of these structures have been associated with either pain, depression, or both. The mPFC, AMYG, and DRN have traditionally been shown to be key regions involved in the expression of affective behaviors

following stress (Maier et al., 2006, 2010). The NAc and VTA may play important roles in the motivational aspects of depressive behaviors and have been shown to modulate neuropathic pain (Ren et al., 2016). Electrical stimulation of the PAG has been demonstrated to modify affective behaviors including sucrose intake in SPT and exploration in OFT (Wright et al., 2011). The comorbidity of pain and depression is also correlated to decreased HIPP volume that is thought to impair cognitive functions including attention, information processing, and memory (Frodl et al., 2006).

Moreover, we would look to see how specific neurotransmitters including dopamine (DA), serotonin (5-HT), glutamate (Glu), gamma-aminobutyric acid (GABA), and brain-derived neurotrophic factor (BDNF) are affected following the induction depressive-like symptoms by chronic neuropathic pain. These molecules are closely associated with the appropriate structures mentioned previously and have been shown to be involved in both pain and depression. Might these molecules be affected differently in chronic pain-induced depression in comparison to LPS-induced depression?

Lastly, we would seek to study morphological changes in neuronal structures – particularly variations in dendrites and dendritic spine density, as these are key structures involved in neuroplasticity. Qiao et al. (2016) recently observed regional distinctions in spine density following stress-induced depression: dendrites in the HIPP and PFC atrophied and spine density decreased but dendrites in the NAc and AMYG exhibited increased spine density.

Examining these forementioned regions, molecules, and structures will help develop a holistic understanding of the chronic pain-induced depression by considering different theories of depression including the GABA/Glu and neuroplasticity hypotheses of depression.

Despite speculations against the inflammatory view of depression (Raison et al., 2014), the evidence supporting the role of glial cells and cytokines in a multitude of both human and animal studies of depression is substantial. Understanding the cellular and molecular neuroinflammatory mechanisms underlying chronic pain, depression and their complex relationship may help to develop novel treatment options that treat chronic pain and depression not as separate pathologies but rather as a single, collective ailment.

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