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Neurochemistry of Nocebo Conditioned Hyperalgesia in Mice

Course: PSYC 495 (Psychology Research Project 2)

Term: Winter 2022

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Date Submitted: May 17th, 2022

Abstract

Nocebo conditioned hyperalgesia occurs when one's experience of pain sensation towards a specific stimulus increases as a result of heightened anxiety and negative expectations or conditioning. When nocebo conditioned hyperalgesia is present, the anxiogenic neurochemical CCK is potentiated, and prior research has successfully blocked nocebo conditioned hyperalgesia by antagonizing $CCK_{A/B}$ receptors. The present study aimed to broaden our understanding of the neurochemistry of the nocebo conditioned hyperalgesia effect by experimenting with CCK antagonists, such as Proglumide ($CCK_{A/B}$ antagonist) and a novel drug, LY-225910 (CCK_B antagonist). Our test subjects were inbred mice of both sexes and were segmented into various groups. Conditioning would be assessed over a two-day period, where after the first day of testing, mice would be injected with Acetic Acid (conditioning stimulus) and then on the following day, would be placed in either the same context or different context. Pain was administered thermally and mice were injected intraperitoneally with the pharmacological agents on the second day. The results we obtained were significant and indicated that antagonizing both A & B CCK receptors can eliminate nocebo conditioned hyperalgesia and that a marked sex difference was also observed, as only males were eliciting signs of nocebo conditioned hyperalgesia. These results replicate numerous studies that inquired about sex differences in pain and nocebo conditioning, and further clarify the link between CCK and nocebo conditioned hyperalgesia. Future research should aim to establish which parts of the brain containing CCK_B receptors might be responsible for counteracting nocebo conditioned hyperalgesia, as no specific brain regions have yet been identified when investigating these effects.

Key words: nocebo hyperalgesia, hyperalgesia, conditioning, pain, anxiety, CCK, CCK antagonists, sex differences

Introduction

Nocebo Hyperalgesia & Placebo Analgesia

The nocebo hyperalgesia effect[1] is when the administration of a non-hyperalgesic substance and/or pain stimulus results in pain sensation increasing due to negative expectations and/or conditioning (Colagiuri, et al, 2015). It is the exact opposite of placebo analgesia; where the administration of a non-analgesic substance and/or non-painful stimulus decreases pain/produces analgesia due to positive expectations and/or conditioning (Colagiuri, et al, 2015). These effects have been studied for over half a century and are extremely well documented in both animals and humans (Frisaldi, et al, 2015). It is important to note that neither nocebo hyperalgesia nor placebo analgesia are isolated experiences. They are deeply intertwined with otherwise independent biological and psychological factors, such as the natural history of a disease, fluctuation in symptomology, response biases (expectations/conditioning), genetic composition, and what is being treated (e.g., treating chronic low-back pain versus a migraine; Schedlowski, et al., 2015). All of these separate, yet overlapping factors affect each other greatly and therefore make an organism's receptivity to nocebo hyperalgesia/placebo analgesia heterogeneous.

Despite this wealth of knowledge, placebo analgesia has received far more research attention than its nocebo counterpart. A quick search on Google Scholar will reveal a stark contrast in scholarly publications. At the time of writing, searching "placebo analgesia" nets nearly 300,000 results, whereas typing in "nocebo analgesia" only brings up 3000 publications.

[1] There are many forms of nocebo hyperalgesia, however, the one we are investigating is the nocebo hyperalgesia effect that arises through a pain stimulus being classically conditioned. Thus, the specific term is "Nocebo Conditioned Hyperalgesia", however, it is a rather long term and is without a direct synonym. So, in the interest of reducing redundancy and increasing readability, it will be used interchangeably with terms such as, "Nocebo Hyperalgesia", "Nocebo Conditioning", "Pain-Conditioning/Conditioned-Pain", and "Conditioned Hyperalgesia". Technically these are different terms, but they all refer to "Nocebo Conditioned Hyperalgesia" in this paper.

A common explanation as to why this large publication disparity exists is due to the inherent ethical limitations surrounding experiments that exacerbate pain in their subjects (Colloca & Benedetti, 2007; Frisaldi, et al, 2015; Benedetti, et al, 2006). Regardless of the reasoning behind this asymmetry, the paucity of data surrounding nocebo hyperalgesia is not a trivial matter. Understanding the fundamental mechanisms of these psychological experiences is of crucial importance for clinical matters, as research data direct practitioners on how to administer medication, conduct surgeries, and above all else, minimize harm.

Conditioning and Pain

A key process for nocebo hyperalgesia to take place is for there to be a conditioning effect taking place. Besides expectations and anxiety, classical conditioning (CC) is the third factor involved in eliciting nocebo hyperalgesia, independent of the formation of expectations and anxiety (Brascher et al., 2018). The reason for CC's prominence in nocebo hyperalgesia is because the pairing of a conditioned stimulus (CS) with an unconditioned stimulus (UCS) leads to a learned association/conditioned response (CR; Bąbel et al., 2017; Thomaidou et al., 2020). In this case, nocebo hyperalgesia is the CR, pain is the UCS, and whatever neutral stimulus (e.g., a tone or light) is repeatedly paired with the UCS is the CS producing the CR (nocebo hyperalgesia). As such, the increase in pain (nocebo hyperalgesia) is the conditioning factor taking place.

Research in both humans and rodents has demonstrated the significant role that CC has in nocebo hyperalgesia (Brascher et al., 2017; Herrnstein, 1962; Hayashi, et al., 1980; Nolan et al., 2012). In humans, Brascher and colleagues (2017) were able to induce a nocebo hyperalgesia effect without verbal suggestions, suggesting that CC has an independent role in nocebo hyperalgesia. For rodents, Nolan and colleagues were able to model an operant pain model in

rats (Nolan et al., 2012), and conditioned drug effects were found in mice (Hayashi, et al., 1980). There is no clear consensus as to exactly how CC produces nocebo hyperalgesia, however, a commonly circulated hypothesis is that being conditioned to a pain stimulus sensitizes the animal/human to said stimulus. This sensitization thereby allows for a hyperalgesic effect to take place and exacerbate the pain response due to the conditioning mechanisms and not the pain itself.

