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Effect of creatine on nociception in a mouse model of inflammatory pain

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ABSTRACT

The objective of this study was to evaluate creatine as an anti-nociceptive compound in an animal model of thermal and inflammatory pain. Creatine has the structural potential to interact with acid-sensing ion channel 3 (ASIC3), which have been involved in pain sensation modulation. Our hypothesis was that creatine will interact with ASIC3 leading to decreased nociception. Male and female C57BL/6J mice were supplemented with creatine (6.25g/kg diet) and tested for thermal hyperalgesia and inflammatory pain response. The latency to withdraw the tail during the thermal hyperalgesia test was unaffected by sex or diet. For the formalin test, males and females responded differently to the stimulus, and the female mice supplemented with creatine seemed to recover faster than the controls. These preliminary data suggest a potential effect of creatine and sex on inflammation-based nociception and can be used as a stepping stone for the development of ASIC-based therapeutics.

EFFECT OF CREATINE ON NOCICEPTION

IN A MOUSE MODEL OF INFLAMMATORY PAIN

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

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in Partial Fulfillment of the Requirements

For the Degree of

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By

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CHAPTER I

INTRODUCTION

Pain is a condition that afflicts millions of people every year and although there are many treatments available, they come with side effects that can be difficult to manage. There is a need to find novel compounds that may be able to reduce pain sensitivity with lesser side effects. The goal of this practicum was to assess the potential of dietary creatine to reduce nociceptive sensitivity in a mouse model of inflammatory pain. Creatine has been shown to interact with neuronal channels involved in pain sensation, positioning it as a good candidate for nociceptive modulation. In the laboratory of Dr. Nathalie Sumien, nociceptive assays were utilized to test the changes in pain perception of mice in response to an inflammatory stimulus, when they were either fed a control or the control diet supplemented with creatine. This study will provide a stepping stone for further studies exploring the role of creatine in pain management.

CHAPTER II

INTERNSHIP SUBJECT

Background and Literature Review

Pain

In the United States, 116 million adults (1) suffer from conditions that cause acute or chronic pain. According to the Institute of Medicine of the National Academy of Sciences, $635 billion are lost every year in the treatment of these conditions and as lost wages due to absent workers with debilitating pain (1,2). Pain is a useful tool for the body as it alerts the brain of any tissue damage that has occurred, which can be repaired by the immune system. Experiencing a painful sensation consists of two components: the sensory component called nociception and perceptive component called pain. Perceiving pain occurs in the brain as a response to the stimulation of nociceptive neurons (3). Nociceptive neurons in the periphery of the nervous system express nociceptors which can sense potentially noxious (painful) stimuli. Nociceptive neurons are afferent neurons that fire action potentials when their nociceptors are activated in response to thermal, chemical or mechanical stimuli (4) that can threaten the body. The cell bodies of the afferent neurons are found in the dorsal horn of the spinal cord, where the sensory input of the skin, muscles and organs is collected and transmitted to supraspinal structures of the brain (thalamus, amygdala, anterior cingulate cortex and rostral ventral medulla) where noxious stimuli are processed (5).

While pain perception is important, pain signaling can become independent of physical sensory input leading to an increased perception that does not match the actual stimulus presented. This phenomenon causes nociceptive signaling to produce an inflated response to

either noxious or innocuous stimuli causing hyperalgesia (elevated pain sensitivity to painful stimulus) or allodynia (elevated pain sensitivity to a normally non-painful stimulus) (4,6).

Nociceptor signaling depends on the ability of a neuron to produce an action potential in response to noxious stimuli. The action potential initiation relies on the activation of ion channels which are expressed in the plasma membrane of nociceptors and hence have a direct effect on the level of pain perceived (4). One type of channels present in the neuronal membrane are acid-sensing ion channels (ASICs), which have roles in modulating pain sensation, mechanosensation and synaptic plasticity (5,7)

Acid-sensing ion channels and pain

Acid-sensing ion channels (ASICs) are trimeric, membrane-bound channels that belong to the epithelial sodium channel/degenerin family (7). There are 6 known ASIC isoforms: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 (8,9). Channels may be homotrimeric or heterotrimeric and depending on the subunits present in the complex, the channel properties may differ (8). ASICs are voltage independent and are activated by a decrease in the pH of the extracellular environment (10). ASIC activation causes a depolarizing current due to the high permeability of the channel to Na^{+} (8,11). Additionally, some ASICs containing a 1a subunit have permeability to Ca^{+2}, possibly conferring them signaling properties (7,8,12). ASIC currents are active briefly in response to changes in pH and their kinetics vary depending on the subtype and channel subunits present (11). ASIC3 channels are particularly sensitive to minor pH fluctuations, as even slight changes from physiological pH can cause the channel to activate (8,11), and will be the focus of this study.

ASICs show a biphasic current when activated: a peak current (transient) followed by a quick desensitization period (7). ASIC3 show a sustained current that is resistant to

desensitization as long as the environment remains acidic and have a markedly slow desensitization current (window current) as pH reaches physiological levels (5). Window currents arise when inactivation of ASIC3 is incomplete due to slight extracellular acidosis (pH 6.5 at most) (4). Sustained currents have been shown to exist in both homotrimers as well as in heterotimeric ASICs to adjust for channel function during prolonged pH changes (7).

In recent studies, ligands other than protons have been shown to modulate the activation of ASICs. The drug amiloride demonstrated its capability to block ASICs in a non-selective manner (13)**.** GMQ (2-guanidine-4-methylquinazoline), a small molecule with a guanidium group, activated ASIC3 currents in vitro at neutral pH with a lack of a desensitization phase (14). GMQ acts at a site other than the proton-binding site to produce its stimulating response (15). Agmatine and other polyamines have a positive charge that can interact with the electronegative proteins on the ion channels, and it was shown to open ASIC3 at physiological pH level, although not to the same extent as GMQ (15). *Anthopleura Elegantissima* toxin 2 (APETx2), a sea anemone-derived toxin, demonstrated ASIC3-specific blocking effects by decreasing the transient peak current of the channel in an acidic environment (16). The aforementioned studies were crucial in understanding the ability of non-proton ligands to modulate ASIC3 under physiological conditions.

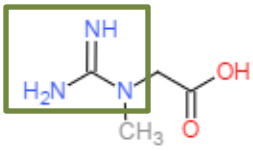
ASIC3 and pain

ASICs expression in peripheral nociceptive neurons and in brain areas dedicated to pain process has led to the thought that ASICs could be involved in pain perception (8). ASIC3 is linked with the modulation of nociception as the protein is highly expressed in sensory neurons in the periphery and in the dorsal root ganglion (DRG) in the spinal cord, where sensory information is gathered (5,17).

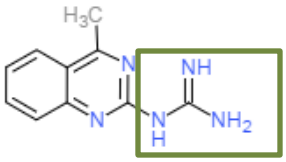
As previously mentioned, the non-proton ligands GMQ, amiloride, agmatine, and APETx2 altered the electrophysiological aspects of ASIC3 and were also involved in the *in-vivo* effects of nociceptive sensitivity (13,15,15,16). Local intramuscular and spinal administration of APETx2 to rats in an acid-induced model of hyperalgesia prevented the development of mechanical hypersensitivity but the ASIC3 inhibitor was not effective at preventing hypersensitivity once the hyperalgesia had already been developed (16). This study highlighted the role of ASIC3 in preventing pain development but not its maintenance. In the same study, inflammatory pain was produced by injection of complete Freund's adjuvant (CFA), local interplantar injection of APETx2 reversed ~98% of the mechanical hypersensitivity (16). In a study evaluating the nociceptive effects of the ASIC3 agonist GMQ, transgenic mice lacking ASIC3 (*asic3-/-*) were injected with GMQ (14). Wild-type mice injected with GMQ in their hind paw displayed an increase in paw licking time compared to their ASIC3 knockout counterparts (14). The increase of the nociceptive paw licking behavior in response to GMQ provided evidence in favor of the involvement in pain and non-proton activation of ASIC3 channels *in vivo*.(14)

Increased tissue acidosis and inflammation cause pain (18). In a human model of acid-induced pain, local amiloride administration after an injection of acidic saline reduced the magnitude of perceived pain (19). In addition, inflammation can also affect ASIC channels. A study measuring levels of expression of ASICs in DRG neurons of male rats, showed that mRNA levels of ASICs (ASIC1a, ASIC2b and ASIC3) increased significantly when measured after inflammation induced by CFA (20). Inflammatory mediators such as NGF (nerve growth factor), serotonin, bradykinin and interleukin-1 caused ASIC expressing sensory neurons to have higher excitability, but only NGF and serotonin had the capability of directly augmenting transcription

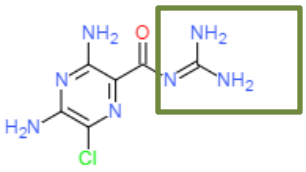
of the gene encoding ASIC3 (21).These inflammatory signals then activate the ASIC3 channels expressed on nociceptors and cascade that initiates pain signaling (5).The previously mentioned non-proton ligands involved in pain modulation of ASIC3, amiloride and GMQ, have a similar chemical structure to the organic acid creatine, with similar guanidium groups that could potentially confer upon them similar interactions with the ASIC3, and therefore affect pain sensitivity.



Creatine



GMQ



Amiloride

Creatine and ASIC3

Creatine is an endogenous organic acid known for its role in cellular metabolism used as a buffer to replenish ATP levels (23). As a dietary supplement, creatine is widely used for enhancing exercise performance (22), additionally, there is a substantial list of both reported and proposed health benefits of its oral consumption: neuroprotective effects (including protection against ischemic damage) and antioxidant properties (23,24). Recent human studies demonstrated that creatine supplementation improved quality of life, sleep and reduced pain associated with fibromyalgia following 8 weeks of treatment along with the patient's regular medications (25). On the other hand, a 16-week creatine diet treatment produced no changes in pain perception in another group of fibromyalgia patients (26). Evidence from human studies is not consistent as every study had a different loading dose, creatine diet duration and different pain measured which could contribute to the discrepancy. Not much is known about the effect of creatine on nociception and preliminary data from our collaborator established that creatine

affects the electrophysiological properties of ASICs, in particular ASIC3. Based on these observations, the aim of the proposed study was to determine whether dietary supplementation of creatine will reduce nociceptive sensitivity in a mouse model of inflammatory pain.

Specific aim

Because of the structure similarity of creatine with non-proton ligands of ASIC3 and the preliminary data collected by Dr. Gonzales' group, we hypothesized that creatine will reduce nociceptive sensitivity in an animal model of inflammatory pain, through an ASIC3-dependent mechanism.

Male and female mice will receive either a control or creatine diet and will then be injected with formalin to induce inflammatory pain, and their nociceptive behavior will be scored.

The results of this study could lead to the repurposing of a known and widely used compound, creatine, with little known side effects and low cost, towards the treatment of pain in many diseases.

Significance

It is important to elucidate the role of creatine in pain perception. The study of non-proton ligands of ASIC3 is still ongoing and the role of creatine in ASIC3 modulation could impact the study of future naturally occurring ligands. There is still much that is unknown about the exact molecular interaction of ASIC channels and their ligands so it is crucial to continue testing compounds that could be used as analgesics in the future. Finding a link between creatine supplementation and nociception modulation through ASIC channels could change the way pain is treated. This project could lead to searching for alternatives to treat pain that do not involve

dangerous surgery or drugs with harmful side effects like opioids or NSAIDs. Creatine is a readily available, affordable supplement that is already in the market with limited confirmed side effects and is an excellent candidate as an alternative or complement to pain management. Finding a link between creatine supplementation and nociceptive modulation through ASIC channels could improve the treatment of pain in order to avoid dangerous surgeries or medications with undesirable side effects.

Materials and methods

Animals

Sixty C57BL/6J 2-3 month old male and female mice were obtained from Jackson Laboratories, and acclimated at the UNTHSC vivarium for one week prior to any manipulations.

Housing, diet and identification

Mice were injected subcutaneously between the shoulder blades with an identification chip (2 x 13 mm biologically inert, Biomark) using a monoject syringe. Weights were taken weekly for the duration if the study. Food intake was measured daily the first week the mice were on their respective diets.

