Activation of Mesolimbic Dopamine Pathway by Pain Relief

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Department of Chemistry and Biochemistry
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5.5.14

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Abstract:

Relief from pain is a natural reward and promotes release of dopamine in brain reward circuits usually characterized as including dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). We hypothesized that pain relief induced NAc dopamine release will activate dopamine receptor expressing neurons in this region. Rats with incision of hind paw received lidocaine, a local anesthetic, to relieve ongoing postsurgical pain. Neuronal activation was investigated using cFos immunohistochemistry, as a neurochemical marker. The number of cFos positive cells in the NAc of the sham-operated rats receiving saline averaged 5.78±1.96. No clear increase in the number of cFos positive cells in the incision lidocaine animals (4.00±3.19cells) could be detected. Attempts to stain neurons for dopamine D2 and D1 receptors were not successful. This study was inconclusive regarding neuronal activation as measured neurochemically with cFos.

Introduction:

Pain is a neurobiological perspective of discomfort or suffering, caused by an aversive stimuli. The sensation of pain can be classified into three different types of subcategories. The first type of pain is the early-warning physiological protective system within the body which helps to minimize the damage that occurs when it comes into contact with an unpleasant stimulus, such as touching a hot stove. Another type of pain can heighten sensory sensitivity in order to allow healing of damaged tissues, this discourages physical contact or movement in the area that has been affected. Lastly, there is pathological pain which is known as the disease state of the nervous system. Pathological pain is closely linked to the dysfunction of the sensory signals in the central nervous system which causes people to feel pain due to the amplification of

sensory signals (1). When humans experience pain, they try to decrease the level of discomfort they feel.

When the sensory sensitivity is heightened or the sensory signals are amplified, pain relief can occur from stimuli that are normally innocuous (e.g., touch stimuli). In the setting of more intense ongoing pain, current therapy relies heavily on the use of drugs like morphine, originally extracted from opium, to produce pain relief. Unfortunately, opiates such as morphine and codeine have side effects such as nausea and vomiting, sedation, respiratory depression, constipation, delirium and addiction (2). Hence, researchers have worked to discover alternative ways to subdue the discomfort caused by the pain. The first step was to understand how humans were able to perceive pain. Basbaum et al, demonstrated that when a stimulus generates acute pain, adaptive changes occur in the peripheral and central nervous system to enhance pain signal leading to hypersensitivity (3). The primary sensory neurons are connected to a cell body in the dorsal root ganglion and have a central axon that synapse on post-synaptic neurons in the spinal dorsal horn to relay information to higher levels of the central nervous system. The first neuron in this pathway is the peripheral nociceptor (4). Signals are then sent to the brain, usually via the thalamus, to cortical areas process the information and generate meaningful experiences such as pain (5).

Once, the researchers were able to decipher parts of the neuro-circuitry of pain, they decided to try to manufacture non-opioid pain relief medication that somehow affected the somatosensory nervous system. Researchers produced medications that could reduce pain by decreasing the excitation or increasing inhibition in the nervous system. However, that caused the same problems as any other opioid medication, and also had a lower reduction of pain than opioids. Hence, the next pharmacologists introduced analgesics that targeted ion channels and

receptors that transduced harmful stimuli into electrical activity (4). In essence, the idea behind the pain relief drug was to block the action potentials that sent signals from the site of injury to the brain. However, clinically, the manufactured pain relief drugs are effective mostly for acute pains or migraines. Most chronic pains caused by illnesses such as cancer, still use opioids as a means of pain relief (2). In order to produce a more effective non-opioid drug that had similar effects of pain relief as an opioid without the adverse side effects, researchers investigated the pain relief pathway used by both types of pain relieving drugs. What they discovered was that the reward pathway and pain relief were very intimately linked (6).

Research has shown that pain and reward are opposing interacting processes that are fundamental to life. For example, pain can be decreased by pleasure such as palatable foods, and pleasure can be diminished by pain. Neuroimaging studies in humans demonstrate overlapping, brain circuits that mediate pain and reward including the anterior and posterior insula, amygdala, anterior cingulate cortex, and nucleus accumbens. Becker et al., studied rewarding stimuli for the hedonic and motivational effects. Hedonic experiences are effects that are linked to a "liking" affect, and the motivational effects are the "wanting" affect. Pain relief has been closely linked to reward due to the fact that there are rewarding qualities in relief of any aversive state to change into a less aversive state (7, 8). The major brain reward pathway is the mesolimbic dopaminergic pathway. Dopamine is a neuro transmitter that is released by nerve cells in order to send signals to other nerve cells. The dopamine is processed in the brain as a signal for pleasure or the state of happiness (9). Navratilova et al (2012), tested the hypothesis that relief of pain induced averseness will produce dopamine release in the nucleus accumbens (NAc).They used animals with incisional injury to the hind paw to demonstrate that peripheral nerve block will produce activation of the mesolimbic reward pathway as well as motivated behavior that can be detected

using the conditioned place preference (CPP) assay. The CPP reflected negative reinforcement, due to removal of pain-induced averseness, revealing the presence of pain. The study also demonstrated that the CPP was due to dopamine release in the NAc as the behavior was prevented by antagonizing dopamine receptors in the NAc and they additionally demonstrated the requirement for activation of the ventral tegmental area (VTA) that sends dopaminergic projections to the NAc. Thus, pain is reflected by motivated behavior (i,e., CPP) and relief of pain is reflected by activation of neurons in the VTA and dopaminergic signaling in the NAc (6). The NAc, therefore, plays a major role in the pain relief pathway. It is key to understand how the dopamine affects the NAc region during pain relief, so further study can be done on creating a drug that can be used clinically to treat patients in pain.