Nocebo Hyperalgesia and Anxiety

Emotions are a central component of the nocebo hyperalgesia effect, with anxiety being a primary emotion for this effect to occur. Anxiety's role in nocebo hyperalgesia is rather comprehensive. An anxious state is usually marked by emotional apprehension that is largely driven by fear, and this mental state physiologically arouses (i.e., muscle tension, increased stress) and behaviourally inhibits (avoidance behaviour) the organism experiencing the emotion. Additionally, anxiety can be felt/experienced towards a real event/thing (i.e., a predator), and can also be felt towards a learned and/or conditioned stimulus (e.g., being taught to fear snakes; fearing a whistle after it was repeatedly paired with an electric shock; Gray & McNaughton, 2003; Panksepp, 2004). Furthermore, anxiety can exacerbate one's perception of pain, and as research shows, many of the neural pathways identified for anxiety overlap with the neural pathways responsible for pain signaling and perception; suggesting that they can heavily influence each other (Ploghaus et al., 2001; Zhuo, 2016; Hunter & McEwen, 2013). In sum, anxiety can be defined as an apprehensive, avoidant, and fearful emotional state that can exaggerate an organism's perception of actual or anticipated negative events/things. In the context of pain research, many scholars would agree that pain fall's under the category of a negative event (Frisaldi et al., 2015; Raja et al., 2020). With this working definition in mind,

anxiety's role in nocebo hyperalgesia becomes much clearer. The experience of pain (a negative stimulus) can engender negative expectations about said stimuli, thereby, opening the door for a nocebo hyperalgesic response to take place as a result of the anxious state. In other words, if the pain stimulus results in an organism experiencing anxiety, this pain-anxiety sensitizes the organism to be receptive to a hyperalgesic response because they expect the stimulus to be similarly, if not more painful, should the stimulus reappear.

Neurochemistry of Nocebo Hyperalgesia/Placebo Analgesia

Placebo analgesia is modulated in part by the descending inhibitory pathways involving endogenous opioidergic and dopaminergic systems (Benedetti et al., 2005). In 1978, Levine and colleagues blocked placebo analgesia by administering the opioid antagonist naloxone in humans. Subsequent human studies have showcased that variability in the genes modulating opioid receptors/mechanisms is associated with an individual's placebo analgesic response (Hall et al., 2012; Peciña et al., 2013). Furthermore, Proglumide ($CCK_{A/B}$ antagonist) can potentiate placebo analgesia, as it enhances opiate analgesia (Benedetti et al, 1995; Benedetti, 1996; Benedetti et al., 1997; Katsuura & Ioth, 1985; Watkins et al., 1985). This finding has been replicated numerous times in both animals and humans (Benedetti et al., 1997; Martin, et al., 2019).

On the other hand, the neurochemical triad of nocebo hyperalgesia is centered around the function of dopamine, opioid and CCK systems (Benedetti, et al., 2005). In the case of placebo analgesia, the neuronal signaling of dopamine and opioid neuronal are increased, and CCK's neuronal signaling is decreased. The opposite takes place for nocebo hyperalgesia; where the activity of dopamine and opioid systems are reduced, and CCK activity is increased (Kleine-Borgmann & Ulrike Bingel, 2018). As stated above, CCK has particular importance when it

comes to researching nocebo hyperalgesia. Benedetti and colleagues (1997) demonstrated that nocebo hyperalgesia can be eliminated with CCK antagonists that target either A or B receptors, but the same could not be done with naloxone. Given that antagonizing the opioid system did not yield any effects on nocebo hyperalgesia, but exclusively inhibiting $CCK_{A/B}$ receptors completely eliminated nocebo hyperalgesia, it is probable that increased CCK firing engenders nocebo hyperalgesia. Thus, the study of nocebo hyperalgesia necessitates the understanding of how anxiety and CCK interact with each other and produce a negatory experience of pain.

CCK, Anxiety and Nocebo Hyperalgesia

CCK is an anxiogenic system and was first known to induce anxiety since 1969 (Rehfeld, 1992). Subsequent research has showcased that exogenously administering it to humans can result in panic attacks (Benedetti, et al., 1997; Powell & Barrett, 1991; DeMontigny, 1989; Abelson & Nesse, 1990). CCK receptors are broadly distributed throughout the brain and body and are divided into two specific categories based on their receptor subtypes: CCK_A (peripheral subtype) and CCK_B (central subtype). CCK_A receptors are mainly located in the gastrointestinal tract (GI-tract), with a small number of receptors found in discrete brain regions, such as the posterior hypothalamus, nucleus tractus solitarius, interpenduncular nucleus and area postrema (Hill et al., 1992). Hence, CCK_A is categorized as the peripheral subtype because the majority of CCK_A receptors are located outside the brain. Conversely, the primary location of CCK_B receptors are found in the brain, with the highest densities being in the cerebral cortex, limbic system, striatum, hypothalamus, ventral tegmentum, and dorsal raphe nuclei (Innis & Snyder, 1980; Honda et al., 1993). CCK_B receptors have also been located in peripheral organs, but not to the degree of CCK_A receptors, and as such, CCK_B remains as the central subtype (Wank et al., 1992). These distinctions are of high importance when studying CCK's effects on anxiety in both

humans and animals because their receptors are not equally distributed, and yet, both A/B receptors have been shown to reduce anxiety when antagonized (Kõks, 1999). Additionally, it is important to note that as of right now, no specific brain region with either A/B receptors has been identified to specifically reduce or increase anxiety. Therefore, any drug that targets CCK receptors is binding to all CCK receptors within the brain, and the results obtained from labs investigating CCK are more generic than they are specific.

While both A/B receptors are involved in the reduction and potentiation of anxiety, many scholars agree that CCK_B receptors have a more important role compared to CCK_A. Earlier studies have showcased that pharmacological interventions on either receptor are dose-dependent. This means that at certain doses, a CCK antagonist will reduce anxiety levels, and in other instances, it will not (Lang et al., 1995). More specifically, the CCK_B receptor antagonist L-365,260 (L-3 for short) has a bell-shaped curve in reducing anxiety, as administering more than 10µg/kg of L-3 does not further reduce anxiety (Chopin & Briley, 1993). Similar results have been demonstrated with the CCK_A receptor antagonist Devazepide (Hendrie et al., 1993). Additionally, rats injected with Caerulein (CCK agonist) had their neophobia dose-dependently reduced by both L-3 & devazepide (Kõks et al., 2000). However, a key finding of this study is that the CCK_B receptor subtype played a more important role in anxiety regulation than CCK_A. One dose of L-3 induced anxiolytic effects in the rats, which was not the case for Devazepide. Koks and colleagues' findings have been replicated, which further solidifies CCK_B's role in attenuated and/or increasing anxiety compared to CCK_A (Harro et al., 1995; Bradwejn et al., 1994; Andre et al., 2005).

