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Exploring the Potential Role of Reactive Astrocytosis in Cognitive Impairments from Early Life Seizures

Harriet Milligan

Honor Thesis

University of Vermont
Abstract

Childhood epilepsy is often associated with extreme cognitive deficits later in life. Using the repeated flurothyl model, there is the potential to study the behavioral deficits and discover the underlying cause of the cognitive deficits. Reactive astrocytosis, which has been connected to other disease models, was assessed as a potential cause for the behavioral deficits. A twenty-seizure flurothyl protocol was used on C57BL/6J mice at postnatal day ten. Mice either went through a behavior battery once developed or be used for immunohistochemistry one day post seizure, using the glial fibrillary acidic protein (GFAP) marker for reactive astrocytosis. Images were then analyzed using a thresholding code in MATLAB. The mice showed both fear extinction deficits and increased anxiety-like behavior. No differences in astrocyte intensity were found when comparing the seizure and control animals. This suggests reactive astrocytosis may not cause the cognitive deficits observed, but this could be due to intensity not correctly quantifying the differences is astrocytes or a different biological cause all together.
**Introduction**

Epilepsy is one of the most prevalent childhood neurological disorders and causes a high burden for the child and their family (*NEUROLOGICAL DISORDERS public health challenges*, 2006). It is estimated that over 2.2 million people are living with some form of epilepsy in the United States, and onset often occurs in childhood (England, Liverman, Schultz, & Strawbridge, 2012). Many children who have epilepsy also have many cognitive and behavioral deficits, even if the seizures are treated or go into remission, therefore negatively impacting their quality of life (England et al., 2012). Around 80% of school-aged children with active epilepsy were found to have a behavioral disorder and/or cognitive impairment, with attention-deficit/hyperactive disorder (ADHD) and autism spectrum disorders (ASD) being the most common (Reilly et al., 2014). It was also found that a majority of children with epilepsy showed delayed global development and deficits in adaptive behavior (Reilly et al., 2019). Mental health conditions, including anxiety have also been associated with epilepsy (Holmes, 2015; Reilly et al., 2014). By using a rodent epilepsy model, these cognitive and behavioral effects can be studied. The flurothyl model has been used to induce epileptogenesis in mice and study the behavioral effects and underlying mechanisms (Kadiyala & Ferland, 2017; Kadiyala et al., 2016).

**Flurothyl Model**

Early life seizures (ELS) are often associated with cognitive deficits later in life. Flurothyl, a volatile chemoconvulsant and GABA<sub>A</sub> antagonist, is used to assess the effect of seizures in animal models (Ferland, 2017). Repeated flurothyl exposures in young mice can cause behavioral deficits and likely epileptogenesis, i.e., changes in the brain that result in the onset of chronic epilepsy (Dichter, 2006). Such epileptogenic models have been proposed as the most effective in searching for new treatments (Löscher, 2002). There are number of traditional
rations for using flurothyl as a chemoconvulsant. First, since flurothyl is highly volatile, the animals are able to inhale the chemoconvulsant in a chamber, which eliminates the need for an injection. Second, unmetabolized flurothyl is quickly eliminated by the body, which reduces the ability for residual chemicals to confound results. Third, the length of the seizure can be controlled by opening the chamber as exposure to external air can ease the seizure (Ferland, 2017). Furthermore, flurothyl can induce cognitive deficits without causing gross anatomical differences or cell death (Holmes, 2016; Holmes et al., 2015; Landrot, Minokoshi, Silveira, Cha, & Holmes, 2001). However, the mechanism by which flurothyl causes cognitive deficits are unknown. In this study, we hypothesized that ELS triggers a response of astrocytes in the brain, as one potential mechanism of cognitive impairment.

*Astrocytosis*

Like the rest of the body, the central nervous system (CNS) has an elaborate immune response to various insults and injuries. This response shows the complexities of the CNS as well as the importance of glial cells. The immune response to trauma or infection usually stems from leukocytes, but chronic damage to the CNS, as found in many diseases, involves astrocytes and microglia (Liddelow & Barres, 2017). Astrocytes make up around 30% of the cells in the CNS, and their star-like shape allows them to have a variety of integral roles, including the regulation of synapses through formation, function, and elimination (Allen & Lyons, 2018).