Mice were randomly assigned to one of the two experimental groups: control group was fed a control diet (Purina LabDiet® cat #: 1813505) or creatine group was fed the control diet supplemented with 6.25 g of creatine /kg diet (Purina TestDiet®; cat #: 1816777-201) (Table 1). The creatine supplemented was creatine monohydrate ≥98% purchased from Sigma Aldrich (cat #C3630). Animals had ad libitum access to food and water, and were group housed by sex and diet assignment (3-4 animals per cage). Animals were placed under a 12 hour light/dark cycle and all housing and procedures were approved by the UNTHSC IACUC.

Thermal hyperalgesia

The tail immersion test was used to examine thermal hyperalgesia, which models acute pain. Mice were allowed 10 minutes to acclimate to the testing room. Mice were lightly restrained and the distal portion of the tail was immersed in a water bath at 52°C for a maximum latency of 10 seconds. The latency to flex the tail during immersion was recorded. Mice received 3 trials with 1 minute between each. The average latency over the 3 trials was measured and analyzed.

Inflammatory pain

Formalin test is used to model inflammatory pain generated by damaged tissue in response to the injection of the noxious agent formalin (27). Mice were allowed 10 minutes to acclimate to the testing room and more specifically to the chamber. Each mouse was lightly restrained and injected subcutaneously in their right hind paw with 30 µl of 4% formalin solution, using a 500 µl U-100 microfine syringe with a 27G needle. Immediately after injection, each mouse was placed in the test chamber (plexiglass box 11 x 8.5 x 15 cm) with mesh flooring elevated 45 cm from the table, supported by PVC tubes and a mirror placed at a 45° angle under the mesh for observation of nociceptive behaviors) and observed for 60 minutes. Behaviors were recorded for 60 minutes. The time spent licking the injected paw was averaged over 10 minute periods and analyzed.

Statistical analysis

The effects of diet and sex on body weights and nociceptive response to formalin were assessed using three-way analysis of variance (ANOVA) with weeks or time-bin as the repeated measure. The effects of diet and sex on food intake and thermal hyperalgesia were tested two-

way ANOVA with sex and diet as between-group factors. Planned individual comparisons between different sex groups and diet groups were performed using a single degree-of-freedom F test involving the error term from the overall ANOVA. The alpha level was set at 0.05 for all analyses.

Results and discussion

Results

Weekly body weight

Body weights were measured weekly from the time of arrival until the last behavioral test and are presented in Figure 1 A. Overall, male mice weighed more than females and gained weight during the study. The mice on the creatine diet weighed the same as the controls, regardless of sex. A repeated measure analysis of variance yielded a main effect of Sex as well as a main effect of Weeks and an interaction between Weeks and Sex (all ps<0.05). There was no significant effect of Diet on body weight (all ps>0.643).

Food Intake

The amount of food consumed by the mice was measured daily for one week and the results are presented in Figure 1 B. Overall, male mice consumed more food than females, regardless of which diet they were assigned to. There were no major differences in food consumption between the control mice and the creatine-supplemented mice, regardless of sex. An analysis of variance yielded a main effect of Sex, but no main effect of Diet or an interaction between Sex and Diet (all ps>0.076).

Tail immersion test

The latency of a mouse to remove its tail from hot water was averaged across three trials and presented in Figure 2. There was no difference in latency between sex groups and diet groups. This observation was supported by an ANOVA yielding no main effect of Sex, Diet or an interaction of Sex and Diet (all *p*s> 0.393).

Formalin test

The duration of paw licking behavior was recorded for 60 minutes and averaged in 10 minute time bins. The results are reported as the percentage of time spent licking and presented in Figure 3. Overall females spent more time licking their paws than males, which was more apparent during the last 30 min of observation corresponding to the inflammatory phase of the formalin test. This was supported by an ANOVA yielding a main effect of sex throughout the 60 min and for the last 30 min (all *p*s <0.05). The male mice fed creatine had similar response compared to controls, while the female fed creatine had lower paw licking times during the last 20-30 minutes. The ANOVAs for the 0-60min or the last 20min of recording did not yield a significant effect of Diet or an interaction between Sex and Diet (all *p*s > 0.052).

Discussion

The major findings from this study were that short-term creatine supplementation did not affect the response of male and female C57BL/6J mice to an intense thermal stimulus, but seemed to affect the nociceptive licking behavior of females in the formalin test.

Male mice were heavier than the females and gained weight over the duration of the study (3 weeks), while the weight of the female mice remained stable. These differences are

consistent with what is reported by Jackson Laboratories. At the age used in this study, C57BL/6J males gain on average 2 g of body weight in approximately 3 weeks, while females in the same age range only gain 1 g (28). In addition, Jackson Laboratories showed that males weighed 6 to 7 g more than females of the same age (28), similar to what is observed in our study.

In our study, supplementation with creatine did not affect the body weight of the mice, which is in contradiction with previous published reports (29,30). Duarte *et al.* reported that male mice that were given a dose of creatine equivalent to 20 g per day (for humans) for six days, gained 4% more body weight than their control diet counterparts (29). The dose given was five times the one in our study (equivalent to 5 g per day for humans) and could account for the lack of an effect on body weights. In another study by Razia *et al*., female albino mice were on a creatine-supplemented diet (1% and 3% creatine) also gained weight, however they were supplemented for 10 weeks (30), while in our study we only exposed the mice to the diet for a total of 3 weeks. Dose and duration are decisive factors in whether body weight will be affected. Based on our data and literature review, a higher concentration for a longer duration seemed to increased body weights of the mice than what was used in our study.

Food intake data showed that males consumed more food than females over the course of 1 week. As stated, male mice gained more weight than female mice of the same age (28). The male mice were at a different growing stage than the females which suggests that the males consumed more food to keep up with their rapid growth.

The data for the tail immersion test showed no significant interaction between nociceptive sensitivity and diet or sex. The tail immersion test is designed to provoke a quick reflex reaction (31) which does not involve inflammatory mediators. The temperature of the

water at 52°C was considered to be a high intensity pain stimulus (32,33) directed at stimulating thermal and nociceptive receptors on the mouse tail. While the role of ASIC3 in pain has mostly been linked to inflammatory condition, in which acidification of tissues and inflammatory intermediates modulate the channel (34), some reports have indicated a potential role in thermal sensitivity as well. Chen *et al.* used male ASIC3 knockout and wild-type mice to observe the nociceptive differences using an automated tail-flick apparatus (radiant heat) to test for thermal hyperalgesia on the tail and found that both groups responded similarly to the test (33), as did the mice in our experiment. Another study reported that male ASIC3 knockout mice had a reduced tail withdraw latency in the tail immersion test compared to their wild-type counterparts (32). While our primary focus was ASIC3 and that ASIC3 have been involved mostly with acid and inflammatory pain (8) , it is not inconceivable that creatine could also interact with other ASICs and therefore affect different pain modalities. Therefore, it was important to not just focus on one type of pain but rather survey several types of pain to determine the extent of the effect of creatine on pain.

In the formalin test, there seems to be a difference in nociceptive response between males and females, i.e. the females spent more time licking than males. Interestingly, the effect of sex on pain perception is still being debated both in animals and humans. Many argue that epidemiological research showed that women report pain more often than men in a clinical setting (35), (36), the magnitude and mechanism underlying this disparity remains unclear. Mogil suggested that women may look for medical attention more than men, may be more susceptive to chronic pain illnesses or that they actually do have a lower tolerance for pain than men (37). Several studies in rodents present even more varied data. In a study done with 1 week old mice, males showed longer latencies to withdraw in a thermal paw-withdraw test, while the

females displayed an increased latency to withdraw on the tail immersion test (38). Furthermore, the genotype of the mice has been shown to affect pain sensitivity (39), altering which sex has a higher nociceptive sensitivity in thermal sensitivity assays and morphine anti-nociception effects (39). Therefore sex/gender might be an important factor to consider when considering the effects of potential anti-nociceptive compounds like creatine.

In our study, the female mice supplemented with creatine showed a more rapid recovery in the second phase of the formalin test than the female controls, while there was no difference in the males. Staniland and McMahon study showed no difference in the duration of nociceptive behaviors between the knockout and the wild-type ASIC3 mice (32), which was also done in male mice. It is interesting to note that creatine supplementation affected the second phase of the formalin test which corresponds to the inflammatory response phase. Considering that ASIC3 is activated by acidosis and inflammatory factors, it is sensible to assume that creatine could have an effect on this phase. Several of the pain studies reviewed only included either males or females, but not both, so it became challenging to find data that would cover both of their differences. Additional studies require investigators to consider both sexes for their experiments, especially regarding pain, as analgesic treatments may need to be developed differently depending on the sex/gender of the subject.

Thus far, we have determined that creatine may lower pain sensitivity associated with inflammation, and that it could be due to ASIC3 activation. However, at this point in time, one cannot rule out the potential role of other receptors that have been involved in nociception. The transient receptor potential V1 channel (TRPV1) has been known to participate in nociception due to inflammation (40,32). A study on rats showed that injecting a TRPV1 antagonist prevented and reversed hyperalgesia and secondary allodynia after formalin injection (40).

ASIC3 is not the only channel that mediates inflammatory pain in the body, so it is possible that other receptors activate and could diminish the effect of creatine.

Summary and conclusions

While our data remain preliminary, it seems evident that creatine may have a role as an anti-nociception compound. In order to further assess the effect of creatine on ASIC3, an injection of the ASIC3 agonist GMQ can be administered directly to the hind paw. As previously stated, GMQ has been documented to cause a higher incidence of nociceptive paw licking in ASIC3 wild-type mice compared to their ASIC3 knockout counterparts (14). Placing the mice under either a control or creatine diet and then injecting GMQ would allow us to document any differences between nociceptive behaviors that could be indicative of the specificity of creatine on ASIC3. Furthermore, ASIC3 is involved in many sensory modalities (5) so it would be interesting to add assays that tested mechanosensitivity, like the paw-pressure test, to explore any creatine effects. Adding the vonFrey test for tactile sensitivity could provide additional information on changes in nociception due to creatine. If the vonFrey test could be assessed before and after the formalin injection, we could add an additional nociceptive measure that could support the results obtained by the formalin test alone. As the role of creatine as an anti-nociception compound gets establish, a dose and duration study will be necessary to identify a maximal effect of creatine. Creatine is a safe, relatively inexpensive compound that is readily available and could be used alone or in combination with other treatments to alleviate pain symptoms. Further studies will be needed to determine the mechanism of action of creatine via ASICs, which could lead to the generation of compounds that reduce pain effectively while minimizing the side effects.

Table 1. Composition of control and creatine diets

	Control	Creatine
Creatine, g/kg	N.D.*	6.25
Protein, g/kg	183.00	174.00
Fat (ether extract), g/kg	51.00	46.00
Fat (acid hydrolysis), g/kg	60.00	55.00
Fiber (max), g/kg	45.00	44.00
Gross energy, kcal/g	34.30	34.00
Ash, g/kg	62.00	63.00
Nitrogen-free extract (by difference), g/kg	560.00	573.00

*N.D.: not determined, less than 0.05 g/kg diet based on expected recovery.

Vitamins(mg/kg): Choline chloride, 2000-1988; Niacin, 86-83; Pantothenic acid, 31-30; Pyridoxine, 10-9.93; Riboflavin, 9; Thiamine hydrochloride, 26-24; Folic acid, 2; Biotin, 0.3; Carotene, 1.9-1.5; Vitamin K as menadione,22.2-14.9; Vitamin B12, 51-75; Vitamin A, 8; Vitamin D3, 4; Vitamin E, 45.
Minerals (mg/kg): Calcium, 1210-1150; Phosphorus, 9300-9200; Phosphorus (non-phytate), 6680-6800, Sodium, ; Chlorine, 4500-4800; Potassium, 6000-6100; Magnesium, 2200; Sulfur, 3300-3000; Iron, 369; Manganese, 154-147; Zinc, 84-90; Copper, 10; Iodine, 2.15-2.12; Cobalt, 0.79-1.09; Selenium, 0.33-0.34; Fluorine, 35.7-7.9; Chromium, 1.94-0.56.