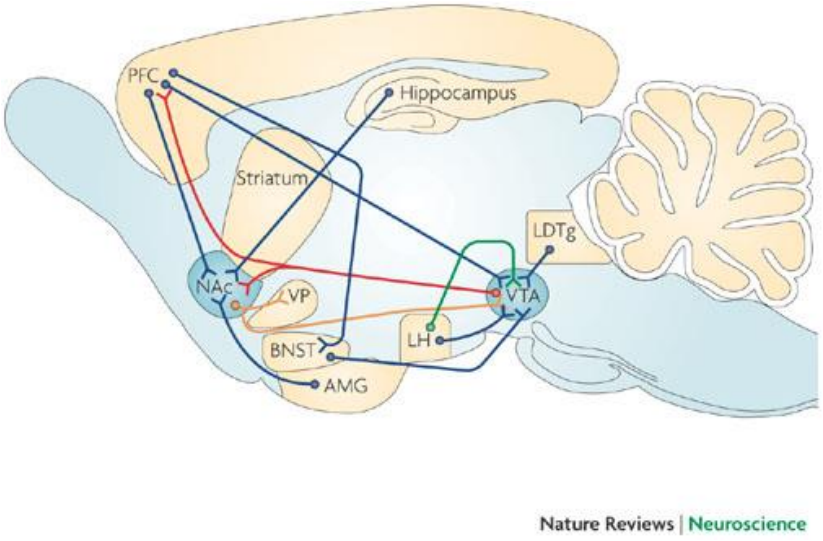


Figure 1: "Mesolimbic dopamine system circuitry" by Julia A. Kauer and Rober C. Malenka.[1] The figure maps the reward circuitry found, specifically the dopaminergic pathway (10).

[1] From "Synaptic Plasticity and Addiction" by Julie a. Kauer and Robert C. Malenka, 2007, *Nature Reviews Neuroscience*. Copyright 2014 by Nature Publishing Group. Reproduced with permission of the image creator. Available at http://www.nature.com/nrn/journal/v8/n11/fig_tab/nrn2234_F1.html.

. It is known that the NAc can receive signals from various parts of the brain: the hippocampal region, basal amygdaloid, prefrontal cortex, midline and intralaminar thalamus, ventral palidum, the VTA, and retrorubral cell groups (10, 11) (Figure 1). The NAc can be anatomically and functionally divided in two major regions: the shell and the core. The NAc shell receives signals from the basolateral amygdala (BLA) and the ventral subiculum, a major output region of the hippocampus and sends projections to the lateral preoptic and lateral hypothalamic areas (12). The core also obtains inputs from the BLA, but it is instead connected to the parahippocampal region. The shell and core can further be divided into many sub regions, and in each sub region there are different inputs and outputs throughout the NAc. Depending on the combinations of neurons activated within the NAc certain outputs signals can be produced from the NAc (13).

The shell is the area of the ventral striatum that is responsible for the control of reward- or drug-seeking behavior that is caused by spatial/ contextual information. The NAc core is in control of similar behaviors, but only when they are caused by "discrete cues" (10). During the pain relief, the rats will release dopamine in the NAc, which will bind to the dopamine receptors on the cell membrane. There are five dopamine receptors, D1 and D5 are known to be "stimulatory receptors" that activates the cell when dopamine binds to the receptor. D2, D3, and D4 are the "inhibitory receptors" which can inhibit a stimulatory cell or inhibit an inhibitory cell. D1 and D2 receptors are known to produce maximal activation of the cell, suggesting that there is a certain type of synergism between the two types of dopamine receptors (14, 15). The NAc shell contains more D1 and D3 receptors while core has more D2 receptors (9). It is unknown if the core or the shell of the NAc is more active during pain relief. By finding whether the shell or core is more active it is possible to see which receptors are more active, and it is possible to map

out the pain relief circuitry with more accuracy. Since the NAc is one of the major areas of the brain related to the dopaminergic pathway, finding out the pain relief circuitry can further the research needed to create a more effective non-opioid pain relieving drug.

In this research we hypothesized that dopamine release after pain relief will activate D1 and D2 receptor expressing cells in the NAc. The rats will be given a one centimeter long incision to the left hind paw to model the post-surgical pain many humans face clinically that heightens sensory sensitivity. The rats will experience pain from the surgery, and will be given either saline (vehicle) or lidocaine the day after the surgery. The lidocaine will give the animal pain relief triggering neuronal activation in the reward pathway. The hypothesis is that activated NAc neurons can be visualized with a neurochemical marker, cFos. Thus, the rats that receive pain relief will more likely have more cFos staining in the NAc. The rats that have more activated neurons might also express D1 and D2 dopamine receptors. We investigated which part of the NAc (shell or core, dorsal or ventral) contains the most D1 and D2 receptor expressing cells.

Methods:

Incision and Lidocaine Injections

Four groups of rats were divided in to two sham/saline, two sham/lidocaine, two incision/saline, and four incision/lidocaine. Each rat was anesthetized with isoflourane, the sham rats did not receive any surgery, and the incision rats were given a one centimeter incision in the left hind paw to stimulate surgical injury and post-operative pain. The rats were given popliteal fossa injections of either saline (vehicle) or lidocaine 24 hours after their surgery. The lidocaine blocks the nerve sending pain signals to the brain, and therefore, provided pain relief.

