Selective Elimination of Microglia via the Cre-Lox System

A Major Qualifying Project

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Abstract

Microglia represent an emerging enigma in the field of neurobiology. While the study of these cells previously focused on their actions in an immune response, it is becoming evident that they must play a significant role in the development and maintenance of a healthy brain. In order to study these phenomena, it is necessary to test new techniques in order to study these systems. One such technique is the cre-lox system. Using cre recombinase, it may be possible to selectively eliminate microglia in the brain through the excision of the CSF1R gene, while simultaneously preserving tissue macrophage populations. In this experiment, microglia were successfully depleted without causing any significant disruption to tissue macrophages located in the liver.

Introduction

The function of microglia in the central nervous system (CNS) represents an intriguing enigma in the field of neurobiology. Previously thought to have function only in the immune response defending the CNS, these macrophages have been discovered to play a part in multiple processes regarding the defense and development of the CNS. Multifaceted as these cells are, their role in many of these processes is still being studied. Part of this investigation requires the testing and validation of individual techniques in order to further the ability to acquire definitive data on the observed variables. One avenue of microglia research seeks to demonstrate the effects on the CNS from of a loss of microglia in the organism. This experiment seeks to demonstrate

the effectiveness of the floxing technique in order to eliminate microglia from the host CNS, without disrupting the presence of microglia in the rest of the organism.

Microglia represent an important function as the exclusive macrophage CNS. As macrophages, these cells are designed to find foreign bodies in the vicinity and engulf them. This phagocytic characteristic may be a key component in understanding the functions of microglia outside of the immune system. Normally this phagocytic capability is discussed in the context of how it comes into play in an immune response. In an immune response, these cells normally attack and engulf foreign material, digest it down into smaller components, and then present these components as antigens on the surface of the cell. (1) These antigens can then be recognized by T cells, which will proliferate to combat whatever assailants are recognized by the macrophage. Microglia perform this role just as any other macrophage might.

So what makes microglia different from other macrophages? The first important disparity differentiating microglia from other macrophages is the point of origin. While all macrophages begin replication in the yolk sac before birth, almost all macrophages proliferate in the red bone marrow and are distributed to the various tissues of the body. Microglia, on the other hand, are generated solely in the yolk sac and are regenerated from the existing colony in the CNS. While most macrophages are derived from monocytes, Microglia develop from erythromyeloid progenitors in the yolk sac of the developing organism. In mice, around embryonic day 8 these embryonic microglia migrate to and colonize the CNS. This early colonization of the CNS is interesting in the scope of observing how microglia are involved in the development and organization of

neuronal tissue in the brain. After the organism is born, no more microglia are being delivered to the CNS, and the present population becomes self sustaining. (2) It is still unclear as to how the microglia maintain their population. Microglia may simply undergo regular cell division, but it is also possible that there is a specific progenitor class of microglia that contribute to the production of new cells, or there may even be a specific state the microglia undergo in which they can proliferate. (21)

In addition to the differing origin of microglia, they also have a unique range of functions in the CNS as well. There are many speculated functions for the microglia, but repeated studies indicate a few likely purposes of the cells. One extremely important finding is the implication of microglia in the process of synaptic pruning. (3) Synaptic pruning is a crucial housekeeping activity in the brain that prioritizes important neuronal connections and eliminates superfluous synaptic connections. This process is crucial in optimizing CNS efficiency. In synaptic pruning, when synapse connecting two neurons is not used frequently enough it is eliminated in favor of strengthening the synapses that are more frequently used. (4) This process is important in allowing the brain to function at it’s normal capacity. Errors in this process have been linked to multiple neurological conditions such as schizophrenia. Proper synaptic pruning is necessary in maintaining the efficiency of the brain, being necessary to retain important information and eliminate superfluous connections. In order for this to happen, some sort of signaling pathway and subsequent mechanism must exist in order to recognize and eliminate the unused synapse. This is where microglia may participate in the process.

There is a classification of proteins known as chemokines that fulfill significant signaling roles in the body. One specific chemokine pathway of interest manages the interactions between microglia and the surrounding neurons. The two protein components of this pathway are CX3CR1, located in microglia, and CX3CL1, also known as fractalkine, located in neurons. These two represent a receptor-ligand pair in a complex series of interactions known as the fractalkine signaling pathway. This is the proposed system through which microglia interact with neurons and their synapses, allowing them to regulate synaptic pruning, initiating cell death, and organizing neuronal growth and wiring. (5)(6) In the process of synaptic pruning, studies provide evidence that the expression of CX3CL1 on the surface of synapses and in the extracellular space may recruit microglia to the site to either strengthen or eliminate the connection. Engulfment assays were performed to determine whether or not the microglia were partaking in phagocytic activity against the neurons to consume and digest the unused synapses. Evidence from these experiments does in fact incriminate microglia as the probable instrument for this process. (7) (8) This is crucial evidence supporting claims and research asserting microglial involvement in neuroplasticity.

Related to this process of synaptic pruning, Microglia are also shown to be involved in the structuring and “wiring” of neurons as well. This may be a very similar process involving microglias’ phagocytic ability, however microglia have also been shown to interact with neurons via multiple trophic factors. (9) Though the exact mechanism may be unclear, microglia are absolutely necessary for the healthy wiring and organisation of the CNS. Multiple studies have been performed to demonstrate that

a loss of microglia has a direct negative impact on the brain. One method used to demonstrate this is by eliminating the macrophage proliferation gene from a mouse's genome. (10)

This gene is expressed by all macrophages, and is responsible for the generation of the colony stimulating factor 1 receptor, or CSF-1R. CSF-1R is necessary for macrophages to receive the signal to begin proliferation. (11) Without this gene, microglia will not express this receptor and will not proliferate. Despite their differences, microglia share this system with other macrophages. This means that if CSF-1R is eliminated from the mouse genome, then microglia will not proliferate. When this is applied, the affected brains do not develop properly. Studies have shown that though the brain retains its normal relative size, the structures within the brain are disrupted. This evidence provides great support to the idea that microglia play a large role in the structural development of the brain. (10) However, using this technique does have ramifications and limitations. By eliminating CSF-1R, this prevents all macrophages in the body from replenishing. This causes compromises the immune response of the host, and generally leads to weakness and frailty of the subjects. The majority of subjects have a vastly reduced life span as well. (11) Since the disruption of this gene on the organismal scale has so many other effects, it makes it very difficult to study. It becomes extremely difficult to draw conclusions on any experiment that uses this technique, as there are so many other consequences that may be influencing the results.

This is extremely problematic when trying to examine the long term effects of an absence of microglia, and eliminates the the ability to modulate when there are or aren't microglia. For example, it would be impossible to see the effects of removing microglia from an already developed brain as the subject that has a CSF1R knockout, as the microglia will be consistently depleted from the start. In addition, since the results would not be indicative of a normal brain as the subject would have developed without microglia to aid in neuronal wiring. Specimens would also likely not survive long enough to provide useful subjects. One solution to this problem is to selectively eliminate CSF-1R primarily in microglia.

This objective can be accomplished by using cre recombinase and loxP sites. This is a technique designed to selectively alter genes in cells that express another gene exclusive to the cell type. This mechanism relies on the protein cre recombinase (Cre). Cre is a protein originally isolated from a p1 bacteriophage that is responsible for genetic reconfiguration. What makes Cre such a useful tool is that it will only interact with DNA that is flanked by loxP sites. LoxP stands for locus of x-over (crossover) p1 bacteriophage, and are regions of 34 base pairs containing symmetrical flanking regions of 13 base pairs and an asymmetrical region of 8 base pairs. (12) When two loxP sites flank a sequence of DNA, depending on their directions Cre can take a number of actions, including deletion, inversion, and translocation. Since loxP sites can be added into a subjects genome, this system makes Cre an invaluable resource in any conditional knockout research, and many studies have already proven it's effectiveness. (13)