The link between nocebo hyperalgesia and CCK is found in anxiety's role for nocebo hyperalgesia. To reiterate, anxiety is coupled with the anticipation of a negative event, and pain

can be perceived as a negative event in the majority of circumstances (Frisaldi et al., 2015; Raja et al., 2020). Hence, when one has a negative expectation of pain and is anxious about it, it opens the door for a nocebo hyperalgesic response to take place when the pain stimulus arrives. This increase in anxiety triggers the activation of CCK, which facilitates pain transmission (Colloca & Benedetti, 2007). Thus, the role of CCK, anxiety and nocebo hyperalgesia is rather clear; anxiety increases CCK signaling, which in turn increases the likelihood that an organism will feel a nocebo hyperalgesic effect from subsequent pain administration. As such, any drugs that potentiate or attenuate CCK signaling will affect how much nocebo hyperalgesia an organism will experience (Andre et al., 2005; Hebb et al., Roach 2005; Lydiard, 1994).

Sex Differences in Nocebo Conditioning

A notable finding within nocebo research is that nocebo conditioned hyperalgesia affects males and females differently. It has been shown that administering pain (UCS) to mice on two consecutive days, within the same context (CS) resulted in them being more sensitive to pain (CR) on the second day when compared to the first day. This conditioned-hyperalgesic effect was completely absent in female mice and was only observed in male mice (Martin, et al., 2019). To further investigate this sex difference, Martin, and colleagues (2019) ran the same pain assays on castrated male mice and ovariectomized females that were exogenously administered testosterone. They concluded that testosterone was a relevant steroid hormone when it comes to nocebo hyperalgesia; as castrated mice did not demonstrate hyperalgesia, but ovariectomized females with exogenous testosterone did (Martin et al., 2019). A thing to consider when evaluating this data is that there is no reason to presuppose that nocebo hyperalgesia would exclusively occur in males. Typically, sex-specific effects of fear-conditioned responses are

driven by estrogen, therefore, if there were any expectations of a sex difference, it would be more likely for it to appear in females than in males (Merz et al., 2018).

The Present Investigation

The present research examined any potential role CCK_B might have in nocebo hyperalgesia. Only a handful of studies examined how CCK_B affects anxiety, without looking into how it might affect nocebo hyperalgesia specifically (Kõks et al., 2000; Harro et al., 1995; Bradwejn et al., 1994; Andre et al., 2005). For our experiment, we were working with a novel CCK_B antagonist, LY-225910 (LY for short), and comparing it to the $CCK_{A/B}$ antagonist, Proglumide, with Saline acting as our control group. The rationale behind using a CCK_B antagonist is to establish receptor-specificity. If Proglumide can cancel out nocebo hyperalgesia by antagonizing A/B receptors, then it is imperative to target specific receptors to narrow down which receptors might be responsible for conditioned hyperalgesia, and its elimination if antagonized.

Mice of both sexes were tested for thermal pain sensitivity on Day 1, and then once the testing would be completed, they would be injected with acetic acid and returned to the cylinder for 30 minutes. The following day, they would either be placed in the same or new context for another day of thermal pain administration and they would also be injected with either Saline, Proglumide or LY. Conditioning would effectively be determined on the second day, and by assessing how their WT would differ within the same context on the second day. Every group would be administered the same pain assays, drugs and have an equal amount of female and male mice, making the differing context the only variable that is distinct between all groups. We hypothesized that a nocebo conditioned hyperalgesia effect would take place exclusively in male mice placed in the same context and that this effect would be counteracted by both Proglumide

and LY. No expectations were made about whether there would be a large difference between Proglumide and LY.

Methods

Animal Subjects

Inbred mice were tested upon, as their genetic composition is more homogeneous compared to their wild-type counterparts. Having genetically homogeneous mice is preferable to genetically heterogeneous ones, as we are observing how pharmacological agents impact our mice, and thus, the reduction of variability that could be caused by differing genes is paramount (Mogil et al., 1996; Mogil, 1999; Mogil, 2012). Both female and male mice were tested upon equally.

Conditioning Paradigm

The conditioning paradigm in our study is identical to the one used in the 2019 study conducted by Martin and colleagues. It goes as follows: mice of both sexes are tested for thermal pain sensitivity on Day 1 within a transparent cubicle. After a sufficient amount of thermal pain has been collected, the mice are intraperitoneally injected with acetic acid and then returned to the cubicle for 30 minutes. Acetic acid acts as the conditioning stimulus due to the tonic pain that mice experience from the acetic acid (Koster et al., 1952). The following day (Day 2), they are either returned to the same cubicle in the same room (same context), or in a cylinder placed in a different room (new context) and tested again for thermal pain sensitivity. Half of the animals were tested first in a cubicle and then in a cylinder in a different room.

Pain Administration

Thermal pain is being administered by a Hargreaves testing assay. This pain test shines a light under the hind paws of the mice and a withdrawal reflex indicates that they are

experiencing pain. Only after they have habituated to the cylinders/cubicles on both days do we administer thermal pain. Mice that have not habituated to their environments were not tested upon, including if they habituated on the first day but did not on the second, or if they dishabituated during the Hargreaves test.

(It is important to note that the Hargreaves test is not the UCS in this instance, acetic acid is. The Hargreaves test simply measures their pain sensitivity (UCR) before and after being injected with acetic acid (UCS). The difference in withdrawal times observed on the second day will indicate if they have been conditioned to the same context (CR).)

Measuring Pain

Pain is being measured through a withdrawal reflex on each hind paw. Essentially, once the mice withdraw their paw from the light/heat source their withdrawal time (WT) is noted down, and we move on to the next mouse and repeat the process for the entire row of mice. Both left and right hind paws were tested equally, and at the very minimum, a minute would have to pass by between a withdrawal reflex in order to alternate between left and right hind paws. Each hind paw was tested six times for their scores to be added into our dataset. Once completed, a difference score is calculated at the end of Day 2 (Difference = Day 2 – Day 1).

Pharmacological Agents

The drugs that were used in this study were two CCK antagonists, and a Saline solution acting as a control. The CCK antagonists were Proglumide ($CCK_{A/B}$ antagonist) and a new CCK_B antagonist, LY-225910 (LY). Injections were administered intraperitoneally on Day 2, after they had already habituated to the same/new context. Once injected with either Proglumide, LY, or Saline, they were left to habituate for another 50 minutes before commencing the Hargreaves test.