Perfusion of the Rat Brain

Perfusions were done two hours post popliteal fossa injection using a solution of PBS (3.2mM sodium phosphate dibasic, 0.5mM potassium phosphate, 1.3mM potassium chloride and 135mM sodium chloride, pH of 7.4) followed by a solution of 4% paraformaldehyde in PBS (PFA) (pH of 7.4). The brains were retrieved and placed in 4% PFA fixative for four hours and transferred into 15% sucrose then into 30% sucrose for another two days. Once the brains were fully saturated, the brains were sectioned with Optimal Cutting Temperature compound (OCT) and sliced onto charged slides on the cryostat into 30μm slices for staining.

Immunohistochemistry for cFos and D1

The brain slice slides were preserved in the -20˚C until the day of staining. The slides were permeabilized using PBS with 0.2% Triton X100 for 10 minutes. Then endogenous peroxidases were quenched with peroxide solution (PBS, 10% methanol, and 0.3% hydrogen peroxide) for 30 minutes. After three washes using PBS and 0.05% TritonX100 (PBSTX), the slides were blocked using PBSTX, 1% BSA, 5% goat serum for 90 minutes. The slides were then placed in primary antibody (rabbit polyclonal anti cFos with a dilution of 1:20,000 dilution) overnight. The next morning the slides were washed three times with PBSTX and placed into secondary antibody (biotinylated anti rabbit from the ABC kit: Vector laboratories) for 90 minutes. During the secondary antibody incubation, the ABC complex was made using the A reagent and B reagent from the kit and were incubated for 30 minutes at room temperature. The slides were then placed into the ABC complex for 60 minutes. The slides were washed and were placed into black boxes to be incubated with TSA-fluorescein (Perkin Elmer) for 10 min.

The slides were kept in a dark room and washed four times before being placed in biotin/avidin blocking for twenty minutes. The slides were blocked again using the PBSTX, 1% BSA, and 5% goat serum for an hour. The primary antibody for rabbit polyclonal anti D1 was added and the slides were incubated overnight. The secondary antibody, ABC complex procedures were repeated, and a TSA Cy3 was added for the second staining.

cFos and D1 microscopy and counting of the cFos cells and D1

The NAc was imaged under the fluorescent microscope using green (fluorescein) and red (Cy3) filter cubes. The images were taken of cFos and of D1 and overlapped on the program imageJ. The number of cFos positive cells within a standard square area in the NAc in the shell was counted using the ImageJ thresholding method. The average number of cells per slice in each treatment group was then calculated and graphed using Prism or Excel.

Microdialysis for Dopamine

Cannulas were placed in the NAc shell of the rats a week before the microdialysis day. The rats were either given incision surgery or left as sham twenty-four hours before the experiment. There were seven animals of each treatment group. The day of the microdialysis, a probe was connected to artificial cerebral spinal fluid (aCSF) as well as a collection tube with antioxidant, to prevent degradation of dopamine, and placed into the cannulas. The samples were collected at thirty minute increments for four hours. The first hour was used to collect waste in order to avoid changes in dopamine release due to handling of the rats during the setup of the experiment. The next hour was used as a baseline, to compare the level of dopamine change due to lidocaine or saline (vehicle) injections. The rats received popliteal fossa injections of either lidocaine or saline after the hour of baseline, and the aCSF was collected for two hours post

injections. The samples collected were all placed into a high-performance liquid chromatography (HPLC), and this instrument separated the different proteins according to different flow rates in adsorbent material. The dopamine levels were corrected using baseline levels.

Results:

The antibodies used in the experiment were commercial rabbit polyclonal antibodies made by Santa Cruz. The antibodies were made by immunizing the rabbit with an antigen containing the clone of the gene of interest. The injections are repeated several times over six weeks. The cell recognizes the antigen as a foreign substance and activates the B cells which differentiates into plasma cells or memory cells. The plasma cells secretes the soluble antibody that was made for that specific antigen and the antibodies bind to the epitopes of the antigens. The antibodies created will also be in the blood stream. Blood sample can extracted, and the red blood cells can be separated from the serum using a centrifuge. The extracted serum is composed of antigen-specific, non-specific antibodies, and other proteins; therefore, the antigen-specific antibody has to be separated from the other polypeptides in the serum. This can be done by using affinity chromatography in which the antigen specific antibodies will bind to the sepharose beads in the column (Figure 2). Low pH buffer can be used to elute the non-specific antibodies as well as other proteins in the serum leaving only the antigen specific antibodies on the beads. By raising the pH of the eluent, the targeted polyclonal antibodies can be obtained. The polyclonal antibodies are then tested by ELISA or Western Blot to make sure that the antibody obtained is indeed the antibody desired. In a polyclonal antibody, the antibodies retained can bind to many different epitopes of the targeted protein. (16, 17).