In order to apply this to research of microglia, a few steps need to be taken. First, strains of mice must be created that have the CSF-1R gene with inwards facing loxP sites. This makes it so all macrophages in the mouse could be targeted by any present Cre. Secondly, mice must also be created that contain the gene for creating Cre recombinase. In order to only be expressed in the desired cells, the Cre gene must be attached to another gene that is expressed only in the desired cell type. In the case of microglia, one of the best options to use is the CX3CR1 gene. Since this gene is expressed solely in microglia, and in sufficient number, this makes it the ideal choice. In addition, the Cre gene must also be produced with an estrogen receptor that will be used as the activator for the Cre. This allows further modulation of when the Cre can excise the CSF-1R gene. Once the two strains of mice are created, they must be bred to have offspring containing both the flanked by loxP (floxed) CSF-1R gene, as well as the CX3CR1 Cre/er gene. The only genotype of these offspring that can have CSF-1R knocked out are those that are homozygous for the CSF-1R floxed gene, and heterozygous for the CX3CR1 Cre/er gene. They must be homozygous for the CSF-1R floxed gene as if only one copy of the gene can be removed, they can still produce the colony stimulating factor 1 receptor. The mice must be heterozygous for the CX3CR1 Cre/er gene as a homozygous mouse as this can lead to certain toxic effects and unexpected genetic translocations. (12)

Once these offspring are ready for testing, a catalyst needs to activate the Cre in order for it to enter the nucleus to perform it's function. When expressed the Cre/er complex resides in the cytoplasm. When these mice are injected with tamoxifen, the

tamoxifen reacts with the estrogen receptor on the Cre/er complex allowing it to relocate to the nucleus and perform it's function. (12) Once there the Cre should excise the floxed gene, which in this case would render the microglia incapable of receiving the signal to proliferate.

This technique has many useful applications in the field of microglia research, and it's application provides our lab a few informative avenues of research. We hope to accomplish a few things by utilizing this technique. This first goal we set out to accomplish is to demonstrate the effectiveness of this technique in reducing and eliminating the population of microglia in the mouse CNS without eliminating macrophages in other organ systems. The second is to gain a more accurate estimate of the time required for the technique to eliminate a sufficient number of microglia in the mouse CNS. Further points of interest include using this technique to observe long term elimination of microglia in the mouse CNS without disrupting macrophages on the organismal level, as well as the examination of how and where microglia repopulate in the brain, as well as the time required to do so.

Goals

The goals of this experiment are based around the depletion of microglia and observing how they may repopulate in the brain. This experiment must show that the cre-lox system can cause significant depletion of microglia, without disrupting other tissue macrophages found elsewhere in the body. It should also provide preliminary

data on where the microglia may begin to repopulate, and identify possible patterns for future investigation.

Methods

Overview:

The goal of this experiment is to show that the Cre-Lox system can effectively eliminate microglia in the brain without significantly disrupting the populations of other tissue macrophages. In this experiment genetically modified mice from Jackson Labs bred to create mice of desired genotypes for these experiments. These mice were then injected over a series of days with tamoxifen to activate their cre recombinase. The mice were then retrieved and perfused at the desired timepoint. The brain as well as other tissues were retrieved for observation. The tissues were fixed using PFA, and then dehydrated with sucrose. The tissues were then sectioned on a microtome, and the gathered slices were used as the samples for imaging. Immunohistochemistry was used to fluorescently stain microglia in the tissues. These stained samples were then observed using confocal microscopy and epifluorescence microscopy. Microglia and macrophages were then counted in these tissues in various regions of the brain, and at multiple magnifications, using the program FIJI, a modified version of ImageJ.

Breeding:

Two strains of mice were acquired from Jackson Labs. One genotype of these mice contained the CSF1R gene with flanking LoxP sites, but no cre-recombinase. The

Second strain of mice had a knock in of the cre-recombinase gene into the CX3CR1 gene. These strains were then crossed, creating breeder mice containing both genotypes. These breeders were then bred to create mouse litters that contained the 3 desired genotypes. The three desired genotypes for this experiment are the knockout genotype: CSF1R Fl/Fl, CX3CR1 er/+, the wild type control: CSF1R Fl/Fl, CX3CR1 +/+,, and the heterozygous control: CSF1R Fl/+, CX3CR1 er/+. The wild type control demonstrates that without the presence of cre, then the CSF1R gene still functions normally. The heterozygous control shows a double knockout of CSF1R is necessary to cause total depletion. Only mice of these 3 genotypes were used in the experiment.

Paradigms:

Two of the paradigms tested are presented in this evaluation. The first paradigm had mice of the desired genotypes being injected with tamoxifen at postnatal days 4 through 8 (P4-P8), with retrieval and perfusion of these mice occurring on P10. The second paradigm consisted of mice that were injected days P1-P5, and retrieved and perfused on day P15. These paradigms were chosen to observe tissues at different time points post injection (2 days and 10 days,) while still showing tissues from juvenile mice.

Genotyping:

Mice were tested for the desired genotypes using a combination of PCR, gel electrophoresis, and ultraviolet imaging.

First mice were identified and tissue samples were gathered. To identify newborn mice, each had one or more of their paws tattooed to mark each individual with a unique identifier. Examples of this are tattooing just the right front paw, indicated by the designation RF, or right-front. These tattoos were performed by hand using a 30 gauge needle.

Each mouse then had a tissue sample taken from it's tail. Less than 1 cm of the end of the tail was removed to be used in the genotyping process. Immediately after tail snipping, pressure was applied to stop the bleeding (if any.)

A tissue digest was then performed on these samples. This is to break down the tissue. Making access to the genetic code possible for genotyping. Tissue samples were subjected to 75 ul of 25nM NaOH/0.2 nM EDTA, and heated at 98 degrees C in a thermocycler for 1 hour. The reaction was then neutralized using 40 mM Tris-HCl. Tubes were then vortexed lightly to homogenize the solution. These samples were then ready for genotyping.

Each sample was subjected to two sets of genotyping. One series tested for the presence of the CSF1R gene, and the other for the CX3CR1 gene. For this, a polymerase chain reaction, or PCR, was performed on each sample. PCR allows for the amplification of desired genes, replicating these sequences to quantities that can be effectively visualized on a gel. For each PCR reaction, there needs to be a sample to act as the template, polymerase to replicate the gene, primers to promote selective replication, and dNTPs to provide the transcript materials.For this experiment, 10 ul of working solution per sample were prepared for each set of genotyping. The working

solution consisted of purified water, 2x Taq RED Master Mix, and the genotype specific primers. (see appendix) 10 ul of working solution was mixed with 2 ul of each sample solution. Positive, wild type, and negative controls were also used. The PCR reaction was then run in a thermocycler. The cycle sequence can be seen in the appendix.

PCR results were then separated using gel electrophoresis. This technique involves running an electrical current through an agarose gel to separate the DNA by size. Since DNA has a negative charge, all the samples should move through the gel towards the positive charge. The gel is porous enough to allow the samples to move through it, while restricting movement enough to hold the samples long enough to allow separation of components. The DNA moving through the gel should separate based on it's size, as the larger the sequence is the slower it will move through the gel. The gel used was a 1.5% agarose gel, diluted in TAE, with ethidium bromide. (See appendix) The ethidium bromide in the gel creates fluorescence in the presence of DNA, allowing for the visualization of the samples. The amplified genes should provide a high enough concentration to create a large contrast between the gene of interest and any background fluorescence, causing the created bands to glow much brighter than the surrounding gel. The gel was also run in a solution of 1x TAE, which provides a medium for the charge to travel to and through the gel.

The PCR results were loaded into this gel, and the genes were separated by size. These results were visualized by exposing the gel to ultraviolet light. When ethidium bromide is exposed to ultraviolet light, it will fluoresce, allowing for visualization of DNA bands. The result shows bands for each sample. The distance these bands

traveled were compared to the ladder containing DNA samples of known sizes, as well as positive and wild type control bands, to discern the genotype of the sample. If the sample generated two bands, that means that both the wild type version of the gene and the modified version of the gene were present for amplification, meaning the mouse is heterozygous for that gene. If the mouse had a single band, they would be homozygous for either the wild type of modified version of the gene. This band was compared to the positive and wild type control bands to discern which gene it represented.