Reactive astrocytosis, or the abnormal increase in the number of astrocytes in an area of the brain, has been shown to occur after trauma to the brain such as traumatic brain injury, stroke or induced seizures (Eng & Lee, 1992), in addition to forming scars around the injury (Anderson et al., 2016). This activation of astrocytes causes secretion of certain neurotrophic factors leading to an inflammatory response and in turn, neuronal death (Brahmachari, Fung, & Pahan,
2006). In these conditions, astrocytes show enhanced expression of glial fibrillary acidic protein (GFAP) (Eng & Ghirnikar, 1994). Reactive astrocytosis and the role of GFAP has been shown to be involved in insults to the CNS (Brahmachari et al., 2006), but has yet to be examined in using the flurothyl ELS model.

*Previous work on cognition and development in the flurothyl model*

The flurothyl model in rats has shown behavioral deficits, including decreased behavioral flexibility, impaired spatial cognition and sociability, and changes in prefrontal cortex (PFC) structure and function (Holmes, 2016; Holmes et al., 2015; Kleen et al., 2010). The flurothyl ELS model has not previously been studied in mice, only rat. Mice present the advantage of having more genetic manipulation as there are a wide variety of genetic tools able to be used. For example, tools are available to assess the effects of gene knock outs or find potential genes of interest when comparing strains of mice. Our group has adapted the repeated flurothyl model, to assess whether the cognitive deficits in rats are also found in mice.

Preliminary data demonstrate cognitive deficits due to early life seizures. Therefore, we wanted to assess whether astrocytosis occurs after ELS. This involves using immunohistochemistry (IHC) to look at certain proteins predicted to be altered by the repeated seizures, specifically GFAP for reactive astrocytosis (Liddelow & Barres, 2017).

In order to consistently analyze IHC images, particularly for astrocytes, we took a computational approach to score GFAP images. Due to the star shape of astrocytes, most automatic cell counters are unable to differentiate individual astrocyte cells. Thus, researchers resort to methods such as hand counting, which can be unreliable and highly variable, or average pixel intensity, which is subject to variability through background fluorescent and is not as specific to the astrocytes. Automatic counters are also highly variable in their goals, with some
focusing on the size of the reactive astrocytosis or glial scar and others focusing on staining intensity (Ostergaard & Jensen, 2013). As we expect ELS to dramatically increase GFAP expression in astrocytes, we developed software to automatically quantify the fluorescence intensity in an image as a proxy for the number and size of astrocytes.

In the current study we will be looking at the effect of repeated flurothyl seizures on behavior and analyzing reactive astrocytosis. We hypothesize that the ELS animals will show deficits across the behavior battery and will show an increase in GFAP staining in PFC and hippocampus sections compared to controls.

**Materials and Methods**

**Subjects**

C57BL/6J mice were received from Jackson Laboratories and were bred in house to maintain an experimental colony. Mice were housed in standard caging in the University of Vermont's animal facility, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice followed a 12-hour light/dark cycle and had *ad libitum* access to food and water. The Institutional Animal Care and Use Committee at UVM approved all animal procedures, which also complied with the National Institutes of Health guidelines for care and usage of laboratory animals. Pregnant dams were used from the experimental colony, pups were weaned at postnatal day 21.

**Flurothyl Protocol**

Mice were designated into two groups: ELS or control. Mice in the ELS experiment started seizures on postnatal day 10. Mouse pups received 4 seizures a day for a period of 5 days, resulting in a total of 20 seizures. During the seizures, both experimental and control animals were removed from the mother. Seizures were administered in a clear, airtight plastic container,
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with eight compartments, inside a fume hood. Flurothyl was injected through plastic tubing, inserted through the lid of the container to drip onto an absorbent patch of filter paper taped under the opening of in the lid. Several pups were each placed into separate compartments of the plastic container and 0.06 mL of flurothyl was injected into the chamber. When half of the pups in the chamber exhibited signs of a tonic seizure, characterized by full body rigidity, the lid of the chamber was removed to allow for venting. Once the pups exhibit normal motions and vocalizations, they were all placed back with the mother. The interval between seizures were at least one hour. All pups were handled with nitrile gloves. All pups were weighed daily during the seizure induction period. After weaning at postnatal day 21, all dams were euthanized and did not take part in the experiment. Mice were allowed to grow into adulthood for behavioral assessment or euthanized for IHC.