Statistical Analyses

The nature of this experimental design involves both a between- and a within-subjects design. Our statistics were analyzed with repeated-measures ANOVAs, following the confirmation of normality and sphericity, post-hoc analyses were based on the Bonferroni test. Our independent variables were Sex, Drugs and Context, with the Days acting as the repeated measure. For the Day variable, either the difference score or simply analyzing the WT on either day was marked as the dependent variable. Data are presented as mean ± SD, with the level significance being $p \leq .05$.

Results

Difference between Day 1 and Day 2

In the Saline group, we had an N = 126 mice, with the sexes being split fairly equally (61 males (M for short) and 65 females (F for short), and N = 56 in the New Context (26F/30M) and N = 70 in the Same Context (35F/35M). The within-subject differences in WT between Day 1 and Day 2 were significant regardless of context or sex ($F_{(1, 122)} = 18.962$, $p < .001$). WT on Day 2 is much lower when contrasted to Day 1, indicating that the mice are experiencing heightened pain sensitivity.

Contextual Conditioning

The same saline sample was analyzed here, in which WT was being compared in the different contexts. The between-subjects effects were significant ($F_{(1, 122)} = 5.912$, $p = .016$), signaling that there is a difference in WT simply based on the context (New Context vs Same Context). As for the within-subjects differences, our results signal that the Day and Context (Day*Context) interact with each other significantly ($F_{(1, 122)} = 11.033$, $p = .001$). When

accounting for both days and same/different contexts, there is a significantly different WT, suggesting a significant day-by-context interaction effect is taking place.

Sex Differences in Withdrawal Time & Conditioned Hyperalgesia

When measuring sex differences, we looked at the saline group's WT to see what the differences might be. The between-subjects effects were nonsignificant ($F_{(1, 122)} = 1.763$, $p = .187$), meaning that both sexes have comparable WT. Our within-subjects differences were not statistically significant, signaling that there is no Day*Sex interaction taking place ($F_{(1, 122)} = 1.546$, $p = .216$). These results showcase that both sexes react equally to the thermal pain assay with their WT being faster on the second day, but no significant differences are to be found between the sexes.

Additionally, we investigated for a potential Sex*Context interaction effect and found none, as our results were nonsignificant ($F_{(1, 122)} = .864$, $p = .354$). This means that only one sex is being affected by the context and not the other. Additionally, when taking into account the day with the sex and context (Day*Context*Sex), a very significant difference was observed ($F_{(1, 122)} = 9.681$, $p = .002$). A three-way interaction effect is found here: WT decreases between Day 1 and Day 2, with differing contexts and both sexes.

Diving deeper into the sex differences, we split the Saline group into separate male and female categories and looked into how WT differed within each sex and if the different contexts affected WT. Our female group had an N = 61, with 26 in the New Context and 35 in the Same Context. When evaluating the within-subjects comparison looking at the differences in WT from Day 1 and Day 2, a highly significant result was yielded ($F_{(1, 59)} = 13.141$, $p = .001$). When adding context as another factor (Day*Context) in the within-subjects analysis, there is no interaction effect to be found ($F_{(1, 59)} = .019$, $p = .892$). The former results demonstrate that in

either context, a significant difference in WT is found between Day 1 and Day 2. As for the latter Day*Context analysis, no interaction effect is found as well, meaning that WT differences found in female mice do not differ because of them being in a New or Same Context, different WTs are only occurring on different days. Looking in between the New vs Same Context groups, a significant difference was found ($F_{(1, 59)} = 5.747$, $p = .020$), indicating that WT between Day 1 and Day 2 is significant, regardless of the context.

Our male group had an N = 65, with 30 in the New Context and 35 in the Same Context. Between contexts comparisons did not net us statistically significant differences between the two groups, ($F_{(1,63)} = 1.117$, $p = .295$). This tells us that there is a different WT occurring between the two contexts due to an asymmetry in WT between both contexts. As for evaluating WT within-subjects, we can see that significant results were yielded when looking at the differences in Day 1 and Day 2 ($F_{(1,63)} = 5.843$, $p = .019$), along with a significant Day*Context interaction effect ($F_{(1,63)} = 24.982$, $p = < .001$). These results differ from the female ones, suggesting that there is an interaction effect with the sex, and context taking place. Furthermore, for the males, the New Context has a longer WT than the Same Context, suggesting that nocebo conditioned hyperalgesia is taking place.

Drug Differences

The two drugs we will be comparing to Saline are Proglumide ($CCK_{A/B}$ antagonist) and LY (CCK_B antagonist), and the variables to be evaluated are the whole sample and different contexts. Starting with evaluating our whole sample N = 372 (188F, 184M; 171 New Context (88F, 83M), 201 Same Context (100F, 101M)), no Sex*Drug interaction effect was observed, but a significant Context*Drug interaction effect took place ($F_{(2,360)} = .297$, $p = .743$; $F_{(2,360)} = 4.812$, $p = .009$). A nonsignificant sex-by-drug interaction, but a significant context-by-drug

interaction effect suggests that antagonizing CCK does not greatly impact either males or females (analyzing for both contexts), but it does eliminate any conditioned hyperalgesia arising from the Same Context.

In the New Context, our total sample size is N = 171 (88F, 83M; 57 LY, 58 Proglumide, 56 Saline) and we have a significant result for a difference between Sex WT ($F_{(1,165)} = 8.814$, $p = .003$), with nonsignificant difference Drug WT ($F_{(2,165)} = .906$, $p = .406$), or Sex*Drug ($F_{(2,165)} = 1.091$, $p = .338$), indicating no interaction effect. The Bonferroni post-hoc test displayed nonsignificant differences between LY ($M = 1.47$, $SD = 6.8$,), Proglumide ($M = 1.87$, $SD = 5.8$) or Saline ($M = .29$, $SD = 4.83$), with all the p-values > .436, indicating that there were no differences between any of the drugs in the New Context condition.