Figure 1. Effects of short-term creatine supplementation on body weight (A) and food intake (B) of male and female C57BL/6J mice. Each value represents the mean ± SEM, n=14-15 for body weight and n=2-5 for food intake. $* p < 0.05$, compared with treatment-matched females.

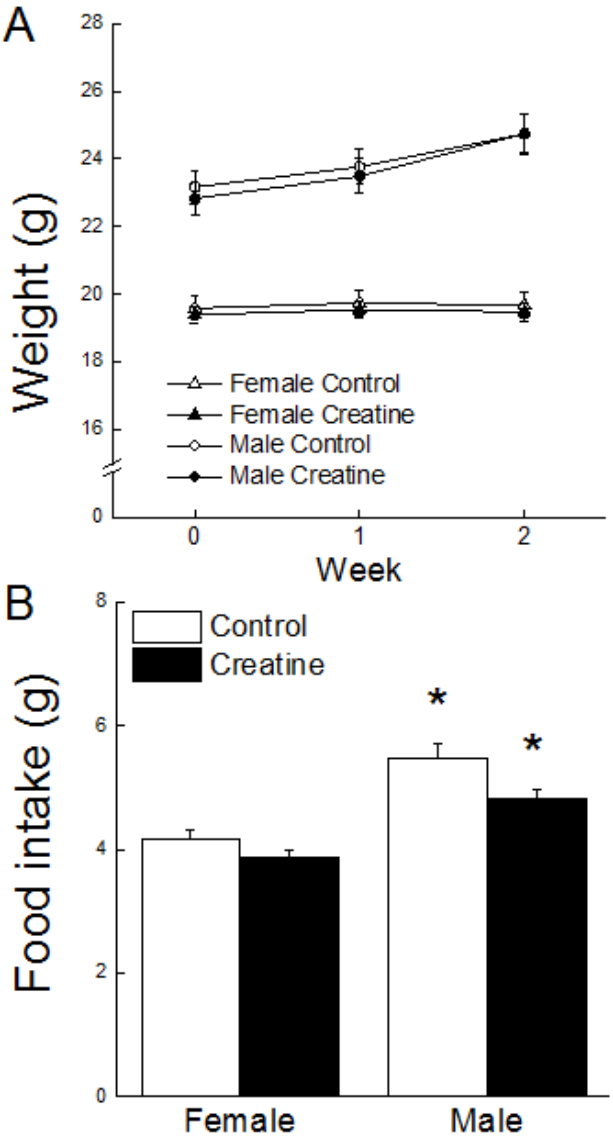

A
Weight (g)
28
26
24
22
20
18
16
0
0
1
2
Week
Female Control
Female Creatine
Male Control
Male Creatine
B
Food intake (g)
8
6
4
2
0
Control
Creatine
*
*
Female
Male

Figure 2. Effects of short-term creatine supplementation on nociceptive response of young male and female C57BL/6 mice in a tail immersion test. Each value represents the mean ± SEM, n=15.

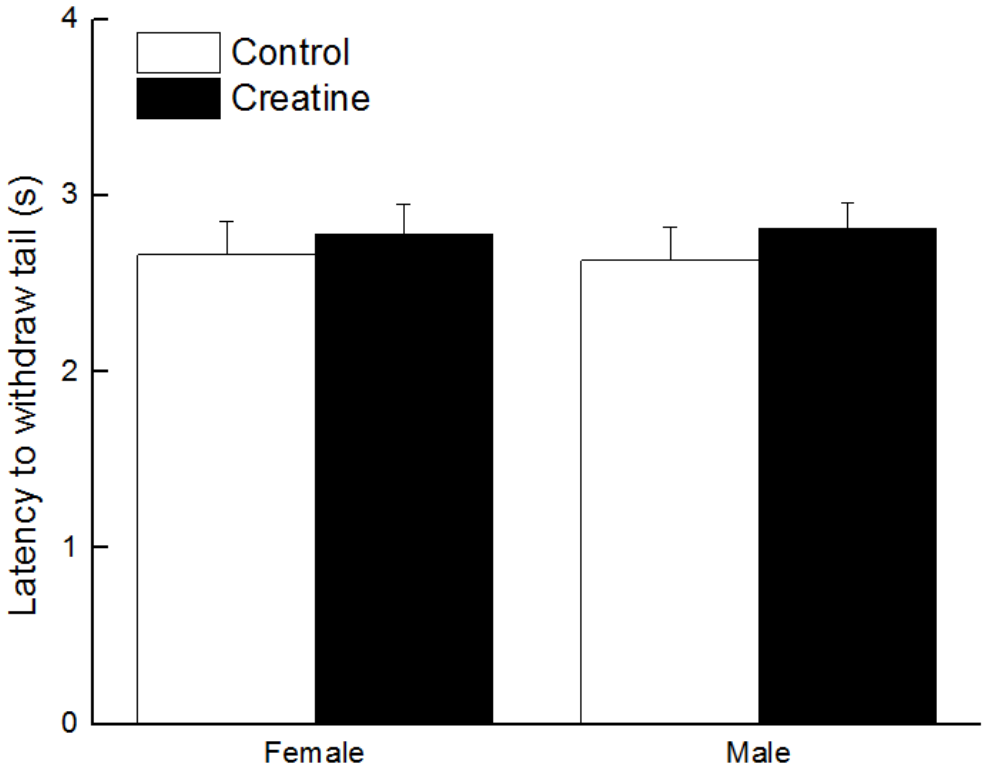
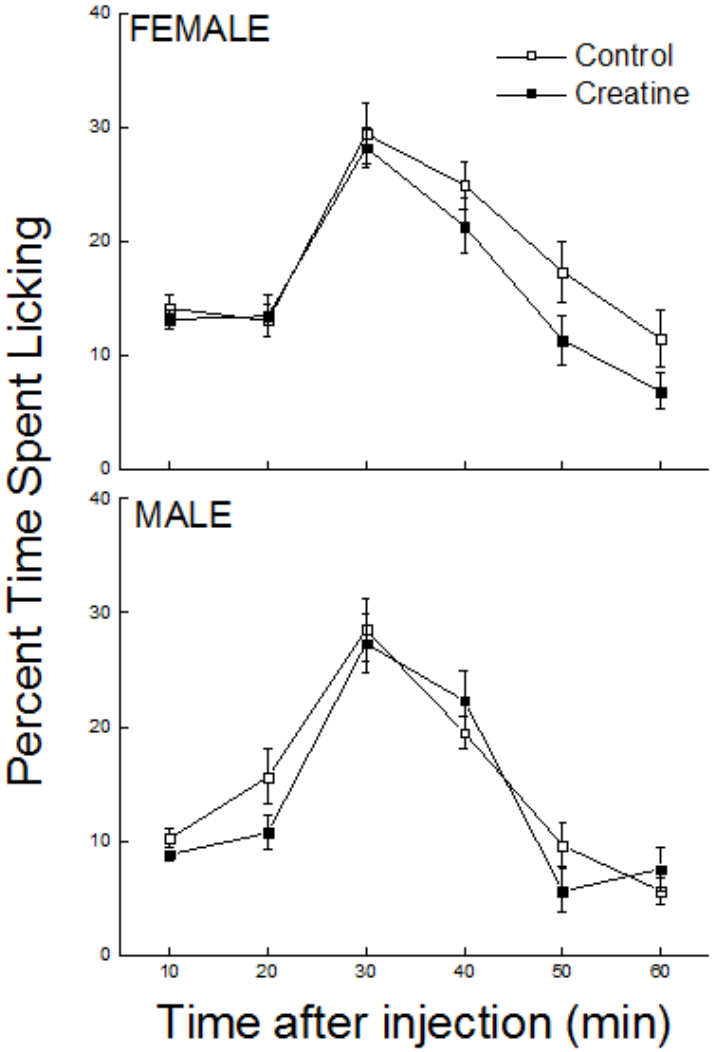

Control
Creatine
Latency to withdraw tail (s)
0
1
2
3
4
Female
Male

Figure 3. Effects of short-term creatine supplementation on nociceptive response of young male and female C57BL/6 mice in a formalin test. Each value represents the mean ± SEM. For Both female groups and control males n=15, creatine males n=14


FEMALE
Control
Creatine
MALE
Percent Time Spent Licking
Time after injection (min)
0
10
20
30
40
50
60

CHAPTER III

INTERNSHIP EXPERIENCE

Internship site

The internship was at the Pharmacology and Neuroscience Department at the University of North Texas Health Science Center in Dr.Sumien's laboratory. The lab specializes in functional assessment of rodent behavior as well as tissue biochemistry. The lab is comprised of 4 graduate students and 2 research technicians that work on both individual and collaborative experiments.

Journal summary

While interning in the laboratory of Dr.Sumien, I was exposed to many different techniques to assess animal behavior in both mice and rats. I was able to learn how to execute tests for spatial learning and memory, reflexes, balance, strength, spontaneous activity, active avoidance learning and cognitive flexibility. Furthermore, I was also exposed to biochemical tests used to measure protein concentration in the brain and test to genotype the mice. In addition, I learned how to dissect a mouse (peripheral tissues: heart, liver, muscles), and how to dissect a rodent brain into 6 distinct regions (cerebellum, cortex, hippocampus, striatum, midbrain and hindbrain). All these techniques were learnt in the context of group projects, however I was assigned my own project to carry out during my internship. My project was high-risk, in the sense that we only had electrophysiological data collected by our collaborator, Dr. Gonzales, to support our hypothesis. Furthermore, the techniques that I was to use to test my hypothesis had not been developed in the lab. However, Dr.Sumien provided me with all the

tools and the support I needed to complete all the tests and analyze the data appropriately. I was able to develop my project independently and learn how to use different statistical analyses.

With Dr. Sumien and Dr. Gatch's help, we developed the proper techniques to be successful in measuring nociceptive behavior in my animals. I carried out my project and collected enough data to present at Research Appreciation Day at UNT Health Science Center.

Throughout my internship I obtained experience in laboratory research, experimental design and data analysis that will be vital in continuing my career in science. Although I carried out an individual project I was able to learn many techniques from this lab and participate in collaborative projects which helped me develop as a scientist.

APPENDIX A

Internship journal: 6th August 2014

Morning

- Talked with Dr.Sumien about the protocol and how it could possible be amended to reflect changes in the administration of creatine, instead if gavage, just give dose in food
- Was given a short tutorial in Origin graphing software and SYSTAT statistical and Graphing software
    - Learned how to input and organize data
    - Learned how to run descriptive statistics , ANOVA and pairwise comparisons
    - Learned how to make a graph

Afternoon

- Was shown animal procedure for food intake
    - Weigh the amount of food let over to calculate the total food eaten by the animals
- Was shown and performed tagging of mice
    - Using large needle, the tag is inserted subcutaneously
- Helped refill food in mice cages and was shown how to visually sex mice

Internship journal: 7th August 2014

Morning: Plucking for genotyping

- Plucked mice fur for genotyping (for mice bred at UNTHSC)
- Extracted DNA from mice fur (PCR for genotyping) using NaOH and centrifuging
    - Gene is GCLM (Glutamate-cysteine ligase)
        - Possible outcomes
            - Wild-type
            - Heterozygous
            - Knockout

Internship journal: 8th August 2014

Morning:Plucking for genotyping

- Plucked fur from remaining animals with the same procedure as day before
- DNA extraction from hair (PCR for genotyping) with the same procedure as the day before

Internship journal: 11th August 2014

Morning and afternoon: Euthanization day

- We euthanized 20 mice by cervical dislocation

- I collected about 0.5ml of blood from the tail by placing the animal on a hot plate, in a holder and making a slit close to the base of the tail with a blade
    - The blood is collected in a 1.6ml tube which has 2 drops of the anticoagulant K_2 EDTA
    - After tubes of blood were collected, they were centrifuged at 2500g for 10 minutes at 4°C
    - The supernatant was removed with a pipette and put in a new 1.6ml tubeand placed in liquid nitrogen
- Another student preformed the cervical dislocation on the animal and removed the head
- A 3rd student was doing the organ extraction
    - Heart
    - Kidneys
    - Liver
    - Skeletal muscle from the hind legs
- All samples were stored at -80°C

Internship journal: 12th August 2014

- Morning and afternoon : Rat rotorod→ we ran 20 rats for 2 sessions each (1 morning and 1 afternoon)
    - Rotorod experiment with rats
        - Placed 3 rats on a rod at a time
        - Recorded the times at which each rat fell
            - During the experiment, check that time increases as the trial number increases, as the rats should get better at the task as they gain more experience
        - Rotorod is an experiment to evaluate motor function as the animals must have balance and coordination to perform the test. Additionally, learning is involved in the process and the latency of animals to fall should increase with progressive trials. This test is done to evaluate motor skills after an experimental treatment.