Tamoxifen Injections:

Once the genotypes of the mice were confirmed, the mice of the desired genotypes were selected to participate in the 2 experimental paradigms. These mice were all to be injected with tamoxifen at the designated timepoints. For each injection the mice were injected with 50 ul of tamoxifen working solution. The tamoxifen working solution consists of 1 ug/ul tamoxifen dissolved in corn oil. This solution was prepared in a 5 ml eppendorf tube by diluting a stock solution of 20 ug/ul tamoxifen in corn oil. Preparation of the stock solution was originally done by sonicating the tamoxifen in the corn oil to dissolve the particles. However, this process led to a high mortality rate in the injected mice of all genotypes. One possible reason for this is the sonication probe may have sheared plastic from the side of the preparation tube into solution. It's also possible that the heat of sonication could have altered the compounds in solution to become toxic. A new preparation method was devised in which tamoxifen was dissolved

overnight in a rotator in an incubator. This led to a drastic decrease in mortality, and became the prefered method of preparation. (See appendix)

When the mice were injected, they were held by the scruff of their neck and the tail. They were then injected intraperitoneally with tamoxifen using a 30 gauge needle. The injection site was always on the ventral posterior quadrant of the abdomen.

Perfusions:

When subject mice were of the appropriate age, they were retrieved for perfusion. Perfusion is a technique of flushing the cardiovascular system of blood with another solution. This process is important for two reasons. The first is that it removes blood that may contaminate the sample brains with cells from other parts of the body. The other reason is that it allows for the fixation of the brain using PFA. By inserting PFA into the brain using the cardiovascular system, more effective fixation can be performed. Tissue fixation using PFA is important as it helps prevent the degradation of the tissue. This is critical in preserving the tissue.

For this process, mice were anesthetized using 2.5% avertin injections. (See appendix) Approximately 0.6-0.8 ml of avertin were injected to induce an unconscious state. Mice were tested for sensation before the dissection by squeezing the feet forcefully with forceps. Once it was guaranteed that the mouse was unresponsive, the hands and feet were secured to a styrofoam surface using push pins. THe mouse was oriented ventral side upwards, with limbs outstretched. The skin of the abdomen was then removed with dissecting scissors to expose the rib cage. The ribcage was then cut

on the lateral aspects of the abdomen. Forceps were used to raise the ribcage, and dissecting scissors were used to disconnect the ribcage from the diaphragm and all other connective tissue to expose the heart and lungs. The ribcage was then fully removed. Microdissection forceps were then used to cradle and raise the heart out of thorax. A butterfly needle was then injected into the left ventricle of the heart. This needle was used to first slowly inject 12 ml of 1x PBS. (See appendix) The needle was then used again to perfuse 12 ml of 4% PFA. (See appendix) Kidney, liver, lung, and spleen tissue was then extracted and left to fix overnight in 5 ml of 4% PFA at 4 degrees celsius. The head of the mouse was then removed. An incision was made in the skin at the base of the occipital bone. The skin was then peeled forward to expose the skull. The skull was cut at the base of the occipital bone and forward through the midline along the sagittal plane. The skull was then peeled to the side exposing the brain. Microdissection forceps were then used to lift the brain by its base from the skull. The cerebellum and olfactory bulb were often retrieved, but later removed. The brain was then fixed overnight in 5 ml of 4% PFA at 4 degrees celsius.

Tissue Washing and Dehydrating:

After the tissues were allowed to fix overnight in PFA, they were then washed using 0.1M Phosphate Buffer, or PB. (See appendix) Tissues were washed in 3 sequential baths of PB, with each wash being no less than 1 minute. This step is important to remove the PFA as the fixation was finished, and in order to handle the brains the potentially carcinogenic PFA should be removed. After washing, brains were placed in 30% sucrose (See appendix) for dehydration at 4 degrees celsius. In order to

slice the brains, they need to be flash frozen. In a fully hydrated brain, this would lyse the cells and render the tissue useless. Sucrose is used to create a concentration gradient that promotes the dehydration of the tissue, allowing the tissue to be flash frozen. These tissues should float on the sucrose, as when they are hydrated they should be less dense. As they dehydrate though, they become denser and sink. Once the tissue is at the bottom of the sucrose solution it should be ready for slicing.

Microtome slicing:

The sample tissues were then sliced on a freezing microtome. This device uses a wedge shaped blade with a fine edge to slice tissues. The vertical setting of the blade can be adjusted by a set distance, which allows for consistent slice thickness. The base plate of the microtome is preemptively chilled using dry ice. Tissues were then flash frozen on dry ice and mounted on the base plate using OST. This solution is viscous at room temperature, but freezes rapidly at colder temperatures, securing the tissue in place. Slices were then taken of the tissue sample at 30, 40, and 50 um in thickness. 40 um proved to be the most reliable slice thickness while still being as thin as possible.

For the liver, kidney, lung, and spleen, the orientation of the tissue was not a largely important factor in mounting. Brains, however, were sectioned to provide coronal cross sections. The cerebellum and olfactory bulbs were removed, and brains were mounted with ventral side upwards. Slices from all tissues were preserved in 3 ml of 0.1 M PB in 24 well plates, each well containing between 3 and 6 slices. These cells were stored at 4 degrees celsius until staining.

Immunohistochemistry:

Tissues were then stained using immunohistochemistry. This process involves using antibodies to specific targets to attach fluorescent compounds to tissues for imaging. This process works by taking advantage of the multiple regions of an antibody. An antibody has both a variable and a conserved region. The variable region is different depending on the antigen that it is targeted against. The conserved region remains the same depending on the animal the antibody is derived from. By using two stages of antibodies, the cost of staining can be greatly reduced.

The way this works is that the primary antibody applied is specific to an antigen. The antibody will then bind this antigen. The secondary antibody will be used to target the conserved animal region of the primary antibody. These secondary antibodies can be made with multiple kinds of fluorophores attached. Since they bind the conserved region of the primary antibody, they can be used in multiple different kinds of staining, conserving cost. The secondary staining also has an amplifying effect, meaning that even low primary levels of antibody binding can lead to a noticeable amount of fluorescence.

The first step in staining the tissue using immunohistochemistry is to transfer the samples into a fresh 24 well plate with 0.3 ml of 0.1M PB per well. The PB was then decanted, and 0.3 ml of PBTGS (See appendix) was applied. PBTGS contains goat serum, and is responsible for blocking and permeabilizing the tissue. Permeabilization allows for larger molecules like antibodies to enter the cells of the tissue. Blocking using goat serum is used to prevent the nonspecific binding of the secondary antibody. Since

goat serum is used in the blocking phase, secondary antibodies are all goat derived. The tissue was left to incubate at room temperature for 30 minutes to 1 hour. At the end of this process, PBTGS was decanted from the samples. 200 ul of primary antibody diluted in PBTGS were then applied to each well. Concentrations of these antibodies in PBTGS were dependent on the antibody used. (See appendix) For brain tissue, antibodies to CD11b and Iba1 were used. In liver tissue staining, antibodies to F480 were used. Once primary antibodies were applied, tissues were covered and left to sit overnight on a rocker at room temperature.

The next day, primary antibody solution was decanted. Tissues were washed 3x5 minutes with 0.3 ml of 0.1M PB. 250 ul of secondary antibody, diluted 1:1000 in PBTGS, were then applied to each well. Antibodies selected were specific to the animal the primary was chosen from. (See appendix) The antibodies were also selected fluorophores that fluoresce at 488 nm, which results in a green fluorescence. Since these antibodies are light sensitive, they must be covered as much as possible. Tissues were incubated on the rocker at room temperature covered in tin foil for 1-2 hours. At the end of the incubation the secondary solution was decanted from the tissue. The samples were then washed 3x5 minutes with 0.3 ml of 0.1 M PB. Samples were then covered in tin foil then stored at 4 degrees celsius until plated.

Plating:

In order to visualize the stained slices, they had to first be mounted on a slide. Using a pipette with the tip cut to provide a wider opening, slices were moved from the well plate onto a microscope slide. Using a compound light microscope for better field of

view, slices were spread onto the the slide using two paint brushes. It is important to make sure all of the slices are unfolded so the coverslip can lay flush and the tissues are all in the same horizontal plane. Excess liquid was decanted off the slide using a pipette and kimwipe. Slides were allowed to partially dry so that only the tissue remained moist. DAPI fluoroshield was then applied to both stain nuclei in the tissue, preserve the fluorescence of the antibody fluorophores, and to help anchor the tissue for coverslip mounting. After application of the coverslip, The edges of the coverslip were coated in clear nail polish to secure the coverslip and create a seal, sterilizing and preventing contaminants from entering the slide. Slides were then stored at -20 degrees celsius until ready for imaging.