For the Same Context, our sample size was a bit larger with N = 201 (100F, 101M; 60 LY, 71 Proglumide, 71 Saline), and our results differ from the New Context. No differences were found between the sexes ($F_{(1,195)} = .000$, $p = .995$), contrasted with a significant differences appearing in our drug variables ($F_{(2,195)} = 5.657$, $p = .004$), with no Sex*Drug interaction effect found ($F_{(2,195)} = 1.332$, $p = .266$). The Bonferroni post-hoc test demonstrates significant differences between LY ($M = .60$, $SD = 5.12$), and Saline ($M = 3.10$, $SD = 4.49$), $p = .015$, and Proglumide ($M = .61$, $SD = 5.38$) and Saline ($M = 3.10$, $SD = 4.49$), $p = .011$. This means that in the Same Context, WT on Day 2 was significantly lower in the Saline group when compared to the LY or Proglumide groups.

Sex & Drug Differences

For the females injected with LY (N = 62; 33 New Context, 29 Same Context), significant WT difference scores were observed between Day 1 and Day 2 ($F_{(1,60)} = 4.852$, $p = .031$), but no significant Day*Context interaction occurred ($F_{(1,60)} = 1.748$, $p = .191$).

Additionally, no statistically significant between-subjects effect was found ($F_{(1,60)} = 1.043$, $p = .311$). The Proglumide group (N = 65; 29 New Context, 36 Same Context) mirrored the LY group's results, with WT differences between Day 1 and Day 2 being significant ($F_{(1,63)} = 5.541$, $p = .022$) and no Day*Context interaction taking place ($F_{(1,63)} = .520$, $p = .473$). A significant difference was revealed in the between-groups analysis ($F_{(1,63)} = 7.317$, $p = .009$ (Figures 1 & 2)).

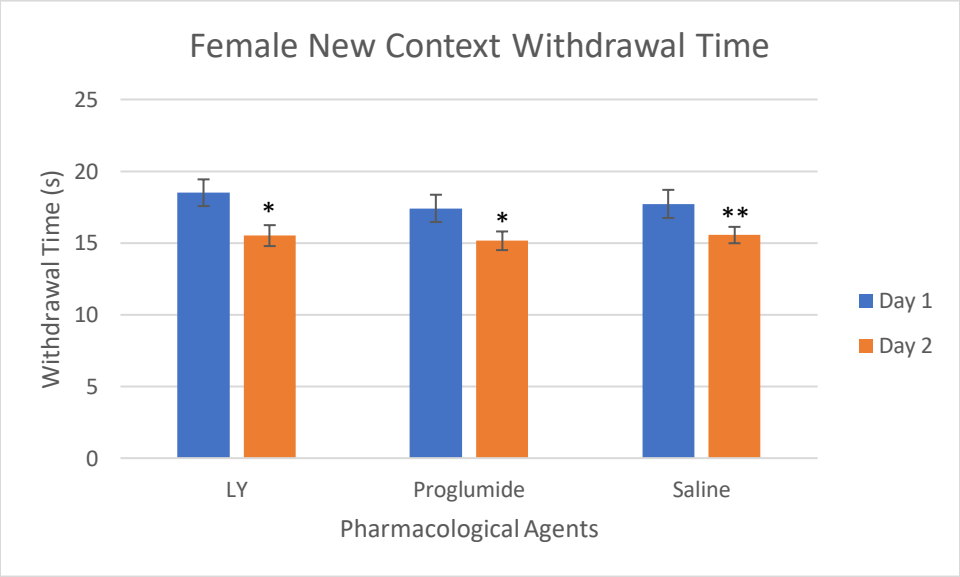

Figure 1. Withdrawal time in the Female New Context group, with Day 1 represented as the Blue Bars, and Day 2 displayed as the Orange Bars. Each WT difference score obtained is significant, with one asterisk (*) being $p < .05$, and two (**) as $p < .01$. The average Day 1 WT for LY, Proglumide and Saline are as follows: LY, $M = 18.5$, $SE = .93$; Proglumide, $M = 17.41$, $SE = .95$; Saline, $M = 17.72$, $SE = .98$. Average Day 2 WT are as such: LY, $M = 15.5$, $SE = .72$; Proglumide, $M = 15.15$, $SE = .65$; Saline, $M = 15.55$, $SE = .57$.

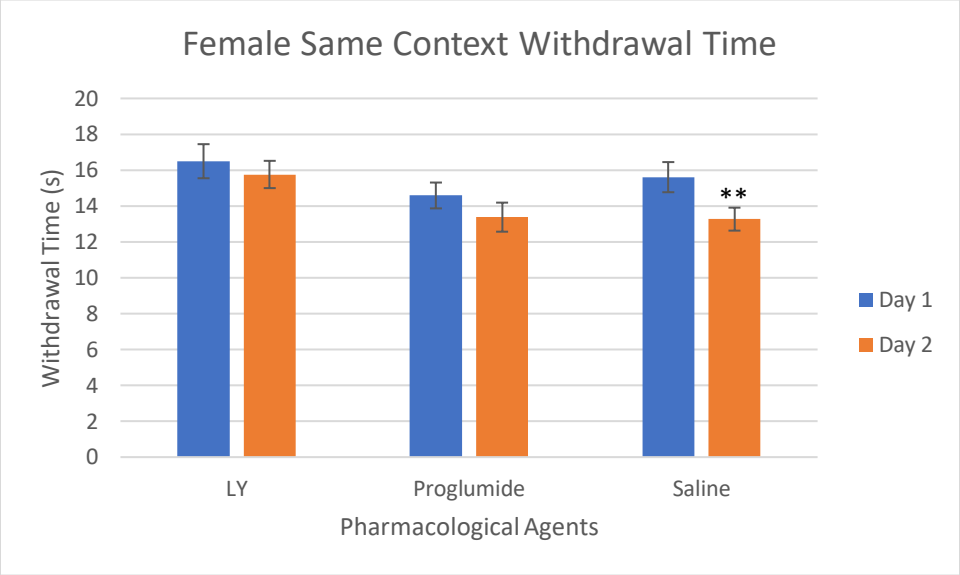

Figure 2. Withdrawal time in the Female Same Context group, with Day 1 represented as the Blue Bars, and Day 2 displayed as the Orange Bars. Only the Saline group yielded a significant WT difference score, with two asterisks' (**) being $p < .01$. The average Day 1 WT for LY, Proglumide and Saline are as follows: LY, $M = 16.5$, $SE = .95$; Proglumide, $M = 14.59$, $SE = .72$; Saline, $M = 15.61$, $SE = .84$. Day 2's averages are observed as: LY, $M = 15.76$, $SE = .76$; Proglumide, $M = 13.38$, $SE = .81$; Saline, $M = 13.27$, $SE = .64$.