One of the main differences between polyclonal and monoclonal antibodies is that polyclonal antibodies bind to many epitopes of the antigen, and monoclonal antibodies only bind

to one antigen. The monoclonal antibodies bodies are made by injecting mice with an antigen, again with the clone of the gene of interest, then after the six weeks the plasma cells are extracted from the spleen tissue and combined with immortal cells to create hybridoma cells. The hybridoma cells deposit the single antibody that recognizes only one epitope (16). During this experiment, polyclonal antibodies were chosen over monoclonal antibodies due to the fact that polyclonal antibodies were cheaper and less sensitive to antigen changes than monoclonal antibodies. The polyclonal antibody would also have a higher amount of cFos positive cells and D1 receptors because, the polyclonal could bind to more than one epitope. The cFos is a basic leucine zipper that dimerizes and binds to DNA. There are four binding sites where the antibody can bind to, and they are all on the N-terminus of the protein (Figure 3). Therefore, the polyclonal antibody could bind to all four sites on the cFos protein. If it was a monoclonal antibody it could have only bound to one binding site. In order for the antibody to bind to all the cFos positive cells, the incubation period for the primary would have been much longer, and even then not all the cells would have been bound by an antibody. The one reason monoclonal antibody could have been a better choice is due to the fact that, monoclonal antibodies have decreased background on the staining, because there would be less nonspecific binding (18).

The cFos positive cells were counted using the ImageJ program using the "Analyze Particles" method with a NAc square. The square took a section of the shell allowing for the number of cFos positive cells in the area to be counted, shown in Figure 4. Less cFos positive cells were found in the NAc of rats who underwent incision surgery and received popliteal fossa injection of lidocaine (Figure 5). The incision rats showed less cFos expression than the sham rats in the shell. The sham rat with lidocaine had an average of 7.14±3.33 cells, the sham rat with

saline averaged about 5.78±1.96 cells. The incision rats with lidocaine averaged 4.00±3.19cells, incision with saline had 3.38±1.0 positive cells (Graph 1).

In order to verify that dopamine is secreted in NAc shell, a microdialysis was performed. The dopamine samples were taken for thirty minutes over a two hour period. To understand how the dopamine is detected on the HPLC, standards of dopamine samples were made at 2.5pg/µL, 5.0pg/µL, 10.0pg/µL, 20.0pg/µL, 40.0pg/µL, 80.0pg/µL, and 160.0pg/µL, and used to create a standard curve of dopamine. Graph 2 shows that the area under the curve obtained at the dopamine peaks increases as the dopamine concentrations increase. The graph also indicated that at levels of dopamine below 0.5pg/µL would produce unreliable results because it was composed of only noise from the HPLC machine. The r^2 obtained from the standard curve of 0.9999 indicates that the data obtained fitted the regression line and was accurate. This standard curve was used to see how the dopamine levels increased in the rats that underwent microdialysis. There was increased dopamine release in rats with incision and lidocaine injection. The incision and lidocaine model showed a 63.27% of dopamine levels at sixty minutes compared to baseline levels. At sixty minutes the incision and saline rats, sham and lidocaine rats, and sham and vehicle rats dopamine levels all decreased by 6.95% , 5.10%, and 10.62% compared to baseline (Graph 3A). Graph 3B shows that the incision lidocaine model had a 4655.76% change of dopamine overall. The data support the hypothesis that there is increased dopamine expression in the NAc shell when a rat in pain receives pain-relieving treatment.

Once, the secretion of dopamine was confirmed in the NAc shell, the brain tissue samples from each of the testing group were double stained for cFos and D1 receptor. Figure 6A indicates that there was cFos staining; however, Figure 6B shows that the D1 receptor staining only had some background. Therefore, the overlay shows only cFos positive cells (Figure 6C). The

staining of D1 receptors will need to be optimized by keeping the sample slices in primary antibody in cold room temperature for 48 hours or even 72 hours. The antibodies may not have had ample time to bind to the D1 receptors due to the slow interaction time caused by lower temperatures in the cold room. On a separate tissue D1 receptor staining did work when the tissue only received single staining of D1 as seen in Figure 7. It is possible that in double staining, using primary antibodies from the same animal may cause lack of staining. It is possible to conclude that there is dopamine release in the NAc shell, but whether the D1 or D2 receptors are activated during the release was not verified.

Figures and Graphs:

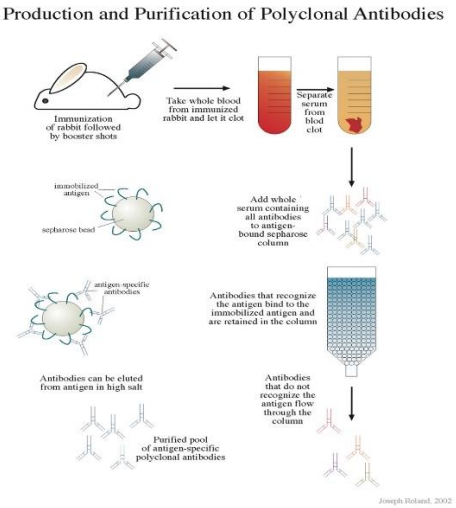


Figure 2: "Production and Purification of Polyclonal Antibodies" by Dr. Joseph Roland, 2002.[2] The image shows the sequence of the production of a specific polyclonal antibody using a rabbit (19).

[2] From "Production and Purification of Polyclonal Antibodies" by Dr. Joseph Roland, 2002, *Overhead*. Copyright 2008 by Cytographica. Reproduced with permission of the image creator. Available at http://www.cytographica.com/overheads/antibody.jpg

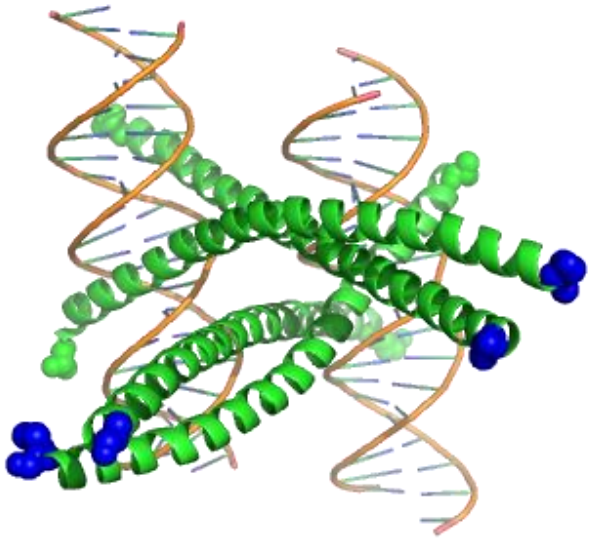


Figure 3: The cFos protein (PDB 1FOS) structure and the epitope binding sites. The blue spheres indicate the N-terminus sides of the protein where the polyclonal antibody can bind.