Imaging:

Stained slides were then imaged using both confocal microscopy and epifluorescence microscopy. Epifluorescent microscopy is a technique that involves reflecting lower wavelength light from a high powered light source to shine through the slides. This is ideal for measuring tissue that is fluorescently tagged in the blue or green spectrums. Since both DAPI and the green fluorophores used in this experiment are of blue and green fluorescence respectively, this is an ideal method to quickly visualize the tagged tissue. Confocal microscopy has some advantages over this technique. This method uses lasers of specific wavelengths to excite the fluorescent labels. This can yield a much higher resolution image. However, in this experiment both techniques used showed nearly identical results. Since the main goal objective was to only count the number of microglial cells, both yielded sufficient resolution to do so.

Anylysis:

The images acquired from microscopy were then analyzed in a program called FIJI, a modified version of the program ImageJ. This program allows for the splitting of fluorescent channels and modification of visual settings like contrast or brightness. This makes it easier to modify the image to accentuate cells for counting. The FIJI program also come equipped with a cell counting tool, reducing the probability of double counting or missing cells. Cell counts were done manually as opposed to using a program to assure that only cells were counted, and background interference was not attributed to the presence of cells.

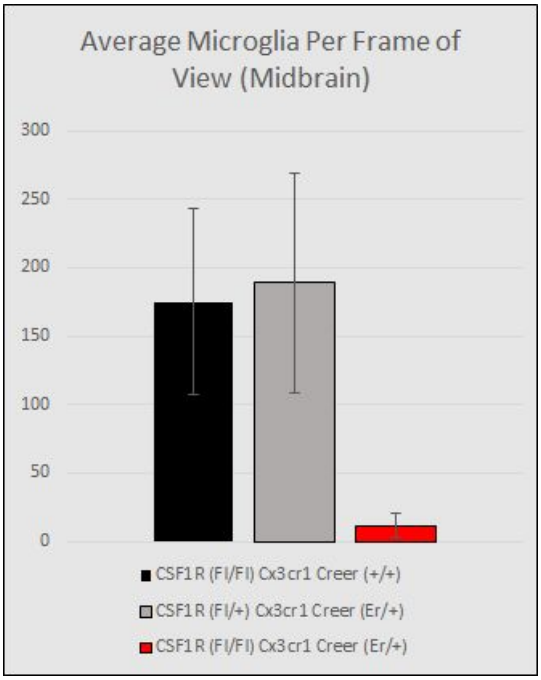
Results

Depletion:

The results of these experiments show very interesting and promising results for the cre-lox technique. Microglia cell counts were compiled from the various genotypes, and it appears evident that there is a significant reduction in microglia throughout to the brain in knockout genotype mice. (figure 1) With the average number of microglia per field of view in knockout mice being only 22, compared to the two control genotypes which boast over 150 microglia per field of view each, it can be seen that in these trials that there was a significant degrees comparing the genotypes. With the only variability between the subjects being the genotypes of the mice, it is a safe assumption that the difference is caused by the cre-lox mechanism. (Figure 1) When examining images of

the tissues as well, it can be seen clearly that there is a marked reduction in both cortex and midbrain tissues. (figure 2)

A.)



B.)

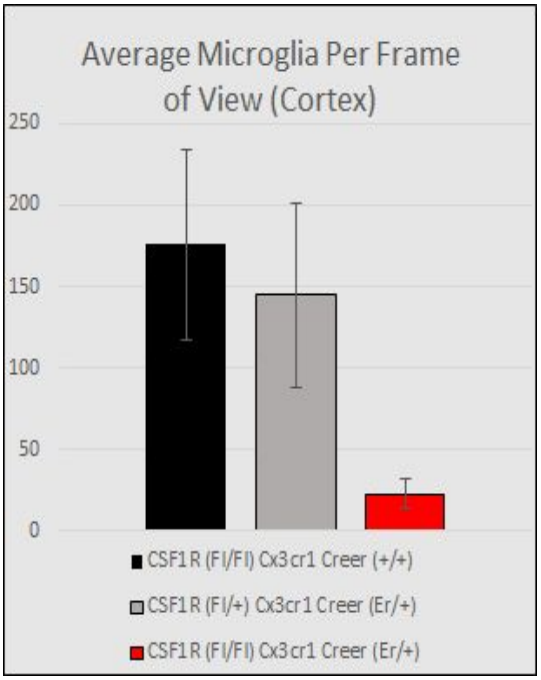
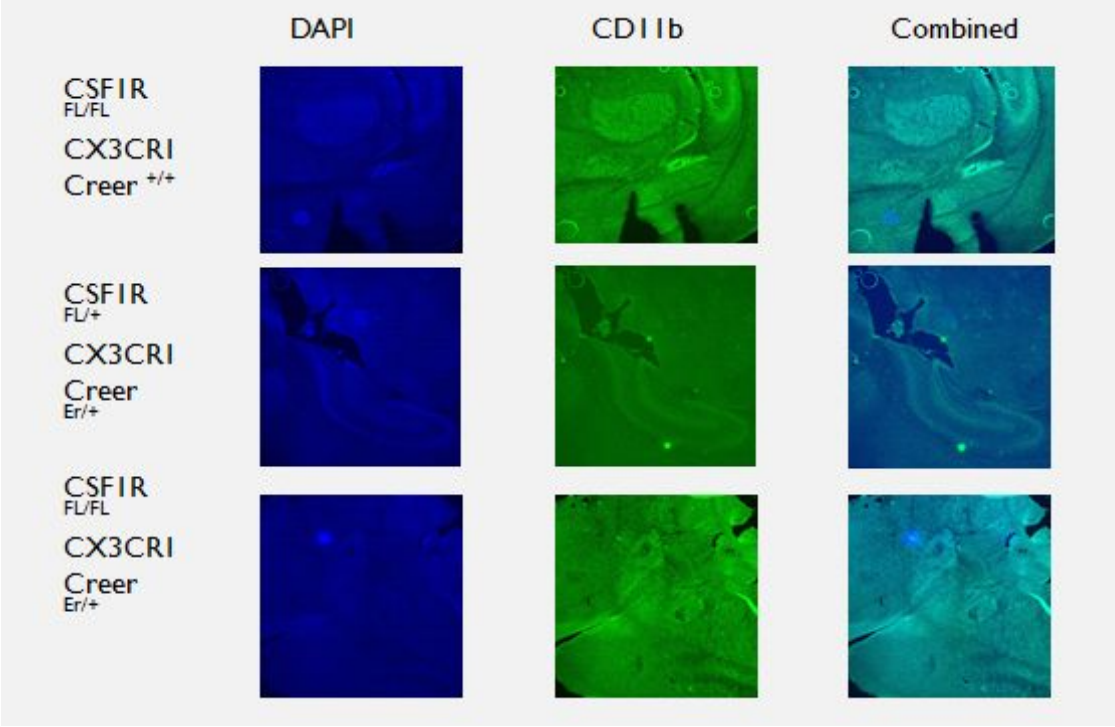


Figure 1: Depletion of microglia in desired genotypes of paradigm 1 mice, measuring microglia per field of view. A.) Paradigm 1 cortex depletion. B.) Paradigm 1 midbrain depletion.

A.)