Conversely, for the male LY group (N = 55; 24 New Context, 31 Same Context), a nonsignificant within-subjects day difference was found ($F_{(1,53)} = .011$, $p = .918$) with a nonsignificant Day*Context interaction ($F_{(1,53)} = .612$, $p = .438$). As for evaluating between contexts, a highly significant score was yielded ($F_{(1,53)} = 12.522$, $p = .001$). In regards to the Proglumide sample (N = 64; 29 New Context, 35 Same Context), the WT differences observed in between Day 1 and Day 2 were not statistically significant ($F_{(1,62)} = 1.212$, $p = .275$) and the Day*Context interaction was also not statistically significant, ($F_{(1,62)} = 1.233$, $p = .271$).

Furthermore, the between context statistics were also nonsignificant, ($F_{(1,62)} = .330$, $p = .568$ (Figures 3 & 4)).

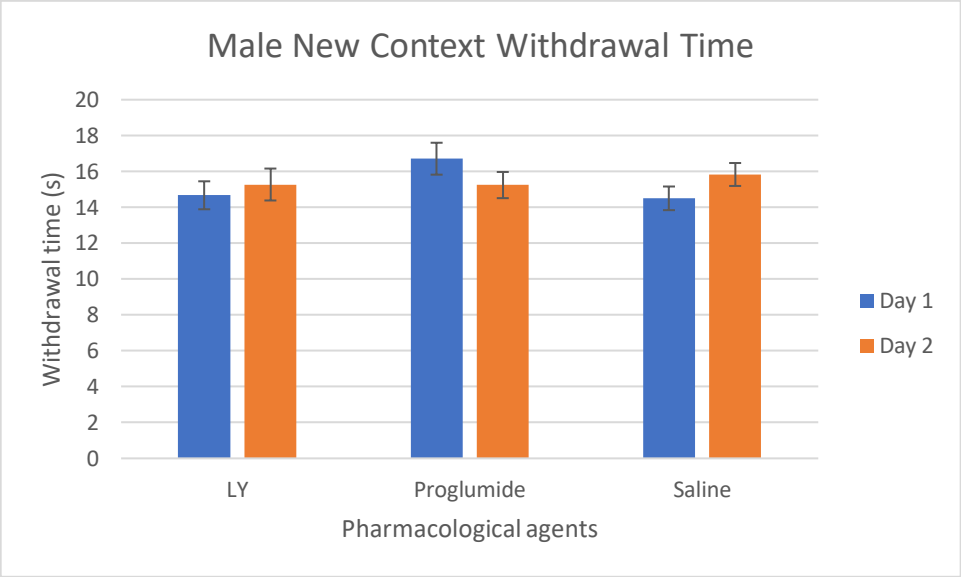

Figure 3. Withdrawal time in the Male New Context groups, with Day 1 represented as the Blue Bars, and Day 2 displayed as the Orange Bars. No significant differences were found between or within the groups. Average Day 1 WT for LY, Proglumide and Saline go as such: LY, *M* = 14.67, *SE* = .78; Proglumide, *M* = 16.71, *SE* = .89; Saline, *M* =14.49 , *SE* = .66. Averages for Day 2: LY, *M* = 15.27, *SE* = .89; Proglumide, *M* = 15.24, *SE* = .73; Saline, *M* = 15.83, *SE* = .64.

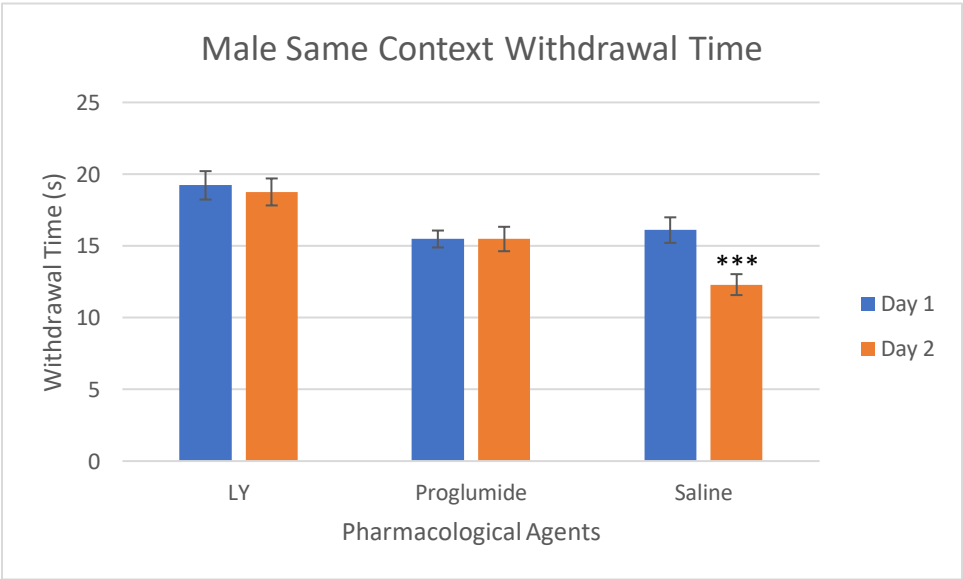

Figure 4. Withdrawal time in the Male Same Context groups, with Day 1 represented as the Blue Bars, and Day 2 displayed as the Orange Bars. Significant differences were exclusive to the Saline group, with three asterisks' (***) equaling to $p = < .001$. Day 1 average WT for LY, Proglumide and Saline are: LY, $M = 19.22$, $SE = .99$; Proglumide, $M = 15.48$, $SE = .59$; Saline, $M = 16.1$, $SE = .89$. Day 2's averages are as follows: LY, $M = 18.76$, $SE = .94$; Proglumide, $M = 15.48$, $SE = .85$; Saline, $M = 12.26$, $SE = .73$.