Internship journal: 13th August 2014

- Morning : Literature search
    - Searched for articles on behavioral methods of assessing pain and investigated the apparatus required for each type of measurement
- Afternoon: Elevated plus maze (EPM) set up
    - Set up the room and learned how to use the computer program ANY-maze to run EPM the day after

Internship journal: 14th August 2014

- Morning: Elevated plus maze (EPM) with mice → ran 20 mice
    - Learned to run mice on the EPM
        - Has closed arms and open arms
        - Measures the amount of time the mice spend in the closed and open arms , as well as the entries into the open arms
        - Elevated plus maze takes advantage of the aversion mice have for open spaces to measure their anxiety levels. When the animals are put in the EPM, they can move into either the closed or the open arms and how long they remain in either indicates their anxiety levels
- Afternoon: Literature search
    - Searched and reviewed articles on creatine's effect on muscle

Internship journal: 15th August 2014

- Morning : Locomotor activity with mice → ran 20 mice
    - Learned how to run mice in locomotor activity chamber
        - The apparatus measures:
            - Vertical activity (rearing) → for examining their motor function
            - Total distance travelled
            - Time spent in the center of the chamber → this is a measure of anxiety, as mice aversion for open spaces will cause them to be in the periphery
- Afternoon: Statistic tutorial
    - Learned how to use SYSTAT statistical software and Origin graphing software to analyze rotorod and elevated plus maze data \

Internship journal: 18th August 2014

- Morning : Straight alley with rats → ran 17 rats for 3 trials per animal
    - Learned how to set up and run rats on the straight alley
    - The time taken for the rats to get from one side of the pool to the platform is measured
    - Straight alley is a way of exposing the animals to water and to the concept of a platform in the maze that they can climb on to
        - The animals should learn improve their times as the trials progress
    - Done the day before doing water maze experiment
- Afternoon: Mice rotorod and rat water maze set up → ran 20 mice for 4 trials per animal
    - Learned how to run mice on rotorod

        - As with the rat rotorod, this is an exercise in motor coordination for mice
    - Learned how to set up the water maze for the rats and how to use the ANY-maze software

Internship journal: 19th August 2014

- Morning : Water maze for rats → ran 17 rats for 3 trials per animal
    - Learned how to run rats on water maze
    - Water maze is a task designed to test spatial learning and memory
    - The animals are placed at different starting points in the pool and they have to swim to find the hidden platform
    - The path length from start to finish is measured
- Afternoon: Protocol review

Internship journal: 20th August 2014

Morning: Internship journal review meeting

Afternoon: Mice rotorod → ran 20 mice

Internship journal: 21st August 2014

Morning: Rat water maze → ran 17 rats

Afternoon: Mice rotorod and statistics

- Only ran 2 animals who hadn't met criteria (no more than a 15% difference between the latency to fall of the last 3 trials)
- Reviewed how to use SYSTAT and Origin to analyze the mouse rotorod data

Internship journal: 22nd August 2014

Morning: Rat water maze + probe → ran 17 rats

- Learned to run a probe trial
    - The probe trial is different from the other trails as the platform is completely submerged so the program is measuring how long the animal spends in the target quadrant and target site as well as how many times the animal crosses the platform location. The probe only lasts 30 seconds and after that the platform is brought back to the surface.

Afternoon: Article search on human studies of the effects of creatine in diagnosed fibromyalgia patients

Internship journal: 25th August 2014

Morning: Brain homogenization

- Learned how to homogenize a mouse brain sample
    - Add buffer and use a Potter-Elvenhjem tissue homogenizer to disrupt the soft tissue and make it into a mix

Afternoon: Brain homogenization

Internship journal: 26th August 2014

Morning: Rat water maze reversal→ ran 17 rats

- Learned how to run a reversal trial
    - The platform is placed on the opposite location to test how fast the animal can learn the platform's new location

Afternoon: Agarose gel electrophoresis of PCR product for genotyping → 13 mice samples

- Learned how to perform agarose gel electrophoresis on PCR product samples of mice DNA and to interpret the gel according to the three possibilities: heterozygous, wild-type, knockout
    - Gel electrophoresis procedure
    - Gel imaging procedure

Internship journal: 27th August 2014

Morning: Rat water maze reversal + probe

Afternoon: Committee meeting and agarose gel electrophoresis of PCR product

- Discussed project and timeline
- Gel electrophoresis of the next batch of 13 samples of mice DNA

Internship journal: 28th August 2014

Morning: Mice motor skills tests→ ran 20 animals

- Learned to execute the following tests:
    - Walk initiation→ measures the animal's latency to move out of a designated area
        - Reflexive behavior
    - Alley turn → measures the animal's latency to turn in a small closed space
        - Reflexive behavior
        - Motor planning
    - Negative geotaxis → latency to orient against gravity
        - Reflective behavior

    - Wire suspension → latency to tread (hind paw wire grab) and latency to fall
        - Motor coordination
        - Strength
    - Bridge balancing → latency to cross bridge or fall
        - Balancing
        - Motor coordination

Afternoon: Test set up and gel imaging

- Set up for the following tests:
    - Straight alley for mice
    - Visible platform for rats
- Imaged gel from next 13 samples

Internship journal: 29th August 2014

Morning: Mice straight alley → ran 20 mice

- Learned to run straight alley for mice
    - Used to get the mice exposed to the water and platform setting before water maze

Afternoon: Agarose gel electrophoresis of PCR product of last 13 mice DNA samples

Internship journal: 2nd September 2014

Morning: Rat visible platform → ran 17 rats

- Learned to run visible platform test
    - Visible platform test is used to asses visual acuity as it uses a small banner on the platform that serves as a cue for the animals to know the platform location
    - It is performed in the same tank as the water maze test
    - There are 3 trials per session and 4 sessions in total (4 days)
    - In each trial both rat and platform placement changes

Afternoon: Mice water maze → ran 20 mice

- Learned to run mice water maze
    - Same principle as rat water maze→ spatial learning task
    - The mice have 5 trials with a 2 minute ITI
    - Only 2 mice are run at a time

Internship journal: 3rd September 2014

Morning: Rat visible platform → ran 17 rats

- Lab meeting: talked about my project

Afternoon: Article search on creatine in the nervous system

Internship journal: 4th September 2014

Morning: Rat visible platform→ ran 17 rats

Afternoon: Mice water maze → ran 18 mice

Internship journal: 5th September 2014

Morning: Rat visible platform

Afternoon: Article search on animal models of pain

Internship journal: 8th September 2014

Morning & afternoon: Rat euthanization

- Observed the euthanization of 15 rats (females that had been ovariectomized)
    - Animals were anesthetized with isoflurane
    - Cardiac puncture was used to remove ~9ml of blood
    - Eyes and brain were removed
        - Brain was cut sagittal to separate the left and right hemispheres and the following areas were separated:
            - Cerebellum
            - Hindbrain
            - Hippocampus
            - Subcortex
            - Cortex

Internship journal: 9th September 2014

Morning: Mouse water maze → ran 18 mice

Afternoon: mouse tagging

Internship journal: 10th September 2014

Morning: Lab meeting

- Presented article: "Functions and effects of creatine in the central nervous system", Robert H. Andres a, Angelique D. Ducray , Uwe Schlattner, Theo Wallimannb, Hans Rudolf Widmer

Afternoon: Mouse water maze + probe day → ran 18 mice

- Learned how to run a probe trial for the water maze
    - The platform is removed for the first 30 seconds of the trial and then placed again for the last 30 seconds.

Internship journal: 11th September 2014

Morning: Plucked GCLM mice for genotyping

Afternoon: PCR of GCLM mice samples

- Learned how to use thermocycler

Internship journal: 12th September 2014

Morning: Agarose gel preparation

Afternoon: Agarose gel electrophoresis of PCR products from previous day and gel imaging

Internship journal: 15th September 2014

Morning & afternoon: Formalin test article review

Internship journal: 16th September 2014

Morning :Formalin test article review

Afternoon: Review on nociception assays and preparation for lab meeting presentation

Internship journal: 17th September 2014

Morning: Lab meeting

- Presented on the formalin test

Afternoon: Tagged, sexed and separated pups from GCLM colony according to genotyping done the previous week

Internship journal: 18th September 2014

Morning and afternoon: Mouse euthanization → euthanized 10 mice

- Collected ~0.5ml of blood by tail bleed
- Learned how to removed brain
    - Brain:
        - Cortex
        - Hippocampus

        - Cerebellum
        - Striatum
        - Midbrain
        - Hindbrain
- Removed organs and tissues:
    - Heart
    - Liver
    - Skeletal muscle

Internship journal: 19th September 2014

Morning: Mice water maze→ 18 mice

Afternoon: Sorted articles and references

- Changed location of lab equipment

Internship journal: 22nd September 2014

Morning: meeting with Dr.Sumien

- Review protocol amendment

Afternoon: Review on pain

Internship journal: 23rd September 2014

Morning: Rat motor skills: Bridge balancing → 15 rats

Afternoon: Water maze probe data tutorial on excel

- Learned how to extract data for water maze and select the appropriate parameters

Internship journal: 24th September 2014

Morning: Mice visible platform → ran 18 mice

- Lab meeting: Presented on the von Frey filament test

Afternoon: Mice visible platform→ ran 18 mice

Internship journal: 25th September 2014

Morning: Rat bridge walking→ ran 15 rats

Afternoon: Article search on the role of ASIC3 in pain

Internship journal: 26th September 2014

Morning and afternoon: Article search on the role of ASIC3 in pain

Internship journal: 29th September 2014

Morning & afternoon: Rat rotorod → ran 15 rats

Internship journal: 30th September 2014

Morning & afternoon: Rat rotorod → ran 15 rats

Internship journal: 1st October 2014

Morning: Lab meeting

- Presented article: "ASIC3 channels in multimodal sensory perception", Wei-Guang Li and Tian-Le Xu

Afternoon:

Internship journal: 2nd October 2014

Morning & afternoon: Learned to run mice T maze

- T maze is an active avoidance task in which the animal's working memory is tested. The animals are placed in a maze in the shape of a T which and has to enter one of the 2 arms. After the mouse has established its side preference in the first trial, it receives a shock every time it walks into its preferred arm. Entering the non-preferred arm causes no shock. This test explores how well the animal can learn to adapt to a new scenario and forget its previous learning.

Internship journal: 3rd October 2014

Morning: Plucked GCLM mice for genotyping

Afternoon: DNA extraction of GCLM fur samples for genotyping

Internship journal: 5th October 2014

Morning: Mice tail suspension test → ran 18 mice

- The tail suspension test is most commonly used to assess antidepressants. The mouse is suspended by its tail and the time the mouse remains immobile is recorded.

Internship journal: 6th October 2014

Morning: PCR of GCLM samples

Afternoon: Sigma Xi grant writing

Internship journal: 7th October 2014

Morning: Prepared gel and ran gel electrophoresis of GCLM samples

Afternoon: Imaged gel from GCLM samples

- Sigma Xi grant writing

Internship journal: 8th October 2014

Morning: Lab meeting:

Afternoon: Gel electrophoresis of 14 GCLM mouse PCR samples and gel imaging

Internship journal: 9th October 2014

Morning: Prepared agarose gel

Afternoon: Gel electrophoresis of 8 GCLM mouse PCR samples and gel imaging

Internship journal: 10th October 2014

Morning: Rat water maze + probe → 15 rats

Afternoon: Article review on other non proton ligands of ASIC3

Internship journal: 13th October 2014

Morning: Prepared agarose gel

Afternoon: Gel electrophoresis of 7 GCLM mouse PCR samples and gel imaging

Internship journal: 14th October 2014

Morning & afternoon: Mice euthanization → 17 mice

- Collected ~0.5ml of blood by tail bleed
- Learned how to remove brain
    - Brain:
        - Cortex
        - Hippocampus
        - Cerebellum
        - Striatum
        - Midbrain
        - Hindbrain

- Removed organs and tissues:
    - Heart
    - Liver
    - Skeletal muscle
    - Kidneys

Internship journal: 15th October 2014

Morning: Lab meeting

- Presented article: "Reversal of acid-induced and inflammatory pain by the selective ASIC3 inhibitor APETx2" Jerzy Karczewski et al.