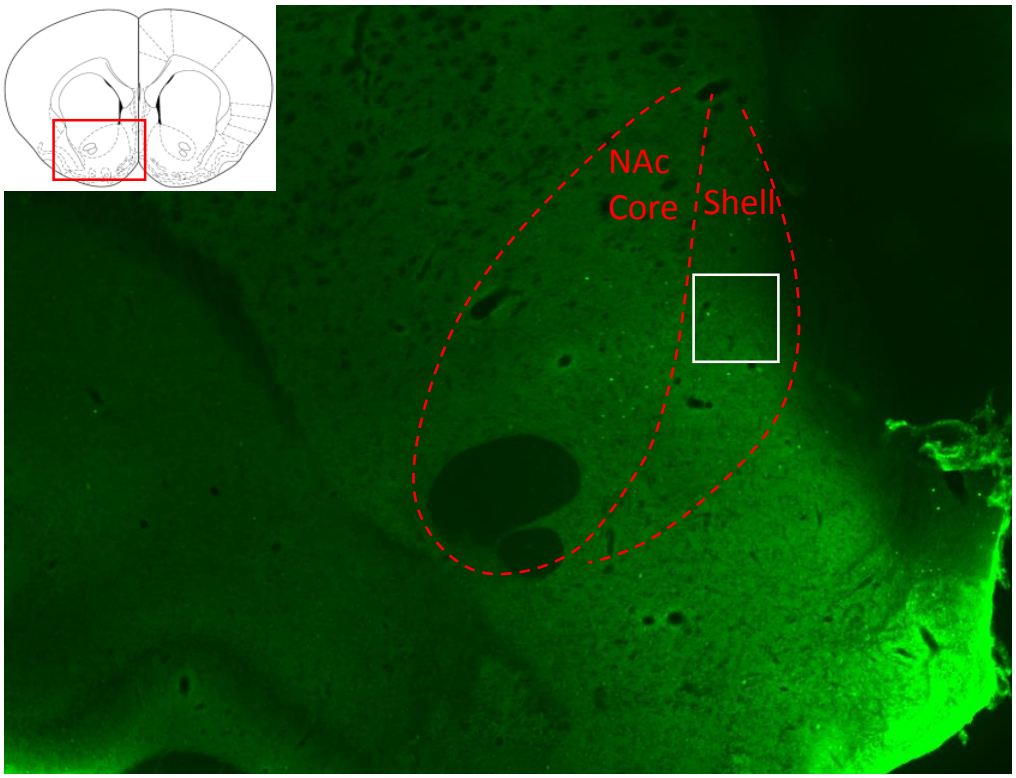


Figure 4: The cFos staining of Nac core and shell. The number of cFos positive cells were counted in the area of the square, using ImageJ and the "Anaylize Particles" method.