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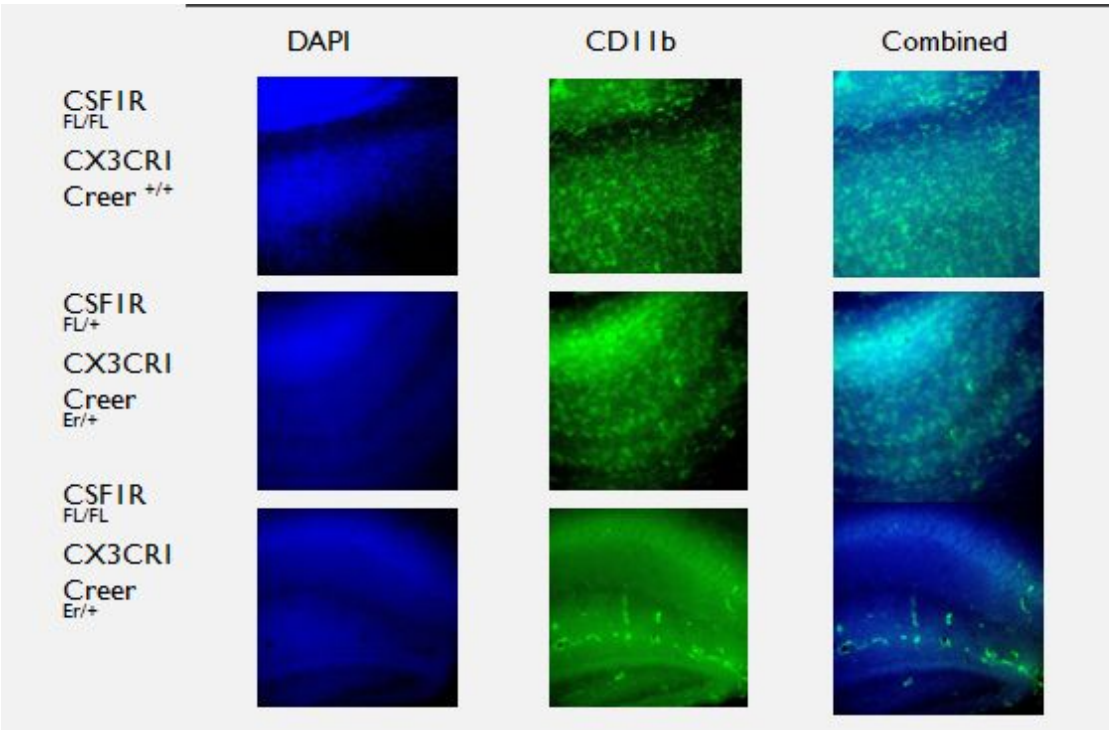


Figure 2: Comparison of paradigm 1 brains of desired genotypes stained using DAPI (blue) and CD11b (green) antibodies. A.) Paradigm 1 tissue samples observed at 5x magnification. B.) Paradigm 1 tissue samples observed at 20x magnification.

Tissue macrophages:

One important criteria in determining the success of this tool was the need to demonstrate that other macrophages in the body were not significantly affected by the application of this tool. For this, liver tissue was stained and imaged, and the number of macrophages were counted. The results from this show that there is almost no difference in the number of macrophages in these tissue samples. (figure 3) It is important to note that only liver staining proved viable for counting, so a generalization for all tissue macrophages cannot be made. However, it is significant to show that there was no reduction in the liver tissue. This provides strong support that the cre-lox system succeeds in selective elimination of microglia, however more testing should be performed to confirm this. The abundance of macrophages can be seen in images taken from both control and knockout tissues. Without counting, the difference between the two genotypes is almost indiscernible. (Figure 4)

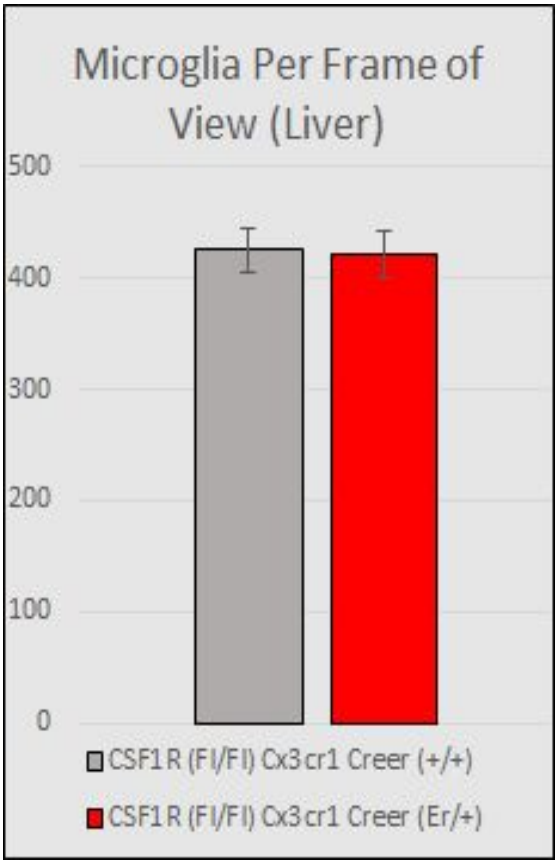


Figure 3: Comparison of tissue macrophage populations in liver samples taken from paradigm 2 mice, measured in macrophages per field of view.

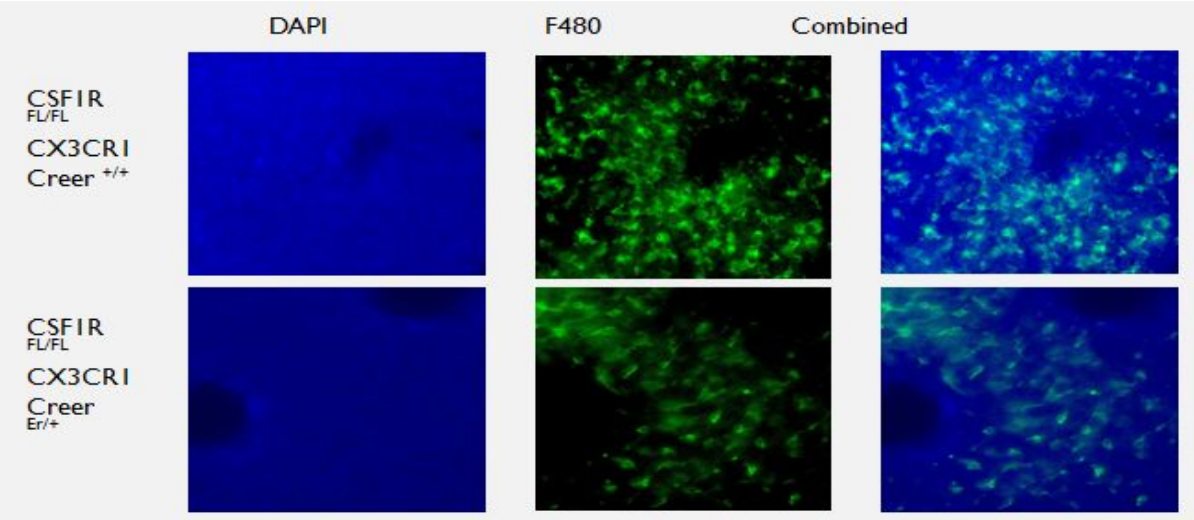


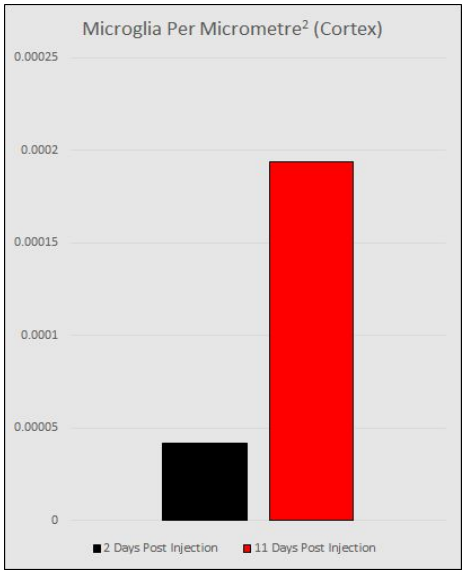
Figure 4: Comparison of paradigm 2 liver samples of knockout and control genotypes at at 20x magnifications, stained using DAPI (blue) and F480 (green) antibodies.

Repopulation:

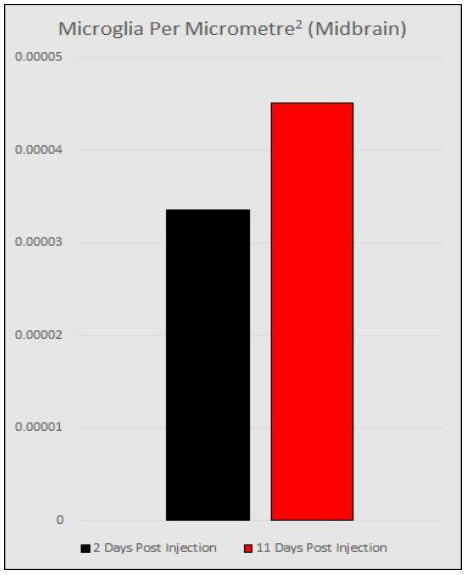
A preliminary examination of repopulation was also performed by comparing the knockout genotypes from paradigms 1 and 2 in different regions of the brain. The reason for this comparison was to give some insight into how and where the process of repopulation in the brain may occur. It is important to note that the results from this cannot be said to be strongly indicative of and single result, and is only a test to provide a potential foothold for where to begin in future examinations. That being said, the results that were obtained are very interesting and if they are representative of a trend could potentially inform a great deal about where future experiments in this field need to be focused. When comparing the two knockout genotypes, both cortex and midbrain were examined. Before examining the samples from these tissues, there were no expectations on where the repopulation may be happening, if any. However, upon

examination, the regions that needed to be compared become extremely evident. THe midbrain regions of both paradigms appeared completely devoid of microglia. However, in the paradigm 2 cortex, there was a consistent high density of microglia compared to the midbrain. (figure 5)The numbers found in these regions contested those of the control mice. (figure 5) This is an extremely interesting result, which warrants further investigation. Images from these tissues support the data acquired by cell counting. (Figure 6)

A.)