Afternoon: Mice tagging practice→ tagged 7 mice

Internship journal: 16th October 2014

Morning & afternoon: Proposal writing

Internship journal: 17th October 2014

Morning & afternoon: Proposal writing

Internship journal: 20th October 2014

Morning & afternoon: Article review on acid pain in humans

Internship journal: 21st October 2014

Morning: Rat visible platform → ran 15 rats

Afternoon: Apparatus revision for upcoming experiment

Internship journal: 22nd October 2014

Morning: Lab meeting

- Presented article: "Acid-induces pain and its modulation in humans" Jones, et al.

Internship journal: 23rd October 2014

Morning: Rat visible platform→ ran 15 rats

Internship journal: 24th October 2014

Morning & afternoon: Tagging practice→ tagged 8 mice

- learned how to clean and prepare new tags

Internship journal: 27th October 2014

Morning & afternoon: Article review optimizing the formalin test

Internship journal: 28th October 2014

Morning: Cleaned lab coats

Afternoon: Article review on additional non proton ligands of ASIC3

Internship journal: 29th October 2014

Morning: Lab meeting:

- Presented on additional non proton ligands of ASIC3

Afternoon: Proposal writing

Internship journal: 30th October 2014

Morning & afternoon: Proposal writing

Internship journal: 31st October 2014

Morning & afternoon: Proposal writing

Internship journal: 3rd November 2014

Morning: Proposal defense preparation

Afternoon: Article reviews for formalin test

Internship journal: 4th November 2014

Morning & afternoon: Proposal writing and defense preparation

Internship journal: 5th November 2014

Morning: Lab meeting

Afternoon: Mice tagging practice

Internship journal: 6th November 2014

Morning: Immunology lecture

Afternoon: Proposal writing

Internship journal: 7th November 2014

Morning: Immunology lecture

Afternoon: DNA extraction from GCLM mice 35 samples

Internship journal: 10th November 2014

Morning: Immunology lecture

- 20 mice arrival at UNTHSC vivarium

Afternoon: PCR of 35 GCLM samples

- Proposal defense preparation

Internship journal: 11th November 2014

Morning: Immunology lecture

Afternoon: Learned intraplantar injection in mice

Internship journal: 12th November 2014

Morning: Immunology lecture

Afternoon: Tagged and weighed 20 mice

- Proposal defense preparation

Internship journal: 13th November 2014

Morning: Immunology lecture

Afternoon: Proposal defense practice

Internship journal: 14th November 2014

Morning: Immunology lecture

Afternoon: Proposal defense practice

Internship journal: 17th November 2014

Morning: Immunology lecture

Afternoon: Proposal defense preparation

Internship journal: 18th November 2014

Morning: Immunology lecture

Afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

Internship journal: 19th November 2014

Morning: Immunology lecture

Afternoon: Gel electrophoresis of 13 GCLM mouse PCR samples and gel imaging

- Proposal defense preparation

Internship journal: 20th November 2014

Morning: Immunology lecture

Afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

- Proposal defense practice
- Weighed 20 mice

Internship journal: 21st November 2014

Morning: Immunology lecture

Afternoon: Arranged mice feeding schedule

- 20 mice started on diets

Internship journal: 24th November 2014

Morning: Immunology lecture

Afternoon: Proposal defense

- 20 mice food intake and weights

Internship journal: 25th November 2014

Morning: Immunology lecture

Afternoon: 20 mice food intake and weights

- Intraplantar injection practice
- Proposal correcting

Internship journal: 26th November 2014

Morning: Immunology lecture

Afternoon: 20 mice food intake and weights

- Intraplantar injection practice

Internship journal: 27th November 2014

Morning: 20 mice food intake and weights

Internship journal: 28th November 2014
Morning: 20 mice food intake and weights

Internship journal: 1st December 2014

Morning: Immunology lecture

Afternoon: 20 mice tail immersion test

- Tail immersion is a test of thermal sensitivity in which the distal portion of the animals' tail is placed in hot water and the latency to withdraw the tail is recorded.

Internship journal: 2nd December 2014

Morning: Immunology lecture

Afternoon: Injection practice

Internship journal: 3rd December 2014

Morning: Immunology lecture

Afternoon: 20 mice weights

- 6 mice formalin test
    - The formalin test provides a measure of inflammatory pain. The animals are injected with a solution of formalin, which causes local inflammation. The animals will show nociceptive behaviors (eg.licking the injected paw) which can be quantified to obtain a measure of pain levels.

Internship journal: 4th December 2014

Morning: Immunology lecture

Afternoon: 6 mice formalin test

Internship journal: 5th December 2014

Morning: Immunology lecture

Afternoon: 8 mice formalin test

Internship journal: 8th December 2014

Morning: 20 mice weights

Internship journal: 9th December 2014

Morning: Data entry and graphing for tail immersion test

Internship journal: 10th December 2014

Morning: Lab meeting

- Presented tail immersion data

Afternoon: Euthanization preparation

- Review of formalin data

Internship journal: 11th December 2014

Morning: Euthanization → 20 mice

- Learned how to remove brain
    - Brain:
        - Cortex
        - Hippocampus
        - Cerebellum
        - Striatum
        - Midbrain
        - Hindbrain

Afternoon: Review of formalin data

Internship journal: 12th December 2014

Morning & afternoon: Video revision and formalin data entry

Internship journal: 15th December 2014

Morning & afternoon: Video revision and formalin data entry

Internship journal: 16th December 2014

Morning: DNA extraction from GCLM mice 15 samples

Internship journal: 17th December 2014

Morning: PCR of 15 GCLM samples

Internship journal: 18th December 2014

Morning & afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

Internship journal: 19th December 2014

Morning & afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

Internship journal: 23rd December 2014

Morning: Tag cleaning and preparation

Internship journal: 5th January 2015

Morning: Protocol review

Afternoon: 20 male mice arrival at UNTHSC vivarium

Internship journal: 6th January 2015

Morning & afternoon: Prepared tags and weight sheets for mice

Internship journal: 7th January 2015

Morning: Lab meeting

- Presented: formalin graphs updated

Afternoon: Tagged 20 mice and took their weights

Internship journal: 8th January 2015

Morning: DNA extraction from GCLM mice 33 samples

Internship journal: 9th January 2015

Morning: PCR of 15 GCLM samples

Afternoon: Report writing

Internship journal: 12th January 2015

Morning: Handled 20 mice

Afternoon: Report writing

Internship journal: 13th January 2015

Morning: Agarose gel preparation and handled 20 mice

Afternoon: Gel electrophoresis of 13 GCLM mouse PCR samples and gel imaging

Internship journal: 14th January 2015

Morning: Agarose gel preparation and handled 20 mice

Afternoon: Gel electrophoresis of 13 GCLM mouse PCR samples and gel imaging

Internship journal: 15th January 2015

Morning: Agarose gel preparation

- Handled 20 mice and took their weights

Afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

Internship journal: 16th January 2015

Morning: Handled 20 mice

Afternoon: Arranged mice feeding schedule

- 20 mice started on diets

Internship journal: 19th January 2015

Morning: Report writing

Afternoon: 20 mice food intake and weights

Internship journal: 20th January 2015

Morning: Report writing

Afternoon: 20 mice food intake and weights

Internship journal: 21st January 2015

Morning: Report writing

Afternoon: 20 mice food intake and weights

Internship journal: 22nd January 2015

Morning: Report writing

Afternoon: 20 mice food intake and weights

Internship journal: 23rd January 2015

Morning: Report writing

Afternoon: 20 mice food intake and weights

Internship journal: 26th January 2015

Morning & afternoon: 20 mice thermal hyperalgesia test

Internship journal: 27th January 2015

Morning: Report writing

Afternoon: 20 mice weights

Internship journal: 28th January 2015

Morning & afternoon: 6 mice formalin test

Internship journal: 29th January 2015

Morning & afternoon: 6 mice formalin test

Internship journal: 30th January 2015

Morning & afternoon: 6 mice formalin test

Internship journal: 2nd February 2015

Morning & afternoon: Formalin test data collection

Internship journal: 3rd February 2015

Morning & afternoon: Formalin test data collection

Internship journal: 4th February 2015

Morning: Lab meeting

- Presented: Thermal hyperalgesia data and preliminary formalin test data

Afternoon: Report writing

Internship journal: 5th February 2015

Morning: Report writing

Afternoon: DNA extraction from GCLM mice 22 samples

Internship journal: 6th February 2015

Morning & afternoon: Euthanization → 20 mice

- Brain:
    - Cortex
    - Hippocampus
    - Cerebellum
    - Striatum
    - Midbrain
    - Hindbrain

Internship journal: 9th February 2015

Morning: PCR of GCLM mice 22 samples

Afternoon: Report writing

Internship journal: 10th February 2015

Morning: Report writing

Afternoon: Formalin data analysis

Internship journal: 11th February 2015

Morning: Lab meeting

- Presented: Redone formalin data

Afternoon: Finished report writing: Introduction

Internship journal: 12th February 2015

Morning: Report writing

Internship journal: 13th February 2015

Morning & afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

Internship journal: 16th February 2015

Morning & afternoon: Gel electrophoresis of 12 GCLM mouse PCR samples and gel imaging

Internship journal: 17th February 2015

Morning: Finished report writing: Methods

Internship journal: 18th February 2015

Morning & afternoon: Report writing

Internship journal: 19th February 2015

Morning & afternoon: Report writing

Internship journal: 20th February 2015

Morning & afternoon: Report writing

Internship journal: 24th February 2015

Morning: 20 mice arrival at UNTHSC vivarium

Afternoon: Report writing

Internship journal: 25th February 2015

Morning & afternoon: Writing RAD abstract

Internship journal: 26th February 2015

Morning: Tagged and weighed 20 mice

Afternoon: Prepared sheets for nociceptive assays and entered weight data

Internship journal: 27th February 2015

Morning: Report writing

Internship journal: 2nd March 2015

Morning: Report writing

Internship journal: 3rd March 2015

Morning: Report writing

Internship journal: 4th March 2015

Morning: Report writing

Internship journal: 5th March 2015

Morning: Report writing

Afternoon: Weighed 10 mice and entered weight data

Internship journal: 6th March 2015

Morning: Report writing

Afternoon: Handled 10 mice and started them on diet

Internship journal: 9th March 2015

Morning: Report writing

Afternoon: 10 mice food intake and weights

Internship journal: 10th March 2015

Morning: Report writing

Afternoon: 10 mice food intake and weights

Internship journal: 11th March 2015

Morning: Report writing

Afternoon: 10 mice food intake and weights

Internship journal: 12th March 2015

Morning: 10 mice food intake and weights

- Weighed and handled 10 mice and entered weight data

Afternoon: Rat rotorod → ran 19 rats

Internship journal: 13th March 2015

Morning: 10 mice food intake and weights

Afternoon: Weighed and handled 10 mice and entered weight data

Internship journal: 16th March 2015

Morning: Rat straight alley → Ran 19 rats

Afternoon: 10 mice food intake and weights

Internship journal: 17th March 2015

Morning: Report writing

- Mice tail immersion test → ran 10 mice

Afternoon: 10 mice food intake and weights

- Entered and analyzed tail immersion data

Internship journal: 18th March 2015

Morning: Report writing

Afternoon: 10 mice food intake and weights

Internship journal: 19th March 2015

Morning: Mice formalin test → ran 6 mice

Afternoon: 10 mice food intake and weights

Internship journal: 20th March 2015

Morning: Mice formalin test → ran 4 mice

Afternoon: 10 mice food intake and weights

Internship journal: 23rd March 2015

Morning: Report writing

Afternoon: Entered and analyzed formalin data

Internship journal: 24th March 2015

Morning: Mice tail immersion test → ran 10 mice

Afternoon: Entered and analyzed tail immersion data

Internship journal: 25th March 2015

Morning: Report writing

Internship journal: 26th March 2015

Morning: Mice formalin test → ran 4 mice

Internship journal: 27th March 2015

Morning: Mice formalin test → ran 4 mice

Internship journal: 30th March 2015

Morning: Rat straight alley→ Ran 24 rats

Afternoon: Report writing

Internship journal: 31st March 2015

Morning: Report writing

Internship journal: 1st April 2015

Morning: Report writing

Internship journal: 2nd April 2015

Morning: Report writing

APPENDIX B

Animal behavior techniques: Mice

Tail immersion test

Each mouse was manually restrained and the distal portion of the tail was submerged in a water bath at 52°C. The latency to flex the tail during immersion was recorded. Each animal had 3 trails with 1 min between each.