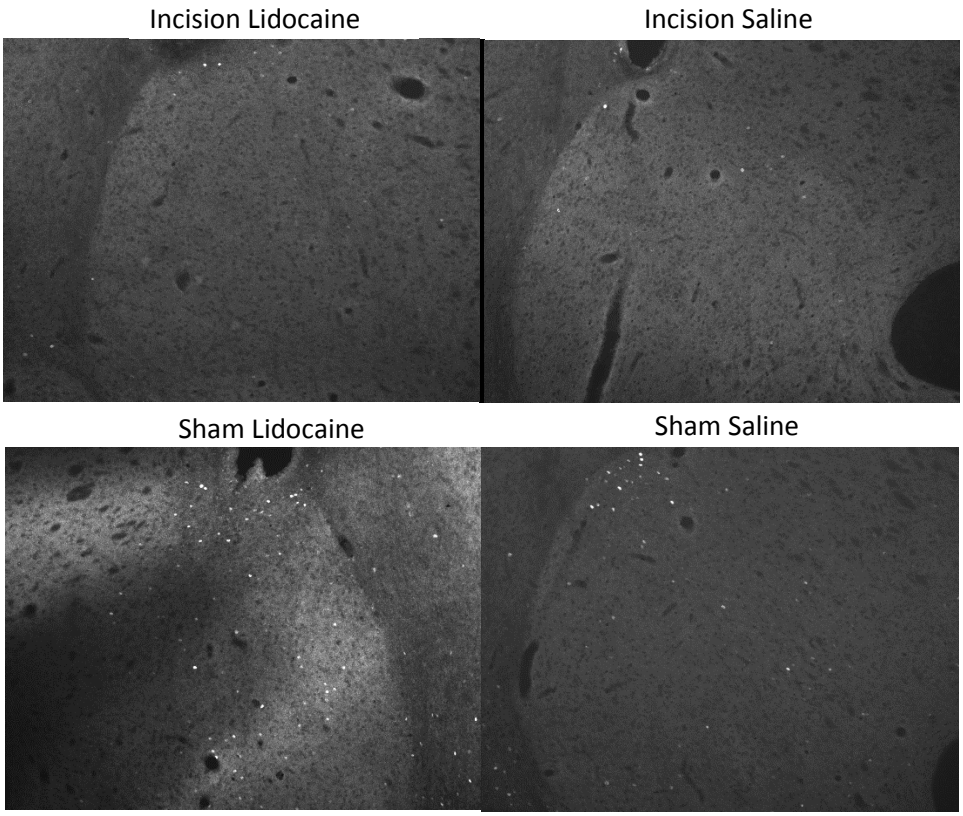
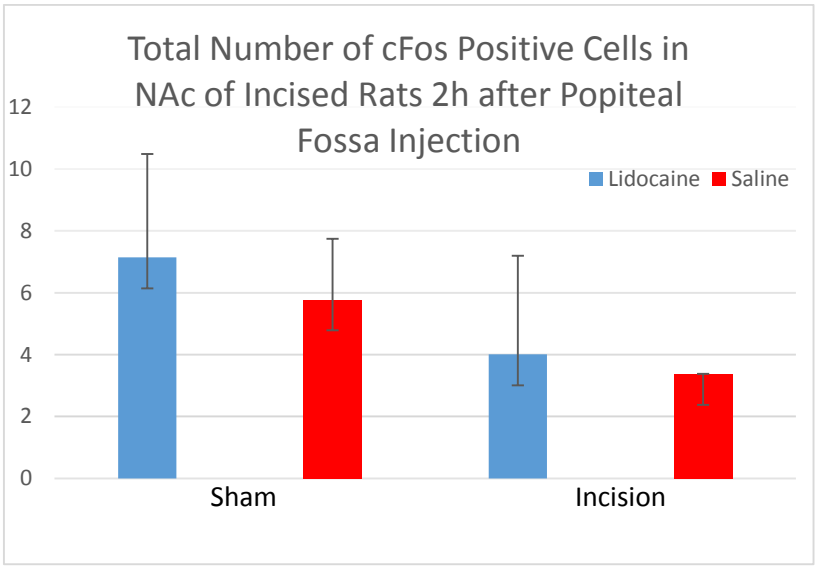
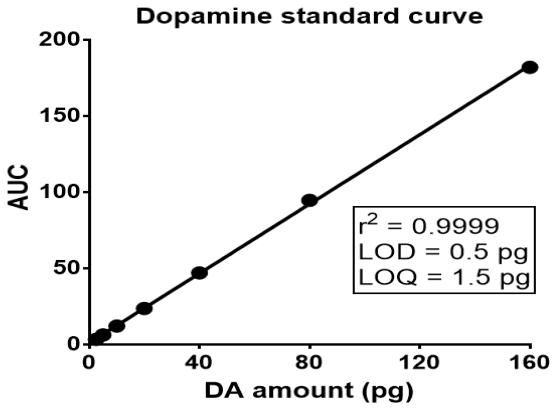


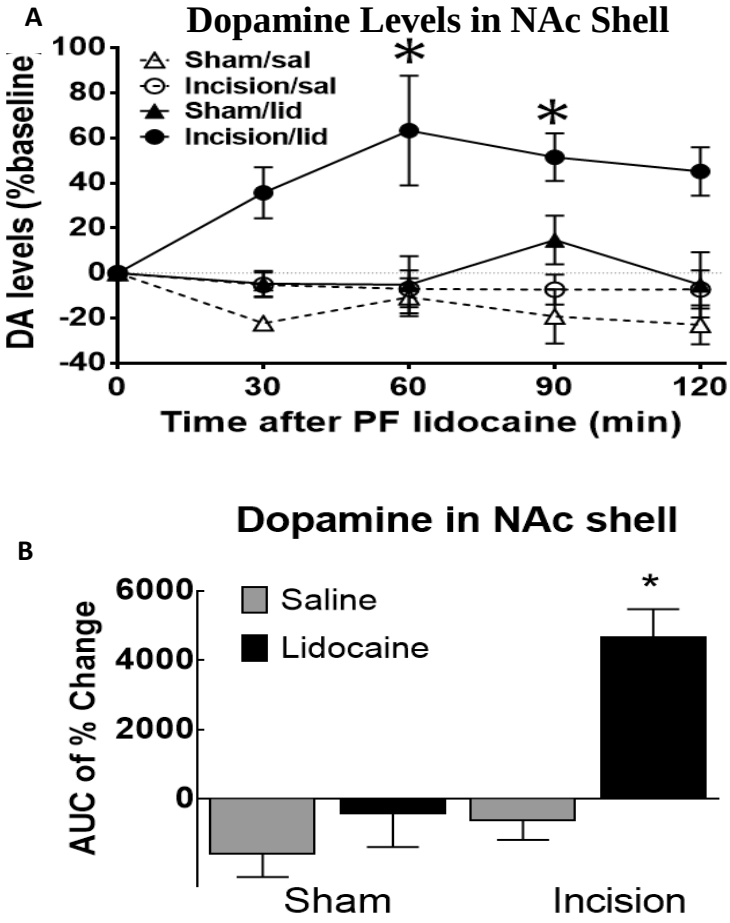
Figure 5: cFos Postive Cells from the Four Different Cases. The top left corner shows the cFos postive cells after pain relief given from lidocaine after incisions. The top right shows the cFos of the rat with incision and saline. The bottom left shows the sham rat with lidocaine, and the bottom right is the image of the NAc of the sham saline rat.