Discussion

This study investigated the nocebo conditioned hyperalgesia effect and how it is mediated by antagonizing CCK_B receptors. Our experimental design replicated the results of several studies. The first replication was the observed sex differences in nocebo conditioning. Martin and colleagues' 2019 investigation on nocebo hyperalgesia demonstrated that conditioned hyperalgesia only manifests itself in male mice. While, not going nearly in-depth into the hormonal and cellular mechanisms behind this male-exclusive phenomenon, the sex differences we obtained from our investigation closely mirror that of Martin and others' study. Our second replication was the numerous studies that investigated CCK's role in nocebo conditioning (i.e., research conducted by Benedetti, Koks, Colagiuri, Colloca, etc). Both Proglumide and LY blocked nocebo hyperalgesia. While not statistically significant, Proglumide did essentially eliminate any nocebo conditioning that male mice placed in the Same Context would have experienced, as their WT on Day 2 was nearly identical to the Different Context group and the same on Day 2 as it was on Day 1 in the Same Context. Moreover, Proglumide has also been proven to block conditioned hyperalgesia in many other studies, therefore, we do not have any reason to be concerned about our non-statistically significant results (Benedetti et al., 1997; Martin, et al., 2019). In short, our results confirm both of our hypotheses about a sex difference

appearing in the conditioned group, and that antagonizing CCK_B receptors would counteract nocebo conditioned hyperalgesia.

Our confidence in our obtained results stems from our rigorous methodology. Firstly, we have a very large sample size to work with (N = 372), with a near 50/50 split in the sexes, different contexts and drug groups. A large sample size that is this evenly split among each group brings in large datasets, that afford us large statistical power in our analyses. Secondly, because we compared LY to another CCK antagonist, and have Saline acting as our control, we have reasonable certainty about LY's effects in nocebo conditioning. By establishing a neutral base without any pharmacological intervention in our Saline group, we can compare and contrast the differences when we do administer our drugs. Furthermore, Proglumide acts as a secondary comparison to LY, since we know from previous research that Proglumide has successfully blocked nocebo hyperalgesia, we can see how large the differences are in this blockade when comparing Proglumide and LY with each other. As mentioned before, the males placed in the Same Context that were injected with LY on 2nd Day had their nocebo hyperalgesia completely eliminated, further solidifying the role of CCK_B in nocebo conditioning.

Digging deeper into our results, the Pavlovian mechanisms behind nocebo hyperalgesia become clearer. Beginning by simply assessing whether or not there was a difference in WT between Day 1 and Day 2 in our Saline group, a significant difference was found in both sexes and contexts, highlighting a three-way Day*Sex*Context interaction effect. This indicates that when it comes to WT, a difference score in WT between Day 1 and Day 2 is significant, WT is not distributed equally among the sexes, and the context plays a role in WT based on the sex and day. While a simple, yet robust finding, it eliminates a potential point variance that might occur had any of the groups had a non-significant difference in WT (i.e., if female mice in the New

Context did not have a faster WT, we would have to factor that in our analysis when applying our experimental manipulations). Knowing that all three variables of time, sex and context interact with each other, allows us to further investigate other points of analyses, such as sex differences, pain-conditioning and how well do CCK antagonists block conditioned hyperalgesia. These results are on par with previous research, further reinforcing the validity of our findings (Benedetti et al., 1997; Bąbel et al., 2017; Thomaidou et al., 2020).

When investigating the sex differences for the Saline group, we find that their differences lie in their receptivity to being conditioned by the contexts. Both females and males had lower WT on Day 2, but their WT is not affected when tested in either New or Same Context, which is not the case for the males. Therefore, only a day-by-sex interaction effect is present in the females. On other hand, males had lower WT on Day 2 and the Same Context, with the New Context essentially maintaining their WT on Day 2. Not only is this a marked sex difference between the two groups, but these results clearly demonstrate a significant three-way Sex*Context*Day interaction effect for the male mice. Given that we established all mice in both contexts had lower WTs on Day 2, the next level of analysis would be determining if there is a conditioning effect taking place within our sample. Indeed, our results suggest that the context is driving the faster WT in the male mice for two reasons, 1) it is not found in either context for our female mice, and 2) male mice only have lower WT in the Same Context. Through these results, we can deduce that the Same Context creates a conditioned hyperalgesic effect because it is absent in all other conditions. Again, this replicates the results found by Martin and colleagues (2019), where nocebo conditioning was exclusive to males. This suggests that there might be biologically encoded differences in pain conditioning between males and females. Further

investigation would need to be conducted in order to determine why this would be the case, as this finding is rather novel, and its mechanisms are unclear.

As for whether our pharmacological agents were able to block these conditioned effects, we obtained significant results for LY and Proglumide's blockade of nocebo conditioned hyperalgesia. Our results showcased that there is no Sex*Drug interaction effect, but a significant Context*Drug interaction effect; signaling that in females there is no heightened anxiety to antagonize and that there is no anxiety to block within the New Context; LY and Proglumide both block the anxiety caused by the conditioned pain occurring within the Same Context, in the male mice. This suggests that CCK_B has a more prominent role in eliminating nocebo hyperalgesia than $CCK_{A/B}$ antagonists because leaving CCK_A receptors untouched did not interfere with LY's anxiolytic effects. Given that the role of anxiety was heavily emphasized upon in our research, it would be fair to assume that benzodiazepines/GABA could have a similar role in eliminating nocebo hyperalgesia. However, previous research by Benedetti and colleagues (2006) noted how CCK's anxiogenic effects differ from those of benzodiazepines. When conducting their investigation, they noticed how both drugs were able to eliminate nocebo hyperalgesia but Proglumide did so without lowering autonomic arousal as Diazepam did. As such, they, and other researchers have concluded that anxiety's role in nocebo hyperalgesia is mediated by CCK and not GABA, and our results do indeed back this claim (Benedetti et al., 2007; Benedetti et al., 2006; Andre et al., 2005).

Splitting males and females into separate groups, and evaluating how both Proglumide and LY interact with both contexts yielded interesting sex differences. Starting in the female Proglumide group, it did not effect either Context, but it did have a significant day result, but this is more likely to be the case as all mice had lower WTs on Day 2 versus Day 1. Additionally,

significant between-group differences were found, further solidifying the sex differences found between males and females. Because the females were not affected by either context, they therefore, do not experience nocebo hyperalgesia, making their CCK levels unaffected by the pain stimulus because they are not experiencing any heightened levels of anxiety. Therefore, antagonizing their $CCK_{A/B}$ receptors yielded no effects, as there is nothing to block/antagonize. On the contrary, the male Proglumide group was affected by the drug. While the results are not statistically significant, the ones in the Same Context had no difference in the WT between Day 1 and Day 2, whereas the ones in the New Context had a lowered WT on Day 2. These results suggest that Proglumide does in fact cancel out conditioned hyperalgesia, as previous research on Proglumide would back this claim, on top of our Same Context group having an equivalent Day 1 and Day 2 score of 15.48s WT on both respective days (Benedetti et al., 1997; Martin, et al., 2019). This allows us to speculate that Proglumide is cancelling out nocebo hyperalgesia, it just happens to be the case that this group in particular has a smaller distribution in WT between the days, making them statistically significant. In regards to the LY group, the results sport a similar trend to the Proglumide group, but the differences between the contexts are grander. These differences tell us that the CCK_B receptors might have a larger role in blocking nocebo conditioned hyperalgesia, since more robust effects were found without antagonizing the CCK_A receptors. Not only can we conclude with reasonable amounts of certainty that LY can successfully eliminate nocebo hyperalgesia, but we can also say that the CCK_B receptor is likely to be the receptor that mediates the nocebo-anxiety link.