B.)



C.)

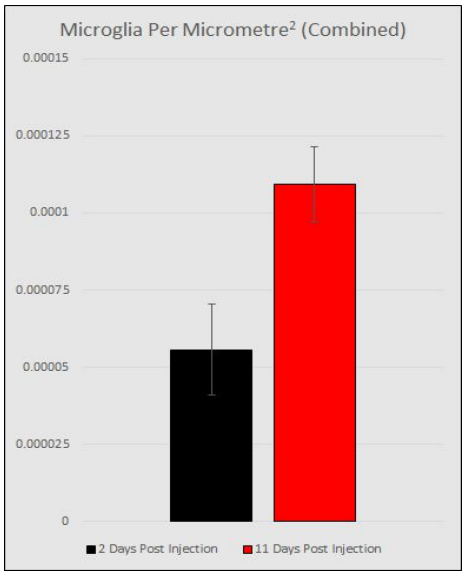
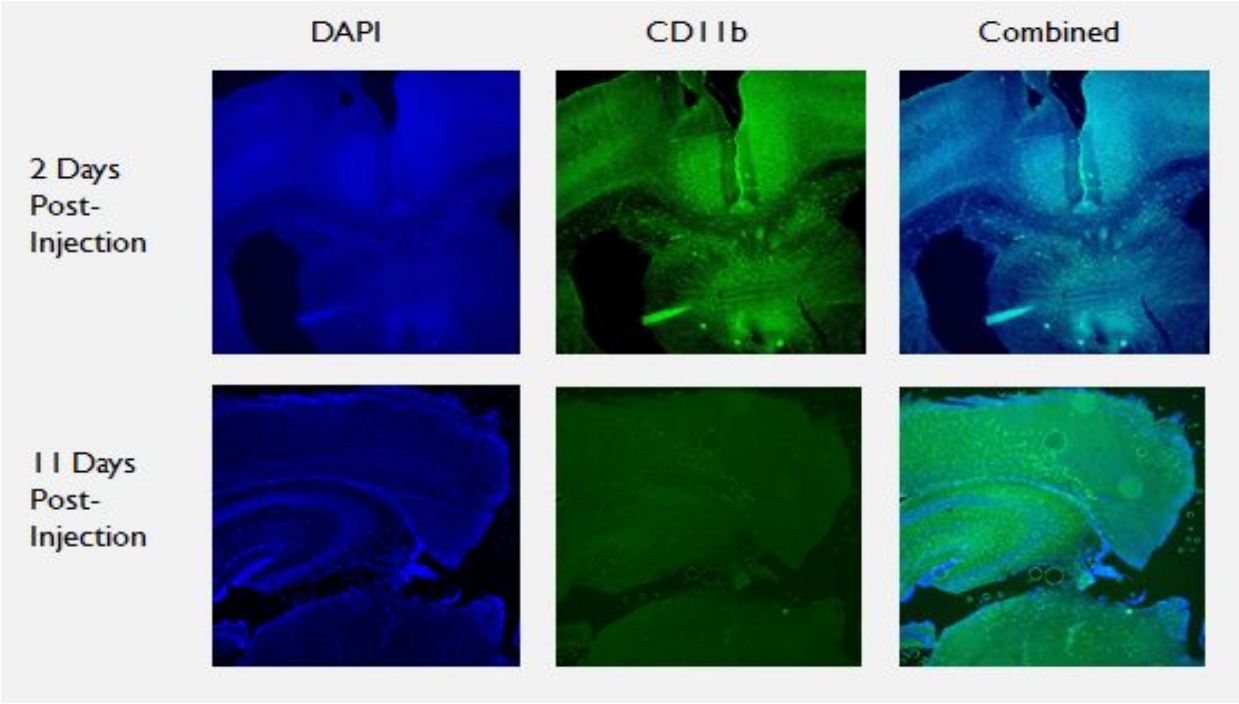


Figure 5: Depletion of microglia in knockout genotypes of mice from paradigms 1 and 2, measuring microglia per square um. A.) Cortex microglia count. B.) Midbrain microglia count. C.) Combined microglia count.

A.)



B.)

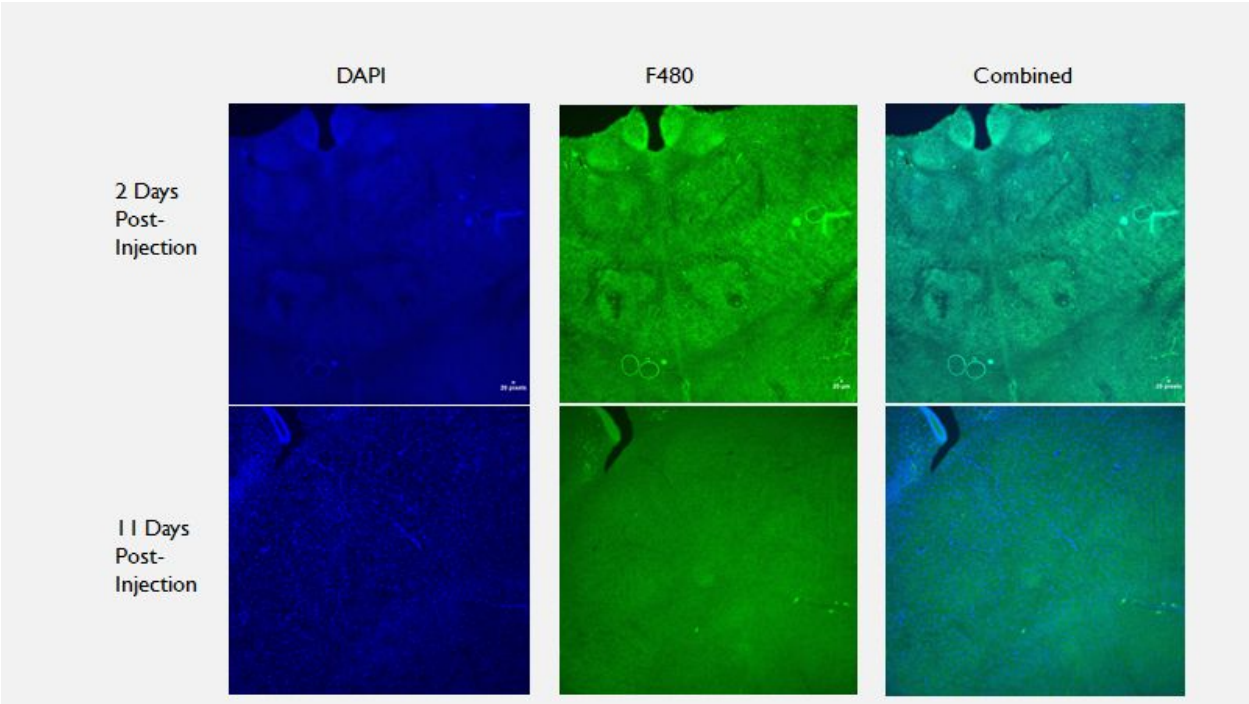


Figure 6: Comparison of knockout mice of paradigms 1 and 2 of cortex and midbrain tissue samples, stained using DAPI (blue) and CD11b (green) antibodies. A.) Cortex tissue samples from knockout paradigms. B.) Midbrain tissues from knockout paradigms.