Graph 1: **The number of cFos positive cells in each treatment group.** The sham rat with lidocaine had an average of 7.14±3.33 cells, the sham rat with saline averaged about 5.78±1.96 cells. The incision rats with lidocaine averaged 4.00±3.19cells, incision rats with saline had 3.38±1.0 positive cells.



Graph 2: The dopamine standard curve determined with different dopamine samples. Different concentrations of dopamine samples were analyzed by HPLC, and the dopamine peaks were quantized by finding the area under the peaks (AUC). The limit of detection was 0.5pg, the limit of quantification was 1.5pg, and the r^2 was 0.9999. The graph shows that with increase of dopamine the (AUC) increased.



Graph 3: The release of dopamine in the NAc shell in each treatment group. In graph A, the dopamine was collected from the NAc shell with a use of a cannula. The aCSF was collected every thirty minute for two hours and analyzed using HPLC. The incision lidocaine (n=7) showed significant release of dopamine at sixty minutes (63.27% baseline) and ninety minutes (51.41% baseline). **Graph B** shows the percent change of dopamine, and the incision lidocaine animals (n=7) had a 4655.75% change and the sham saline rats (n=7) had a -1572.63% change.

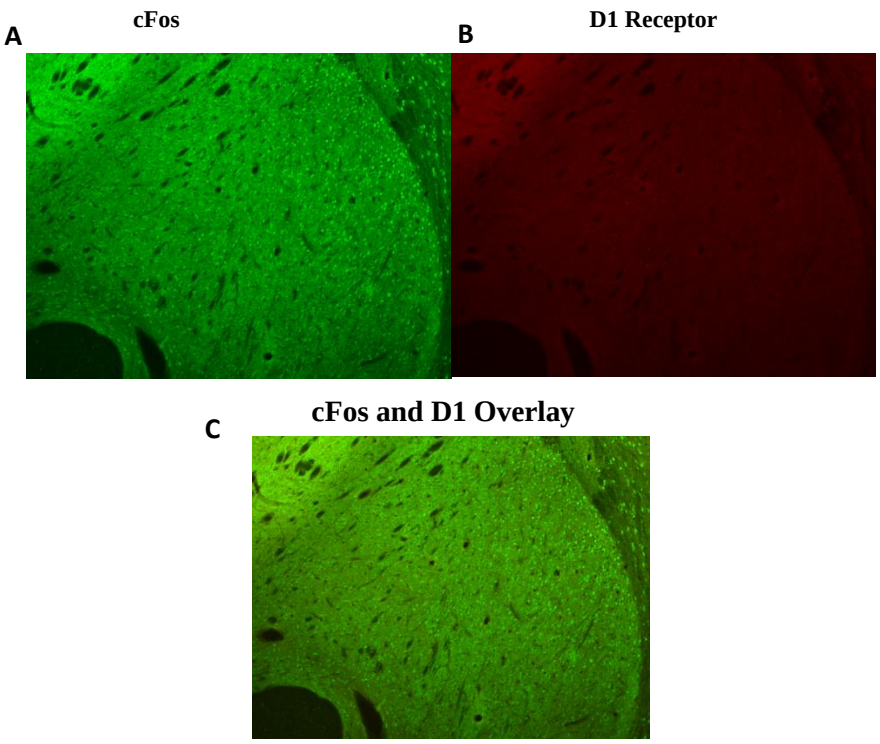


Figure 6: The double staining of cFos and D1 receptor with rabbit polyclonal antibody. Figure 6A shows the cFos staining inside the cell, 6B image is of the D1 receptor staining. Figure C shows the image of cFos and D1 receptor staining overlaid. The D1 receptor did not stain very well, so the overlay only shows cFos staining.

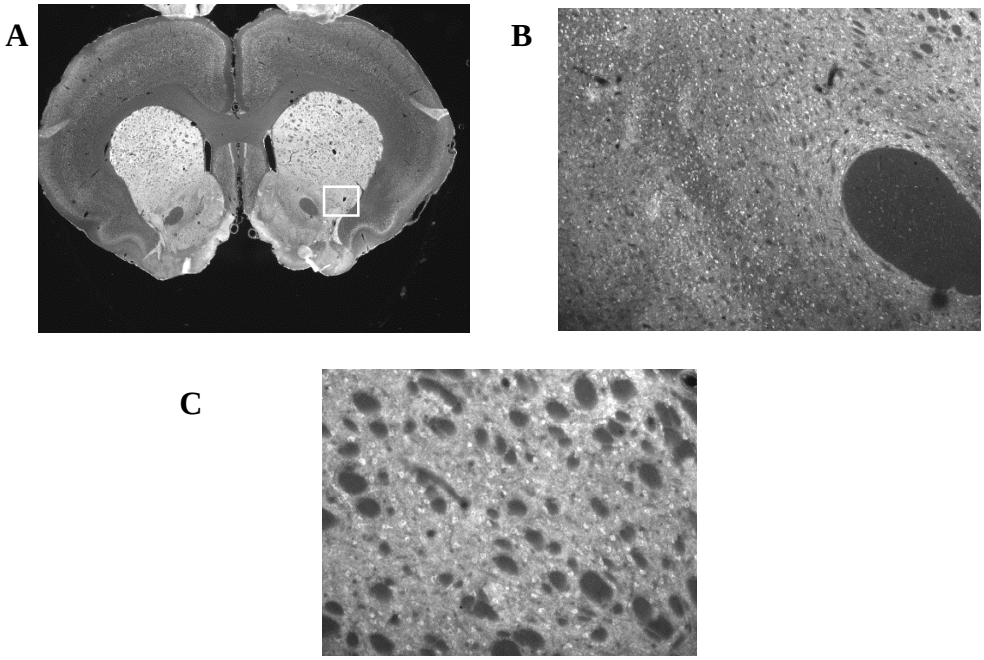


Figure 7: D1 receptor staining done on B-sap animals. Figure A shows the NAc section at 1x magnification, and depicts the anatomy of the NAc. A square section was chosen out of the 1x image and magnified. Figure B is the NAc at 10x magnification which shows D1 receptor staining in the NAc shell. Figure C shows the striatum at 20x magnification verifying that the staining is on the cell membrane instead of the nucleus.