Formalin test

Each mouse was manually restrained and injected with a solution of 4% formalin on the plantar area of the right hind paw. Each animal was placed in a clear acrylic box (15 x 8.5 x 11cm) on top of a wire mesh (1 x 1 mm) elevated 45 cm from the surface. The behavior of each mouse was recorded for 60 min, and the time the animal spent licking its injected paw was used as a measure of nociceptive behavior.

Morris Water maze

The Morris Water Maze is a test used to assess spatial learning and memory. The apparatus consists of a 120-cm diameter plastic tank filled to 34 cm from the top with colored water (white non-toxic paint) kept at 24 ± 1°C. A small, hidden platform (10 x 10 cm), 1.5 cm below the level of the water, is placed in the tank as an escape. A computerized tracking system recorded swim speed and path length to reach the platform (Stoelting Any-maze).
For the pre-training phase, the tank was covered with a black curtain to prevent the mice from exposure to extra-maze cues. Each mouse was placed at the end of a straight alley (10 x 65 cm) with a platform at the opposite end. The animal was allowed to swim until it reached the platform or after 60 seconds had passed. Each animal had two sessions per day (for 2 days), each consisting of five trails with 5 min between each.
After pre-training, the black curtain was removed, and the mice were tested for their ability to learn the location of the platform using spatial cues. Testing was divided into three phases: acquisition (eight sessions with the platform in a fixed location), retention (two additional sessions after a 3 day delay interval), and reversal (four sessions with the platform fixed in the opposite quadrant of acquisition phase). Each session consisted of five trials, at 10-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. Two sessions were conducted per day, separated by a period of at least 2 h, during which the mice were returned to the home cages.
After session 8, a probe trial was given. The platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 seconds, and the trial ended when the mouse successfully located it. On this trial, spatial bias for the platform location was evaluated in terms of the (1) percentage of time spent in the platform quadrant, (2) percentage of time spent within 40- and 20-cm diameter annuli surrounding the platform location, and (3) entries into the platform zone itself.

Visible platform

The visible platform test is used to assess visually cued learning. The apparatus consists of a 120-cm diameter plastic tank filled to 34 cm from the top with colored water (white non-toxic paint) kept at 24 ± 1°C. A small, hidden platform (10 x 10 cm), 1.5 cm below the level of the

water, is placed in the tank as an escape. A computerized tracking system recorded swim speed and path length to reach the platform (Stoelting Any-maze). The hidden platform location was identified by a triangular flag that was raised above the surface of the water (6 cm from the water surface to the bottom of the flag). Eight sessions were administered, each consisting of five trials at 10-min intervals. Each trial, the mouse had to swim to the platform from a different starting point in the tank and the platform was moved to a different location before each trial. Thus, the mice had to learn to associate the location of the flag with the location of the platform.

Rotorod

Rotorod is an experiment used to evaluate motor function (balance and coordination). Each mouse is placed on an accelerating rod apparatus (Omnitech Electronics, Omnirotor treadmill), which consists of a rotating cylinder (3.2 cm in diameter) which is mounted (35.5 cm) above a padded surface. The latency to fall off the rod is recorded for each trail with four trails per session (2 sessions per day separated by at least 2 h). The test was ended when the average latency to fall showed no further improvement over three consecutive sessions.

Locomotor activity

To assess spontaneous locomotor activity, each mouse was placed in a clear acrylic test cage (40.5×40.5×30.5 cm) that was surrounded by a metal frame lined with photocells. The test cage was enclosed in a dimly lit, sound-attenuating chamber equipped with a fan that provided background noise (80 dB). During a 16-min period, movements in the horizontal plane as well as a vertical plane 7.6 cm above the floor were detected by the photocells and processed by software to yield 14 different variables describing horizontal, vertical, stereotypic, and spatial components of spontaneous activity in the apparatus.

Tail suspension

The tail suspension test is used to assess behaviors related to depression, by suspending them upside down in a suspension box. Each mouse was placed on a metal hook that is suspended (30 cm inside the box) by the distal portion of the tail. During a 6 min interval, the time in which the animal was immobile was recorded.

Motor skills battery

Walk initiation: Each mouse was placed on a flat surface and the latency to move one body length is recorded.

Alley turn: Each mouse was placed in plastic dead-end alley (3.5 cm width x 14 cm length) and the latency for the animal to reverse direction was recorded,

Negative geotaxis: Each mouse was placed facing downward on a flat mesh surface that was tilted 45° from the surface. The latency for the animal to turn 90° in any direction was recorded

Wire suspension: Each mouse was placed on a horizontal wire (suspended 27 cm above a padded surface) and allowed to grip it with its front paws. The latency to fall and latency to tread (reach wire with their hind legs) were recorded for two trials per day (four days).

Bridge walking: Each mouse was tested for the latency to fall or reach a safe platform after being placed on one of four acrylic bridges, each mounted 50 cm above a padded surface. The bridges differed in diameter (small or large) and shape (round or square), providing four

levels of difficulty. Each bridge was presented three times, and the measure of performance was the average latency to fall (up to a maximum of 60 seconds) across all bridges.

Elevated plus maze

The elevated plus maze test is used to assess the anxiety level of an animal. The plus maze elevated three feet off the floor, in a dimly illuminated room, with two open arms and two enclosed arms so that the floor is not visible. The mice were placed in the center of the maze facing an open arm and where allowed 5 min to explore the maze, while a computer tracking system (Stoeling Any-maze) monitored their position. The time spent in the closed vs. open arms was measured.

T maze/discriminated avoidance

A T-maze constructed of acrylic (black for the sides and clear for the top) was utilized for the discriminated avoidance task. The maze was divided into three compartments: a start box (10×6.3×6 cm), a stem (17.5× 6.3×6 cm) and two goal arms (14.5×6.3×6 cm), each separated by clear acrylic doors. The maze rested on a grid floor wired to deliver 0.27-mA scrambled shock to the feet. The test consisted of two sessions (one per day, for two days). On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning of the trial. On the first trial of the first session, the mouse received shock in the first arm entered and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 seconds after the opening of the start door if the mouse had not entered the correct goal arm, or immediately upon entry into the incorrect arm. In either case, the shock continued until the correct w entered or a maximum of 60 seconds had elapsed. Upon the mouse's entry into the correct arm, the door was closed (to prevent departure) and, after 10 seconds, the mouse was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training in this fashion continued at 1-min intervals until the mouse had met the criterion of a correct avoidance (defined as running directly to the correct arm within 5 seconds) on four of the last five training trials. The second session of avoidance training was a reversal such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous day. Ability to learn the avoidance problem was considered inversely proportional to the number of trials required to reach criterion in each of the sessions. The latency to reach the goal on the last trial of the first session was assessed.

Animal behavior techniques: Rats

Morris water maze

The Morris Water Maze is a test used to assess spatial learning and memory. The apparatus consists of a 180-cm diameter plastic tank filled to 20 cm from the top with colored water (blue non-toxic paint) kept at 24 ± 1°C. A small, hidden platform (15 x 15 cm), 1.5 cm below the level of the water, is placed in the tank as an escape. A computerized tracking system recorded swim speed and path length to reach the platform (Stoelting Any-maze).

For the pre-training phase, the tank was covered with a black curtain to prevent the rats from exposure to extra-maze cues. Each rat was placed at the end of a straight alley (115 cm length) with a platform at the opposite end. The animal was allowed to swim until it reached the

platform or after 60 seconds had passed. Each animal had two sessions (separated by a minimum of 2 h), each consisting of three trails with 5 min between each.
After pre-training, the black curtain was removed, and the rats were tested for their ability to learn the location of the platform using spatial cues. Testing was divided into three phases: acquisition (four sessions with the platform in a fixed location), retention (one additional sessions after a 2 day interval), and reversal (two sessions with the platform fixed in the opposite quadrant of acquisition phase). Each session consisted of three trials, at 10-min intervals, during which the rat had to swim to the platform from one of four different starting points in the tank. Two probe trials were given, one before session 4 and one after session 7. In the probe trial the platform was submerged to a depth that prevented the rat from climbing onto it. The platform was raised after 30 seconds, and the trial ended when the animal successfully located it. On this trial, spatial bias for the platform location was evaluated in terms of the (1) percentage of time spent in the platform quadrant, (2) percentage of time spent within 40- and 20-cm diameter annuli surrounding the platform location, and (3) entries into the platform zone itself.

Visible platform

The visible platform test is used to assess visually cued learning. The apparatus consists of a 180-cm diameter plastic tank filled to 30 cm from the top with colored water (white non-toxic paint) kept at 24 ± 1°C. A small, hidden platform (15 x 15 cm), 1.5 cm below the level of the water, is placed in the tank as an escape. A computerized tracking system recorded swim speed and path length to reach the platform (Stoelting Any-maze). In the hidden platform location was identified by a triangular flag that was raised above the surface of the water (6 cm from the water surface to the bottom of the flag). Four sessions were administered, consisting of three trials each at 10-min intervals. For each trial, the rat had to swim to the platform from one of four different starting points in the tank and the platform was moved to a different location before each trial. The rat had to learn to associate the location of the flag with the location of the platform.

Rotorod

Rotorod is an experiment used to evaluate motor function (balance and coordination). Each rat is placed on a rotorod apparatus (AccuRotor Rota-Rod,Accuscan) which consists of a rotating cylinder (6.5 cm diameter) which is suspended (35 cm) above a padded surface. The latency to fall off the rod is recorded for each trail, for a total of 4 trails per session (2 sessions per day and 7 days). The test was ended when the average latency to fall showed no further improvement over three consecutive sessions.

Bridge walking

Each rat was tested for the latency to fall or reach a safe platform after being placed on one of four acrylic bridges (60 cm length), each mounted 70 cm above a padded surface. The bridges differed in diameter (small or large) and shape (round or square), providing four levels of difficulty. Each bridge was presented three times, and the measure of performance was the average latency to fall (up to a maximum of 60 seconds) across all bridges.