Bringing all this together, our findings demonstrate that nocebo conditioned hyperalgesia occurs in male mice, and can be blocked by CCK_B antagonists. The present study contributes to the existing literature on nocebo conditioned hyperalgesia, while simultaneously expanding on

the neurochemistry driving this effect. Subsequent studies would want to narrow down how exactly CCK_B receptors interact with conditioned hyperalgesia, and also collect blood samples after testing to assess corticosterone levels. Knowing how much corticosterone is circulating in the blood of mice will allow researchers to have a better understanding of how much stress the animals are actually experiencing when tested upon, and how CCK_B potentially mediates this stress. Exclusively manipulating CCK_B receptors does not allow any researcher to conclude with certainty about the stress the animal experiences; conclusions can only be made about what happens if there is an excess or absence of CCK_B signaling. Similarly, LY is a very generic drug that affects all the brain regions that have CCK_B receptors, which means that so far, we can conclude that they have a role in blocking nocebo hyperalgesia, but we do not know if a particular brain region is more responsible for this blockade than another. As such, to further inquire about how CCK_B affects nocebo conditioning, future research would have to either use CCK_B knockout mice or use the DREADD system. The research that would either design a drug that targets specific CCK_B receptors or tests on mice totally devoid of CCK_B receptors, would better establish CCK_B's role in anxiety and nocebo hyperalgesia, as these neuroscientific techniques are much more specific than general pharmacological agents.

Limitations

Our study had several limitations, in spite of its strengths. The first one is that we never investigated why nocebo hyperalgesia was only present in males. It was hypothesized by the research done by Martin and colleagues (2019), that nocebo hyperalgesia appeared in males because of testosterone and aPKC. While we do similarly replicate their results, we cannot infer with much confidence as to why this effect is male-specific without further investigating into the blood samples of our mice. Second, only thermal pain administration was tested, making our

results limited to thermal pain and thus, not generalizable to mechanical pain assays such as Von Frey (or other forms of pain). Third, we cannot overstate the effects of CCK on blocking nocebo hyperalgesia. For us to get a better understanding of CCK's role in anxiety and hyperalgesia, blood samples would have to be taken before and after testing to measure their corticosterone levels. This would bring in a more insightful perspective on how CCK interacts with anxiety. Fourth, LY and Proglumide are very generic drugs that target all $CCK_{A/B}$/CCK_B receptors, and thus, our results lack specificity. We can conclude that both LY and Proglumide eliminated conditioned hyperalgesia, but our study design does not demonstrate which brain region(s) are being targeted by either drug. Fifth, our experiment only utilized one conditioning trial to evaluate whether or not our mice became conditioned to the same context. It is possible that one conditioning trial is too little for nocebo hyperalgesia to occur in females, and that males are simply more sensitive to conditioning effects, and not necessarily nocebo hyperalgesia alone.

Future investigations into CCK/CCK_B antagonists and nocebo hyperalgesia will have to take these variables into account by collecting blood samples, varying pain assays, and potentially usinh CCK-DREADD/$CCK_{A/B}$ knockout mice to inquire about CCK's specificity within the mouse brain.

Conclusion

In conclusion, our results show strong support for the neurochemistry of the nocebo hyperalgesia effect being mediated by CCK_B receptors. A difference in withdrawal time (WT) in the Saline group was found between Day 1 and Day 2 for both sexes, revealing to us that WT was lower on the second day. When looking into the sex differences between WT, we observed a sex difference being driven by contexts. Both females and males had lower WT on Day 2, but males in the Same Context had significantly lower WT than their male New Context

counterparts. However, this was not the case in either context for the female groups. This clearly indicates a significant day-by-context interaction effect for the male mice and given that the context is driving their WT time, we can conclude that there is a hyperalgesic effect taking place for the males. Both LY and Proglumide were able to block the conditioned nocebo hyperalgesia effect occurring within the males placed in the Same Context.

These results demonstrate that nocebo hyperalgesia occurs exclusively in male mice, and that it is likely to be mediated by CCK_B receptors. To further our understanding of CCK_B's role in nocebo hyperalgesia, subsequent studies would need to invest in alternative methods to target CCK_B receptors in specific brain regions. As of right now, our current understanding of how CCK_B receptors function is largely generic and understanding which CCK_B receptors are most active in which brain region(s) when an animal experiences nocebo hyperalgesia would be the next step for future research. This can be done by either designing a drug that targets those receptors using the DREADD system or by creating a CCK_B knockout mouse to assess whether the absence of anxiety created by CCK gives rise to nocebo hyperalgesia.

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Appendix A

Statement of Contribution

The current project was part of a larger study ran within the MogiLAB and makes up a section of Dr. Skvortsova's Post-Doctorate studies. This project aimed to further explore the novel nocebo-conditioned hyperalgesia effect, by not only attempting to replicate the results of previous studies, but as well as expanding upon the currently scant literature surrounding this phenomenon.

During the whole process, I have received extensive help and guidance from my supervisor, Dr. Jeffrey Mogil, his post-doctorate student, Dr. Aleksandrina Skvortsova as well as working alongside a fellow undergraduate student, Simon Carrier. Throughout the project, I was helping with the data analysis, staying abreast with the literature, as well as conducting ethically permissible pain experiments on the laboratory's mice. The paper was written up entirely by me, and was subsequently reviewed and edited by Dr. Mogil and Dr. Skvortsova before it was submitted. Outside of my research paper, I was involved in several meetings with Dr. Mogil's lab to discuss the findings of our project and how to further improve our study as we collected data. Furthermore, I received ethical certification and training from McGill's Animal Resource Center, CMARC (Comparative Medicine and Animal Resources Centre) to be able to work with the mice for this study.