Discussion

The study of microglia in a healthy central nervous system is a field shrouded in enigma and uncertainty. There are many things that are simply not known about these unique cells, and many things that can only be speculated about. One thing that is certain, however, is that these cells are vitally important, and that their role in the healthy brain may still be underestimated. This experiment demonstrates a trial of a technique that could provide insight and answers to many of the questions that still need to be answered.

The results from this experiment show promise in that the technique is likely effective. However, though the results are promising, more validation is needed. The cre-lox system is well established in it's own right, a fortunate fact that may relieve some of the burden to prove it's worth in this application. With all new techniques, however, there are many aspects that need to be tailored to the situation in which they are applied. During the process of this trial, there was a degree of troubleshooting that had to be performed to enhance the efficacy of the system.

The first hurdle that needed to be overcome is increasing the survival rate of mice in the trial. In the early stages of this experiment, many mice were dieing before the retrieval timepoint. Others appeared sickly and weak as well. Trials were run where the remaining genotypes of each litter that weren't desired for the experiment were injected with only corn oil, and no tamoxifen, to determine whether that was the problem. With an almost zero fatality rate for those mice, it was determined that perhaps

the preparation of the tamoxifen was causing these fatalities. By changing the method of dissolution in the corn oil from sonication to incubation on a rotator, the survival rate became nearly 100% in all trials. It is possible that the sonication was shearing pieces of plastic from the inside of the eppendorf tube, which would then be harmful to the mice when injected. It's also possible that the head from sonication could cause the alteration of the chemicals in solution to become toxic. A third possibility is that the sonication created free radicals in solution that would also be toxic to the subject. Any combination of these three effects could be possible, or there could be an unknown effect of sonication that rendered the solution harmful to the mice.

Another issue to be confronted was the staining of tissue macrophages. While CD11b and Iba1 seemed to stain microglia extremely well, using them to stain the tissue macrophages proved less successful. Since F480 was used to stain liver macrophages in the past, it was implemented as the antibody of choice in this case as well. Though this was only shown to work in the liver in this experiment, future tests should provide more answers on how best to stain for tissue macrophages in conjunction with these techniques.

With these two main issues being addressed, this experiment provides grounds for future experiments. One of the critical questions this technique is being designed to answer is where do microglia replenish from? Microglia have been seen to be dividing on occasion, however it has already been shown that the rate of repopulation is far too high to be attributed to an unmodified pattern of cellular division. (21) The results of the knockout comparison shed some light onto where studies may begin to examine these

repopulation patterns. It appears that in cortex, specifically in the white matter tracts, there is a high density of repopulating microglia at later timepoints. Comparing the brains that were retrieved 2 days after last injection and brains retrieved 10 days after last injection, it appears that at some point between these two times repopulation began at a rapid rate.

Based on these results, there are a host of future experiments that should be performed to narrow down this question. The first experiment that will be crucial to this is to generate a litter of mice that consists only of knockout genotypes. If there is not enough data at that time to establish this technique as effective in depopulating microglia, a litter of non knockout genotypes could be generated as a control comparison. The knockout litter would undergo a series of tamoxifen injections, and every day following the final injection a mouse should be retrieved and examined. This would provide a scale of time points showing an estimation of when and where repopulation will begin. This experiment could be performed again using shorter time points centered around the observed repopulation timepoints.

Another experiment that should be performed is examining the depletion of microglia over time. This should be performed exactly as the previously described experiment, but mice should be retrieved starting on the first day of injection. This should provide information on where microglia are depleted first, and in which regions they remain the longest, if any. It would also be pertinent to examine how long it takes for tamoxifen without the knockout genotype present to affect the health of the subject. Over shorter time points there does not seem to be a drastic effect, however the

long-term effects should be studied first hand to provide references when creating new protocols.

Lastly, more trials should be performed to examine tissue macrophage colonies throughout the body to confirm that the technique does not disrupt other tissues. This can be done upon retrieval, but another possibility is to use punch biopsies to recover tissue from living specimens over the course of the trial. This would be invaluable data, as it comes as close to real time imaging in a host as our technology allows.

With modern technology it is possible to observe many phenomena in real time. One avenue of approach may be to examine the CNS in real time using MRI technology. In it's current state there are significant limitations though. It may be extremely difficult to get an accurate enough image to be able to count microglia, if it's even possible. Though the technology may not be advanced enough yet, real time imaging of microglia in the brain will lend enormously to this field of research. With static images, it is very difficult to say for certain what these cells are doing at any given point in time. Growing these cells in vitro, as well, shows vastly different morphology from microglia found in vivo.

Hindrances of technology aside, the current techniques should prove sufficient to answer many of the broader questions on the topic. While the field is still establishing many of these answers, the array of other research they may assist is large. For example, there are many speculations about how microglia are involved in diseases like alzheimer's. Techniques such as this may prove useful in manipulating alzheimer's systems to find out what role microglia actually perform in these diseases. This applies

to conditions like schizophrenia as well, where microglia are shown to be exceedingly active. Studying their role in these neurological conditions may well inform how they may function in a healthy brain as well. As stated before, the studies of how microglia are involved in synaptic pruning and neuronal wiring are proving hot topics in the field, and will prove to be significant discoveries if confirmed and elucidated.

Conclusions

There are two major conclusions that can be drawn from the data provided in this experiment. The first is that the cre-lox system, when applied in this way, does have to potential to consistently eliminate microglia from the brain. The data shows a clear and drastic decrease in cell counts comparing the control and knockout genotypes which can be seen both through visualization and quantitation. This evidence is sufficient to advise further testing of the technique, and validates using this technique and supporting the results gained from doing so.

The second major conclusion that can be drawn is that the cre-lox system does not severely affect tissue macrophages in the liver. This shows that when the technique is applied, specimens should retain normal function other than the disruption to the microglia population. This experiment should allow for the use of this technique with minimal concern as to the animal's health other than the depletion of microglia.

The last conclusion that should be drawn from this experiment is that though this technique works, there may still be ways to improve it. With new resources becoming available, some of these methods may rapidly become outdated or replaceable. It is

important to continue adapting and perfecting the techniques used here, while still seeking out other techniques to improve the methodology. There are many more questions that need to be answered, but the utilization of the cre-lox system may prove invaluable in bringing to light the enigma that is the microglia.

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Appendix

Appendix I: Genotyping Primers and Cycles

JAX Cx3cr1 CreER	*#Rxns*	*8*	*CycleName:*		*Kapp Cycling*	
Reaction			*Cycling*			
Reaction Component	*Volume (µl)*	*Total Volume (µl)*	*Step #*	*Temp °C*	*Time*	*Note*
ddH2O	*3.1*	*24.8*	*1*	*94*	*2 min*	*-*
2X Taq RED Master Mix	*6*	*48*	*2*	*94*	*20sec*	*-*
20 uM 12266	*0.3*	*2.4*	*3*	*65*	*15sec*	*-0.5 C per cycle*
20 uM 14314	*0.3*	*2.4*	*4*	*68*	*10sec*	*-*

20 uM 16221	*0.3*	*2.4*	*5*	*-*	*-*	*repeat steps 2-4 for 10 cycles*
DNA	*2*		*6*	*94*	*15sec*	*-*
			7	*60*	*15sec*	*-*
Total	*12*		*8*	*72*	*10sec*	*-*
Total w/out DNA	*10*	*80*	*9*	*-*	*-*	*repeat steps 6-8 for 28 cycles*
			10	*72*	*2 min*	*-*
			11	*10*	*-*	*hold*

Expected Results:	***Mutant = 300 bp***					
	Heterozygote = ~300 bp and 695 bp					
	Wild type = 695 bp					

CSF1R fl/fl	***#Rxns***	***14***	***CycleName:***		***sst cre***	
Reaction			***Cycling***			
Reaction Component	***Volume (µl)***	***Total Volume (µl)***	***Step #***	***Temp °C***	***Time***	***Note***
ddH2O	***3.4***	***47.6***	***1***	***94***	***2 min***	
2X Taq RED Master Mix	***6***	***84***	***2***	***94***	***20sec***	
16422	***0.3***	***4.2***	***3***	***65***	***15sec***	***-0.5 C per cycle decrease***
26825	***0.3***	***4.2***	***4***	***68***	***10sec***	
DNA	***2***		***5***			***repeat steps 2-4 for 10 cycles***