Molecular biology techniques

RT-PCR and gel electrophoresis for mouse genotyping

1. Collecting hair samples
    a. Each mouse was restrained manually and hair was removed from its abdominal area using plastic forceps and placed in an 1.5 ml Eppendorf tube
2. DNA extraction from hair preparation
    a. 50 mM NaOH was added to the hair in each tube and each was boiled and chilled
    b. Each tube is centrifuged and the supernatant is removed and stored
3. RT-PCR
    a. A master mixture of primers, Taq polymerase, DMSO and water was added to a each PCR tube with the supernatant
    b. The PCR tubes are placed in a thermocycler for 2 h 30 min
4. Gel electrophoresis and DNA imaging
    a. Samples were mixed with 6X loading dye and were loaded into a 1.5% agarose gel
    b. Ethidium bromide is added to stain the DNA, which can then be visualized with UV transilluminator (Biospectrum Imaging System, UVP)

Bacterial transformation with GAPDH plasmid

1. Place tubes with plasmid cDNA (GAPDH plasmid 0.1 µg/ µl) on ice
2. Add 25µl of frozen competent cells into a 1.5 ml Eppendorf tube
3. Add 2µl of the plasmid cDNA into Eppendorf tube containing competent cells and tap gently to mix
4. Incubate tube on ice for 30 min
5. Heat shock tube for 1 min in a 42°C water bath
6. Incubate tube on ice for 2 min
7. Add 500 µl of LB medium to the tube
8. Shake tube horizontally at 37°C for 1 h at 225 rpm in a shaker incubator
9. Spread 10 and 50 µl of transformation mix on separately labeled LB agar plates containing 50 µg/ml of carbenicilin
10. Incubate at 37°C for 18-24 h
11. Collect 2 colonies from each plate and inoculate them into 2 separate tubes with 3ml LB medium containing carbenicillin
12. Repeat steps 1-11 using a control plasmid

Plasmid DNA isolation of GAPDH plasmid (Wizard plus SV miniprep DNA purification kit)

Production of clear lysate

1. Pipette 1.5 ml of bacterial transformation culture (from: Bacterial transformation with GAPDH plasmid experiment) into a microfuge tube
2. Centrifuge at 3000 rpm for 5 min at room temperature
3. Discard supernatant
4. Add 1.5 ml of bacterial transformation culture to the microfuge tube
5. Centrifuge at 3000 rpm for 2 min at room temperature
6. Discard supernatant

7. Resuspend pellet by adding 250 µl of Cell Resuspension solution (50mM glucose, 25nM Tris, HCl pH8, 10mM EDTA)
8. Add 250 µl of Denaturation Buffer (0.2 M NaOH, 1% SDS)
9. Invert 4 times to mix
10. Add 350 µl Neutralization solution (5 M potassium acetate)
11. Invert 4 times to mix
12. Centrifuge at 14000 rpm for 6 min at room temperature
13. Transfer supernatant to new microfuge tube and centrifuge at top speed for 4 min

Binding of plasmid DNA

14. Insert spin column into collection tube
15. Decant cleared lysate into spin column
16. Centrifuge at 14000 rpm for 1 min at room temperature
17. Discard flow through and reinsert column into collection tube

Washing

18. Add 750 µl of Wash Solution
19. Centrifuge at 14000 rpm for 1 min at room temperature and discard flow through

Elution

20. Dry the spin column for 3 min and transfer the spin column to a sterile 1.5 ml microfuge tube
21. Add 50 µl of nuclease-free water to the spin column and centrifuge at 14000 rpm for 3 min at room temperature
22. Discard the column
23. Use Nanodrop apparatus to measure DNA concentration

Restriction digestion of cDNA clone encoding GAPDH gene

1. Pipette 8 µl of GAPDH plasmid DNA into sterile microfuge tube
2. Add 2 µl nuclease-free water
3. Add 10 µl of digestion mix containing:
    a. 7 µl nuclease-free water
    b. 2 µl 10x buffer H
    c. 1 µl of Pst-1 (10 units/ µl)
4. Mix by tapping the microfuge tube
5. Incubate the mixture for 1 h at 37°C

Agarose gel electrophoresis of restriction digestion

1. Weigh 1 gm of agarose and add 100 ml of 1 x TAE buffer (40mM Tris, 20 mM glacial acetic acid and 1mM EDTA) in a 250ml conical flask
2. Microwave for 1 min, swirling intermittently, until it begins to boil
3. Add 10 µl of ethidium bromide (10 mg/ml) and rock the flask gently for about 10 min until its cool to the touch
4. Assemble gel casting unit
5. Pour the molten agarose into the gel casting
6. Allow get to solidify for 20 min
7. Prepare samples
    a. Restriction digestion samples

        i. Add 4 µl of 6x loading dye to 20 µl of restriction digestion in a microfuge tube
    b. Uncut plasmid DNA
        i. Add 2 µl of uncut of uncut plasmid DNA, 3 µl of nuclease-free water and 1 µl of 6x leading dye in a microfuge tube
8. Add buffer solution to electrophoresis apparatus, just enough to cover the gel
9. Load samples into gel as well as 1kb ladder
10. Run gel electrophoresis at 150 V for 40 min
11. Remove gel from casting box and image using a UV transilluminator

RNA isolation from HNPE cells

1. Remove most growth medium (except for 1ml) from 100mm tissue culture dished containing the pertinent cell (HNPE)
2. Scrape the cells using a cell scraper and transfer to a 1.6 ml microfuge tube
3. Spin down at 3300 rpm for 5 min at 4°C
4. Carefully discard growth medium and aspirate the remaining growth medium with a pipette, being careful not to dislodge the cell pellet
5. Add 1ml of Trizol reagent to the cell pellet
6. Gently aspirate 5-6 times until de cell pellet is completely solubilized
7. Incubate on ice for 10 min
8. Add 200ml of chloroform (ACS grade) and mix by capping and inverting the tubes 3 times (work under a fume hood)
9. Incubate on ice for 5 min
10. If there is a clear separation between the top aqueous phase (colorless) and the bottom organic phase (pink) then continue to the next step. If not, then add an additional 200 µl of Trizol reagent and mix by inverting the tube 3 times and incubate on ice for 5 min
11. Centrifuge at 14000 rpm for 10 min at 4°C
12. Carefully aspirate the aqueous phase (colorless) into a new 1.6 ml tube. If the pink organic phase is accidentally aspirated, dispense it back in the tube and recentrifuge at 14000 rpm for 5 min
13. To the aqueous phase, add an equal volume of isopropanol (approximately 600 µl) and mix by inverting the tube
14. Incubate on ice for 10 min
15. Centrifuge at 14000 rpm for 10 min at 4°C
16. Discard supernatant
17. Add 1 ml of 70% ethanol
18. Centrifuge at 14000 rpm for 10 min at 4°C
19. Carefully discard supernatant and do not dislodge the RNA pellet
20. Air dry pellet for 5 min
21. Add 50 µl of autoclave water to dissolve pellet and tap tube gently
22. Measure RNA levels using a Nanodrop apparatus

cDNA synthesis from RNA isolates of HNPE cells

1. Obtain RNA concentration from RNA isolation experiment

2. Add 13.5 µl nuclease-free water, 2.5 µl total RNA and 4 µl of 5x iscript mix (RNasin, dNTP,AMV reverse transcriptase, 5x RT buffer, $MgCl_2$, KCl) to a PCR tube
3. Incubate on ice for 5 min
4. Place mix in thermocycler (25°C for 5 min, 42°C for 30 min, 85°C for 5 min)
5. Store at -20°C

RT PCR of cDNA synthesis from RNA

1. Make Taq PCR mix
    a. 100 µl 5x Taq buffer
    b. 60 µl $MgCl_2$
    c. 10 µl dNTP mix (10mM)
    d. 10 µl GAPDH sense primer
    e. 10 µl GAPDH antisense primer
    f. 300 µl nuclease-free water
    g. 2.5 µl Taq polymerase
2. Add 49 µl of Taq PCR mix to a PCR tube
3. Add 1 µl of cDNA from cDNA synthesis form RNA experiment
4. For the negative control, repeat steps 1 to 3 with 1 µl of nuclease-free water instead of cDNA
5. Place PCR tubes in thermocycler(amplification program)
    a. Denaturation I at 95°C for 2 min
    b. Denaturation II at 95°C for 30 s, Annealing at 58°C for 30 s and Elongation I at 72°C for 30 s (repeat for 30 cycles)
    c. Elongation II at 72°C for 10 min
6. Store PCR product at 4°C

TA cloning of PCR

Ligation

1. Add 3 µl of nuclease-free water, 2 µl of 5x ligation buffer, 2 µl PCR 21 vector (25 ng/ µl), 2 µl PCR product and 1 µl T4 DNA ligase to a microtube
2. Incubate for 15 min at room temperature
3. Store ligation product at 4°C for 24 h

Transforming competent cells

4. Make X-gal agar plates by adding 32 µl of x-gal to an LB medium agar plate and spread
5. Add 2 µl of ligation product to 50 µl of INVαF' chemically competent E.Coli cells to a microtube
6. Incubate on ice for 30 min
7. Place microtube in a 42°C water bath for 30 s
8. Incubate cells on ice for 1 min
9. Pipet cells into a microtube containing 250 µl of SOC medium
10. Place microtube on a shaker at 225 rpm at 37°C for 1 h
11. Plate cells on x-gal agar plate
12. Incubate at 73°C overnight

Growing clones

13. Use a toothpick to obtain 1 white colony from the x-gal plate and culture it in 3 µl of LB medium containing carbenicilin (50 µg/ µl)

14. Incubate at 37°C overnight

    Plasmid isolation

15. Pipette 1.5 ml of bacterial transformation culture into a microfuge tube
16. Centrifuge at 3000 rpm for 5 min at room temperature
17. Discard supernatant
18. Add 1.5 ml of bacterial transformation culture to the microfuge tube
19. Centrifuge at 3000 rpm for 2 min at room temperature
20. Discard supernatant
21. Resuspend pellet by adding 250 µl of Cell Resuspension solution (50mM glucose, 25nM Tris, HCl pH8, 10mM EDTA)
22. Add 250 µl of Denaturation Buffer (0.2 M NaOH, 1% SDS)
23. Invert 4 times to mix
24. Add 350 µl Neutralization solution (5 M potassium acetate)
25. Invert 4 times to mix
26. Centrifuge at 14000 rpm for 6 min at room temperature
27. Transfer supernatant to new microfuge tube and centrifuge at top speed for 4 min

    Binding of plasmid DNA

28. Insert spin column into collection tube
29. Decant cleared lysate into spin column
30. Centrifuge at 14000 rpm for 1 min at room temperature
31. Discard flow through and reinsert column into collection tube

    Washing

32. Add 750 µl of Wash Solution
33. Centrifuge at 14000 rpm for 1 min at room temperature and discard flow through

    Elution

34. Dry the spin column for 3 min and transfer the spin column to a sterile 1.5 ml microfuge tube
35. Add 50 µl of nuclease-free water to the spin column and centrifuge at 14000 rpm for 3 min at room temperature
36. Discard the column
37. Use Nanodrop apparatus to measure DNA concentration from plasmid isolate

    Restriction digestion and gel electrophoresis

38. Add 2 µl of plasmid isolate into a new microtube
39. Add 18 µl of EcoR1 restriction enzyme mix (15.3 µl autoclave water, 2 µl 10x buffer H, 0.2 µl BSA(10 µg/ µl) and 0.5 µl EcoR1 enzyme)
40. Incubate for 1 h at 37°C
41. Prepare 2% agarose gel
42. Prepare running samples
    a. Restriction digestion samples
        i. Add 20 µl TA plasmid isolate sample and 4 µl 6x loading dye
    b. Uncut plasmid DNA
        i. Add 0.5 µl TA plasmid isolate, 4.5 µl nuclease-free water and 1 µl 6x loading dye
43. Load samples into gel as well as 100 bp ladder
44. Run gel at 120 V for 1 h
45. Remove gel from casting box and image using a UV transilluminator

Sequencing clones

46. Send TA plasmid isolates to be sequenced
47. Obtain forward primer sequence
48. Use BLAST (blast.ncbi.nlm.gov) to confirm the sequence matches the original

Northern blot analysis of RNA isolates from HNPE cells

Denaturing gel

1. Add 1.2 gm agarose, 75 ml deionized water and 10 µl of 10x MOPS buffer (water, 2-N-morphlino-propanesulfonic acid, 80mM sodium acetate, 0.5M EDTA) to a 100 ml Erlenmeyer flask
2. Microwave flask for 1 min, stirring intermittently until agarose begins to boil
3. Cool until it is bearable hot
4. Add 15 ml of formaldehyde
5. Pour gel into casting unit and allow it to solidify

Sample preparation

6. In a 1.6 ml microtube add 13 µl of RNA isolation product from HNPE cells
7. To the microtube, add 1 µl nuclease-free water and 21 µl of RNA sample buffer (500 µl formamide, 182 µl formaldehyde, 100 µl 10x MOPS)
8. Incubate at 65°C for 10 min
9. Incubate tube on ice for 3 min
10. Add 7 µl of loading dye and 1 µl ethidium bromide
11. Tap gently to mix
12. Load 43 µl of mix into each well
13. Run gel at 80 V for 3 h
14. Image gel using a UV transilluminator