Discussion:

Immunohistochemical techniques for cFos staining and counting were optimized to allow evaluation of the effects of pain relieving treatments on neuronal activity in the nucleus accumbens. The results indicate that there is significant amount of cFos expression from sham rats that received lidocaine injection. Unexpectedly, the rats with the least amount of cFos was indicated to be from the rats with incisions and saline injections. Previous studies that also experimented on cFos expression showed that rats with incision surgery that received pain relieving drugs stained more positively for cFos activation. Different outcomes were observed in this experiment from previous work. There were experimental differences, however, as the previous experiment had employed a pain relieving treatment that resulted from morphine microinjection into a cortical region. In the current experiment, rats received a nerve block by a local anesthetic to prevent pain signals from reaching the central nervous system. The reasons why less cFos was observed in the NAc in these rats is not clear and may require an increase in sample size and optimization of timing. This seems especially likely as the microdialysis studies showed increased dopamine release within the NAc with the same local anesthetic treatment in the incised rats (see below). The difference between the cFos expression in incision lidocaine rats and sham saline rats was not significant indicating that cFos expression in NAc is not sufficiently sensitive for evaluation of pain relief. During the time of the experiment, new primary antibody for the cFos was ordered, but the primary did not work as well as the previous primaries. One of the consequences of using the polyclonal antibody was that the animals that produce the antibodies do not live forever; hence, the new animal might produce antibodies that do not bind to as many epitopes as the previous primary. The lack of a good primary may have led to the insignificant cFos expression differences in the incision lidocaine rats and the sham

and saline rats. Another factor which may have affected the staining is the fact that the samples were left in the cold room for only 24 hours in the primary antibody. Since, the cFos proteins are inside the nucleus, it may take longer for the antibodies to enter into the cell and bind to the cFos proteins especially at cold room temperatures. The antibodies will not bind very quickly to the proteins at colder temperatures than in room temperature. Further research is required to increase the sample size of each of the treatment groups, where changes in cFos expression might be detected with pain relieving treatments.

The microdialysis shows that there is increased dopamine release in the NAc shell once the incision animal received pain relief through injection of lidocaine. To further evaluate the extent of the neuronal activity in the NAc shell, D1 and D2 receptors were stained. Unfortunately, D2 receptor staining showed little to no staining, probably due nonspecific binding of the primary antibody. The D1 receptor staining showed better results, but due to a fair amount of background and bleeding of the primary staining into the secondary staining, it was not possible to tell if the staining really was for D1 receptors. There were much more D1 receptor staining in the very caudal part of the brain, but the more proximal the brain slices got, the less staining was able to be seen. It is possible that staining for D1 receptors also needed to be placed in the primary for a longer period, so that the antibody could interact with the receptor better. Scott et al, in their experiment performed immuniohistochemistry and obtained D1 receptor staining; however, their concentration of antibody used was 1:200 (20). The concentration used in this experiment was 1:2000; therefore, there was a tenfold difference in concentration. Increasing the concentration of the antibody may also help.

A Western blot analysis can be done to observe whether or not there are D1 or D2 receptors on the cell membrane. The microdialysis done in this experiment proves that the NAc

secretes dopamine in the shell during pain relief. The Western blot can confirm the existence of D1 and D2 receptors and the levels of the receptor expression in the NAc shell. Then an antagonist of D1 and D2 receptors can be administered *in vivo* to see if the animal still receives pain relief after the block of the dopamine receptors. The cFos positive cells can be stained in order to compare the level of cFos positive cells between the rats that have wild type dopamine receptors and the rats that have blocked dopamine receptors. If the rat does experience pain relief, more cFos positive cells will be verified. Another way to verify whether or not the dopamine receptors are vital to the pain relief circuitry is by determining the effect the block of dopamine receptor has on the dopaminergic pathway. The dopamine receptors send signals to the anterior cingulate cortex (ACC); therefore, a Western blot analysis can be done to measure the signaling proteins sent from the NAc to the ACC, to verify if blocking the D1 and D2 receptors causes the stunting of pain relief.

This experiment did not test whether or not D1 or D2 receptors-expressing neurons were activated during dopamine release in the NAc. In conclusion, further experiments are needed to see if cFos expression in the NAc can be a significant indicator of pain relief activity in the brain of a rat. Increasing the samples size would give a better trend on cFos activation as well as D1 and D2 receptor positive cells. The D1 and D2 receptors can be further stained using primaries that come from different animals than the primary used to stain cFos to avoid back labeling. Better resolution images can also help in indicating whether or not back labeling is really the problem being faced in the double staining. There have been studies showing that pain relief causes dopamine release, discovering whether the D1 and D2 receptors are activated in the NAc can further the knowledge of pain relief pathway.

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