			6	***94***	***15sec***	
Total	***12***		***7***	***60***	***15sec***	
Total w/out DNA	***10***	***140***	***8***	***72***	***10sec***	
			9			***repeat steps 6-8 for 28 cycles***
			10	***72***	***2 min***	
Expected Results:	***Mutant ~273 bp***		***11***	***10***		***hold***
	Heterozygote = ~200 bp and 465 bp					
	Wild type = 193 bp					

Appendix II: Electrophoresis Gel Preparation

1.5% Agarose Gels for the Gel Trays in the Schafer Lab
(NB - these should make gels 0.5cm thick)
7 x 10 cm (the tiny tray)

30mL 1x TAE
.45g Agarose
7.5uL 1% EtBr
Use the small flask, microwave in increments of 15 secs, check and swirl between (usually takes 45s total)

15 x 15 cm (the smaller big tray)

100mL 1x TAE
1.5 g Agarose
25uL 1% EtBr
Use the larger flask, microwave in increments of 30 secs, check and swirl between (usually takes 90 - 120s total)

15 x 25 cm (the larger big tray)

180mL 1x TAE
2.7g Agarose
45uL 1% EtBr
Use the larger flask, microwave in increments of 30 secs, check and swirl between (usually takes 120 - 180s total)

Appendix III: Tamoxifen Preparation

Tamoxifen Catalogue Number: Sigma T5648-1G
Concentration: 50 mg/kg, 50ug/ul
Tamoxifen prepared in 5 ml eppendorf tube
Stock solution generated from Tamoxifen diluted into corn oil.
Add tamoxifen to 20 ug/ul, dissolve overnight on rotator in incubator.
Dilute Stock solution to a concentration of 50ug/50ul to create working solution.

Appendix IV: Avertin Protocol

Materials:

- 2,2,2-tribromoethanol (97%) (Avertin)
- Tert-amyl alcohol, reagent grade

Preparation of stock solution

1. Add 6.2 ml tert-amyl alcohol to 10g of Avertin. Concentration of stock will be 1.61 g/ml
2. Stir Avertin on a magnetic stirrer overnight.
3. Store stock in dark bottle, tightly capped, and at room temperature. The stock solution is stable for 6-12 months.

Preparation of 2.5% Working Solution (To make 50ml)

1. Recommended: pre-treat a 50ml graduated cylinder with 10% HCL to remove detergent residue. Thoroughly rinse the cylinder with Milli-Q water.
2. Add 49.2ml 1X PBS to the cylinder.
3. Add 0.78ml of Avertin stock solution.
4. Wrap with aluminum foil to exclude light.
5. Stir slowly on heat overnight to dissolve Avertin stock solution.
6. Filter the working solution through a 0.2 micrometer filter.
7. Store at 4°C. Working solution is stable for 6 months.

Appendix V: Stock Solutions

Solutions

10X Phosphate Buffered Saline (PBS)

For 1000mL (1L):

To 900 mL ultrapure water on stirrer add:
80g NaCl (sodium chloride)
2.0g KCl (potassium chloride)
14.4 g Na_2HPO_4 (dibasic phosphate)
2.4 g KH_2PO_4 (monobasic potassium phosphate).
Once dissolved adjust pH to 7.4 with 10N HCl. Adjust volume to 1L with additional distilled ultrapure water.

1XPBS
For 1000mL (1L): 100 mL 10X PBS and fill to 1000mL with ultrapure water.

0.2M Monobasic Phosphate Buffer (PB-A)
For 500 mL: Measure 13.8 g Monobasic Phosphate into 400 mL ultrapure water.
Stir over low heat.
Once dissolved, fill to 500 mL with ultrapure water.

0.2M Dibasic Phosphate Buffer (PB-B)
For 1000 mL (1L):
Measure 28.4 g Dibasic Phosphate into 900 mL ultrapure water. Stir.
Once dissolved, fill to 1000 mL with ultrapure water.

0.1M Phosphate Buffer (PB)
For 1000 mL (1L):
95 mL PB-A
405 mL PB-B
Fill to 1000 mL with ultrapure water

10% TritonX 100 (TX-100) Stock
For 10 mL:
Add 1 mL TX-100 to 9mL 0.1M PB
Store at RT

PBTGS
For 5 mL:
-Add 0.5 mL goat serum and 150ul 10%TX-100 to a 15 mL falcon tube
-Fill tube to 5 mL with 0.1M PB.
*Store at 4 degree and use within 3 days.

Appendix VI: PFA Preparation

4% PFA diluted in sterile filtered PBS:
16% EM grade diluted in PBS to 4%: EMS catalog #30525-89- 4.

Appendix VII: Antibodies

Primary Antibodies

Anti Iba1	Glia Box 1	5	Abcam	ab139590	Chicken	1:1000
Anti Iba1	Glia Box 1	6	Abcam	ab107159	Goat	1:1000
Anti Iba1			abcam	ab5076	Goat	1:1000
Anti Iba1	Glia Box 1	2	Wako Chemicals	019-19741	Rabbit	1:500
Anti Mouse CD11b	Glia Box 1	1	AbD Serotec	MCA711G	Rat	1:200

Secondary Antibodies

Goat anti-Guinea Pig IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate			Thermo Fisher	A-11073	Goat	1:1000
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate			Thermo Fisher	A-11029	Goat	1:1000
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate			Thermo Fisher	A-11034	Goat	1:1000
Goat anti-Rat IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate			Thermo Fisher	A-11006	Goat	1:1000
Donkey anti-Chicken (H+L) Alexa Fluor 488 conjugate	Fluorescent Protein Box 1	11	Sigma	SAB4600031	Donkey	1:1000
Donkey anti-Chicken IgY(IgG) (H+L) Alexa Fluor 488-conjugate	Alexa 488 Box #1		Jackson ImmunoResearch	703-545-155	Donkey	1:1000
Donkey anti-Goat IgH H&L Alexa Fluor 488	Alexa 488 Box #1					
Goat anti-Guinea Pig IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate			Thermo Fisher	A-11076	Goat	1:1000
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate			Thermo Fisher	A-11032	Goat	1:1000

Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate			Thermo Fisher	A-11037	Goat	1:1000
Goat anti-Rat IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate			Thermo Fisher	A-11007	Goat	1:1000
Donkey anti-Goat (H+L) Secondary Antibody, Alexa Fluor 594 conjugate			Thermo Fisher	A11058	Donkey	1:1000
Goat anti-mouse IgM (μ chain) Secondary Antibody, Alxa Fluor 494 conjugate			Thermo Fisher	A21044	Goat	1:1000
Goat anti-Guinea Pig IgG (H+L) Secondary Antibody, Alexa Fluor 647 conjugate			Thermo Fisher	A-21450	Goat	1:1000
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 647 conjugate			Thermo Fisher	A-21236	Goat	1:1000
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 647 conjugate			Thermo Fisher	A-21245	Goat	1:1000
Goat anti-Rat IgG (H+L) Secondary Antibody, Alexa Fluor 647 conjugate			Thermo Fisher	A-21247	Goat	1:1000
Alexa Fluor® 647 AffiniPure Donkey Anti-Guinea Pig IgG (H+L)			Jackson ImmunoResearch	706-605-148	Donkey	1:1000
Donkey anti-Goat IgG (H+L) Secondary Antibody, Alexa 647 conjugate	Alexa 647 Box #1		Invitrogen	A21447	Donkey	1:1000
Anti-Mouse IgG (H+L) HRP	HRP-Conjugated Box1	4	Bio-Rad	170-6516	Goat	1:10000
Anti-Rabbit IgG (H+L) Horseradish Peroxidase-conjugated	HRP-Conjugated Box1	3	Bio-Rad	170-6515	Goat	1:10000

Goat IgG Horseradish Peroxidase-conjugated	HRP-Conjugated Box1	1	R&D Systems, Inc.	HAF005	Donkey	1:10000
Rat IgG Horseradish Peroxidase-conjugated	HRP-Conjugated Box1	2	R&D Systems, Inc.	HAF109	Goat	1:10000

Appendix VIII: Tissue Digest

Reagents:

25mM NaOH/0.2mM EDTA

20uL 0.5M EDTA
125uL 10M NaOH
50mL H_2O
pH should be approx 12

40mM Tris HCl

242.28mg Tris Base
15-25mL H_2O
pH to 5.5 with HCl
add H_2O to volume of 50mL