Assembling the transfer components

15. Wet the Whatman filter paper wick with the transfer buffer and make sure the ends of the wick touch the buffer (20x SSC)
16. Cut and place thin parafilm strips on the sides of the gel
17. Place the gel on the wick, so that the wells are facing up
18. Cut to gel size and place the nylon membrane on top of the gel
19. Place 2 sheets of Whatman paper (cut to gel size) on top of the nylon membrane
20. Place a pile of napkins over the Whatman paper (pile should be at least 12 cm high)
21. Place 2 long pieces of tape over the napkins as to apply vertical pressure
22. Wrap the components in a piece of cling wrap
23. Allow the transfer to proceed for 24 h
24. Remove nylon membrane from the transfer components
25. UV cross link membrane for 1 min (255-302 nm)
26. Image membrane using a transilluminator and mark the 5kb and 2kb bands with a pencil for visualizing ease

Prehybridization

27. Prepare prehybridization solution
    a. 20 ml formamide
    b. 5 ml 20x SSC
    c. 2 ml Denhardt's solution
    d. 1 ml Sodium phosphate (1M)

e. 0.2 ml 10% SDS
f. 1.2 ml Nuclease-free water
g. 0.6 ml Salmon sperm (Incubate in boiling water for 5 min and then on ice for 5 min before use)

28. Roll the nylon membrane and insert it into a hybridization bottle
29. Add the prehybridization mix and incubate in a hybridization oven with rotation at 42° for 4 h
30. Obtain labeled cDNA probe (Biotinylated GAPDH cDNA)
    Hybridization
31. Prepare hybridization solution
    a. 10 ml formamide
    b. 5 ml 20x SSC
    c. 0.8 ml Denhardt's solution
    d. 0.65 ml Sodium phosphate (1M)
    e. 0.4 ml 10% SDS
    f. 2 ml Nuclease-free water
    g. 2 gm Dextran sulfate
32. Mix 10 µl of cDNA probe with 400 µl of salmon sperm DNA and incubate in boiling water for 5 min, then transfer to ice for 5 min and add to the hybridization solution
33. Discard prehybridization solution from hybridization tube and add hybridization solution and add hybridization solution
34. Hybridize for 24 h at 42°C in hybridization oven with rotation
    Northern blot exposure
35. Discard hybridization solution
36. Wash nylon membrane twice with 2x SSC
37. Discard solution and wash with 1x SSC
38. Discard solution and wash with 0.2x SSC
39. Gently warm the blocking buffer and 4x wash buffer to 37°C in a water bath until all the particulate is dissolved
40. Add 20ml of blocking buffer and incubate for 15 min while gently shaking
41. Add 333 µl stabilized streptavidin-horseradish peroxidase (1:300 dilution) conjugate to the 20 ml blocking buffer
42. Incubate membrane in the conjugate/blocking buffer solution for 15 min while gently shaking
43. Transfer nylon membrane to a new container and rinse briefly with 20 ml of 1x wash solution
44. Wash membrane four times for 5 min each in 50 ml of the 1x wash solution while gently shaking
45. Transfer membrane to a new container and add 50 ml of substrate equilibrium buffer
46. Incubate member for 5 min while gently shaking
47. Prepare substrate working solution
    a. 6 ml luminol/enhancer solution
    b. 6 ml stable peroxide solution
48. Remove substance from substrate equilibrium buffer and blot an edge with a paper towel to remove excess buffer and place the membrane in a clean container

49. Pour substrate working solution onto the membrane so that is completely covers it and incubate for 5 min at room temperature
50. Remove membrane from working solution and blot to remove excess buffer
51. Expose membrane to a fluorescent camera in a transilluminator to image it

Southern blot analysis of PCR product of cDNA synthesis from RNA

1. Utilizing the gel from RT PCR of cDNA synthesis from RNA experiment add Denaturation solution(1.5M NaCl and 0.5 M NaOH) enough to cover the gel
2. Incubate the gel for 45 min with a constant and gentle shake
3. Rinse gel twice with 300 ml of deionized water
4. Add neutralizing solution (1 M Tris and 1.5 M NaCl) enough to cover the gel and shake gently for 30 min on a shaker
5. Discard Neutralizing solution and rinse gel with 20x SSC

   Assembling transfer components
6. Wet the Whatman filter paper wick with the transfer buffer and make sure the ends of the wick touch the buffer (20x SSC)
7. Cut and place thin parafilm strips on the sides of the gel
8. Place the gel on the wick, so that the wells are facing up
9. Cut to gel size and place the nylon membrane on top of the gel
10. Place 2 sheets of Whatman paper (cut to gel size) on top of the nylon membrane
11. Place a pile of napkins over the Whatman paper (pile should be at least 12 cm high)
12. Place 2 long pieces of tape over the napkins as to apply vertical pressure
13. Wrap the components in a piece of cling wrap
14. Allow the transfer to proceed for 24 h

Prehybridization

15. Prepare prehybridization solution
    a. 20 ml formamide
    b. 5 ml 20x SSC
    c. 2 ml Denhardt's solution
    d. 1 ml Sodium phosphate (1M)
    e. 0.2 ml 10% SDS
    f. 1.2 ml Nuclease-free water
    g. 0.6 ml Salmon sperm (Incubate in boiling water for 5 min and then on ice for 5 min before use)
16. Roll the nylon membrane and insert it into a hybridization bottle
17. Add the prehybridization mix and incubate in a hybridization oven with rotation at 42° for 4 h
18. Obtain labeled cDNA probe (Biotinylated GAPDH cDNA)

Hybridization

19. Prepare hybridization solution
    a. 10 ml formamide
    b. 5 ml 20x SSC
    c. 0.8 ml Denhardt's solution
    d. 0.65 ml Sodium phosphate (1M)
    e. 0.4 ml 10% SDS
    f. 2 ml Nuclease-free water

g. 2 gm Dextran sulfate

20. Mix 5 µl of cDNA probe with 400 µl of salmon sperm DNA and incubate in boiling water for 5 min, then transfer to ice for 5 min and add to the hybridization solution
21. Discard prehybridization solution from hybridization tube and add hybridization solution and add hybridization solution
22. Hybridize for 24 h at 42°C in hybridization oven with rotation

Exposing Southern Blot

23. Discard hybridization solution
24. Wash nylon membrane twice with 2x SSC
25. Discard solution and wash with 1x SSC
26. Discard solution and wash with 0.2x SSC
27. Gently warm the blocking buffer and 4x wash buffer to 37°C in a water bath until all the particulate is dissolved
28. Add 20ml of blocking buffer and incubate for 15 min while gently shaking
29. Add 333 µl stabilized streptavidin-horseradish peroxidase (1:300 dilution) conjugate to the 20 ml blocking buffer
30. Incubate membrane in the conjugate/blocking buffer solution for 15 min while gently shaking
31. Transfer nylon membrane to a new container and rinse briefly with 20 ml of 1x wash solution
32. Wash membrane four times for 5 min each in 50 ml of the 1x wash solution while gently shaking
33. Transfer membrane to a new container and add 50 ml of substrate equilibrium buffer
34. Incubate member for 5 min while gently shaking
35. Prepare substrate working solution
    a. 6 ml luminol/enhancer solution
    b. 6 ml stable peroxide solution
36. Remove substance from substrate equilibrium buffer and blot an edge with a paper towel to remove excess buffer and place the membrane in a clean container
37. Pour substrate working solution onto the membrane so that is completely covers it and incubate for 5 min at room temperature
38. Remove membrane from working solution and blot to remove excess buffer
39. Expose membrane to a fluorescent camera in a transilluminator to image it

Immunocytochemistry

1. Obtain HNPE cells that are grown, treated , fixed and stained directly on the coverslip
2. Aspirate media and add 1 ml of 4% PFA in PBS and incubate for 20 min at room temperature
3. Aspirate fixative and wash with 1 ml of 1x PBS (NaCl, KCl, Na_2HPO_4, KH_2PO_4, deionized water)
4. Discard PBS and rinse three times in 1 ml of 1x PBS for 5 min each
5. Permeabilize cells using 600 µl permeabilization buffer(PBS, sodium citrate, Triton X-100) for 5 min at room temperature
6. Block cells with 600 µl of blocking buffer (1x PBS, 5% normal goat serum, 0.3% Triton X-100, distilled water) for 1 h at room temperature
7. Prepare primary antibody mix and place on a gentle shaker

    a. 15 µl β-actin (Santa Cruz)
    b. 15 µl αβ-tubulin
    c. 7.5 ml Antibody buffer (1x PBS, 1% BSA, 0.3% Triton X-100, distilled water)
8. Aspirate the blocking buffer and add 1 ml of primary antibody mix to cells and incubate under foil for 1 h at room temperature
9. For the control, do not add any primary antibodies
10. Rinse three times in PBS for 5 min each
11. Prepare secondary antibody mix
    a. Donkey α Mouse (ALEXA 555)
    b. Donkey α Rabbit (ALEXA 488)
    c. Antibody buffer
12. Aspirate all PBS and add 1 ml of secondary antibody mix to the cells and incubate them for 1 h at room temperature under a foil cover
13. Rinse three times in PBS and add 1 ml of DAPI (DNA stain) for 5 min
14. Rinse one time with 1x PBS
15. Allow coverslip to air dry
16. Mount coverslip on glass slide with 1 drop of mounting medium
17. Seal coverslip with clear nail polish to prevent drying
18. Use a fluorescent microscope to visualize target proteins on the cell
19. Store in dark at -20°C or 4 °C

<u>RNA interference</u>

1. Obtain HNPE cells grown on a 35 mm dish
2. Mix 5 µl of Lipofectamine 200o in 100 µl serum free DMEM in a microtube and place at room temperature for 5 min
3. Mix 2 µl of C-JUN DNA is 100 µl of SF-DMEM in a microtube and place at room temperature for 5 minutes
4. For the control, mix 2 µl of vector DNA in 100 µl SF-DMEM in a microtube and place at room temperature for 5 minutes
5. Add 100 µl of Lipofectamine mix into C-JUN DNA solution and mix well. Place at room temperature for 15 minutes
6. Add 100 µl of Lipofectamine mix into vector DNA solution and mix well. Place at room temperature for 15 minutes
7. During solution incubation, change media in the HNPE dish to 2 µl of SF-DMEM
8. Add 200 µl of C-JUN mix into HNPE dish dropwise and then mixing carefully using a round motion and an inverted shake
9. Repeat step 8 on a new HNPE dish for vector DNA mix
10. Incubate dishes at 73°C for 6 h
11. Change medium in dish to 2 µl DMEM + 10% FBS +penicillin +streptomycin and incubate the dish at 37°C for 24 h
12. Aspirate all the medium and discard
13. Add 1 ml of Trizol and shake on shaker for 10 min at room temperature
14. Scrape cell lysate and transfer it to a 1.5 ml tube
15. Add 200 µl chloroform and shake vigorously for 15 s
16. Incubate at room temperature for 2-3 min
17. Centrifuge at 16000 g for 15 min at 4°C

18. Transfer the top aqueous layer containing RNA to a fresh tube and add 500 µl of isopropanol and mix by inverting four times
19. Incubate at room temperature for 5-10 min
20. Centrifuge at 16000g for 10 min at 4°C
21. Remove supernatant and wash the pellet with 1 ml of 70% ethanol in DEPC water by inverting the tube four times
22. Centrifuge at 7500 g at 4°C
23. Air dry pellet
24. Dissolve RNA pellet in 90 µl of DEPC water
25. In a new tube, add 45 µl RNA, 5 µl 10x DNase buffer I and 1 µl DNase I
26. Place tube in a 37°C water bath for 30 min
27. Add 10 µl DNase I inactivation reagent and mix by inverting 4-5 times and place at room temperature for 2 minutes
28. Centrifuge at 16000g at 4°C for 1 min
29. Transfer supernatant to new tube and incubate at 65°C in a water bath for 15 min
30. Immediately quench on ice for 2 min
31. Measure RNA concentration with Nanodrop apparatus
32. Calculate volume of RNA sample needed for 500ng of RNA $\frac{500ng}{RNA\ concentration\ ng/\mu l}$
33. Add the following to 3 separate microtubes
    a. 14.7 µl RNA sample, 4 µl 5x iscript RT mix, 1.3 µl nuclease-free water
    b. 5.2 µl RNA sample, 4 µl 5x iscript RT mix, 10.8 µl nuclease-free water
    c. 12.6 µl RNA sample, 4 µl 5x iscript RT mix, 3.4 µl nuclease-free water
34. Place mixed in thermocycler (25°C for 5 min, 42°C for 30 min, 85°C for 5 min)
35. Store at 4°C

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