Behavior Protocol

Animals were tested for behavior during adulthood (postnatal day 60).

Fear Acquisition and Extinction

For fear experiments, an operant box was used which was in a dimly lit and sound reducing chamber. The box had metal bars for the floor to deliver the electric shock. On day 1 for fear acquisition, the mouse was placed in the box and was subject to four 30 second tones with one minute in between. Each tone was followed directly back 2 seconds of a 0.4 mA shock. On the second day, for fear extinction, each animal was placed into the same box and were subject to 30 of the same 30 second tones every minute. These tones were not followed by a shock. Each session was recorded with a webcam and the Microsoft LifeCam program. Between each mouse, the box was cleaned with ethanol. Fear acquisition was scored using AnyMaze software for total time freezing during each time block. Fear extinction was analyzed by hand. A
movement score was assigned at 2, 10, 20 and 30 second time points within each tone and then averaged together to create the score for each tone. The score for each time point was either 0 for freezing or 1 for moving.

_Elevated Plus Maze_

The elevated plus maze is a plus-shaped elevated chamber with two open arms and two closed arms. Animals were placed in the center of the plus and allowed to move freely for 5-minute sessions, which were recorded using a webcam and the Microsoft LifeCam program. The chamber was cleaned with ethanol between each session. Videos were later hand scored, for percent time in the center, open and closed arms.

_Open Field_

The open field chamber was a bare cylindrical chamber, 0.476 m in diameter. The animal was placed in the center of the chamber and allowed to move freely for 10 minutes. Each session was recorded using a webcam and the Microsoft LifeCam program. The chamber was cleaned with ethanol between each session. The videos were then later scored using Any-Maze software and the percentage of time spent in the center and perimeter zones was recorded.

_Social Interaction and Social Novelty_

The sociability chamber was 0.224 m wide and was divided into three sub chambers, each filled with fresh bedding. Each of the dividing walls had a removable door. On the first day, social interaction was assessed. A novel object in a cage was placed in the left chamber and a novel strain- and sex-matched mouse was placed in a similar cage in the right chamber. The novel mouse was not known to any experimental or control mouse. The experimental mouse was placed in the central chamber for a 5-minute acclimation period. After 5 minutes, the doors to the left and right chambers were quietly and simultaneously removed. The mouse was then allowed
to freely move between all 3 chambers for 10 minutes. Each session was recorded with a webcam and the Microsoft LifeCam program. Between each mouse the chambers were spot cleaned as necessary. Videos of the 10-minute experimental session were then scored by hand, where the time in each of the chambers was recoded.

Statistical Analysis

Fear acquisition was modeled using a gamma log generalized estimating equation (GEE) in SPSS. For fear extinction a binary GEE model was used in SPSS. For open field, elevated plus maze, and social novelty and interaction, a Two-Way ANOVA test was performed to assess differences between groups. All figures were created using GraphPad Prism. A standard significance value of p<0.05 was used.

GFAP Protocol

Sample Collection

Two days following the completion of the ELS protocol, animals were anesthetized with 5% Isoflurane and anesthesia was confirmed by a firm toe pinch. The chest cavity was opened to expose the heart and animals were transcardially perfused with 0.9% phosphate buffered saline until the blood was removed, followed by 4% paraformaldehyde. Brains were harvested and placed into 4% paraformaldehyde overnight to post-fix. Brains were cryo-protected for 24 hours, or until the they sank, in 30% sucrose. Once cryo-protected, brains were frozen in OCT prior to cryosectioning.

GFAP Staining

Once frozen, the tissue was sliced using a cryostat. Sections, 40 µm thick, were taken from the prefrontal cortex and hippocampus, and put into PBS in a well plate. They were stored until they were stained.
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First sections were washed in PBS twice for 15 minutes. A blocking buffer solution was made using the following ratios: 89.5% 1x PBS, 0.5% Triton 100 and 10% Normal Goat Serum. After the washes, the sections were put in 1mL of blocking buffer per well, on the rocker for 2 to 4 hours. The sections were then incubated in 500 µL of a 1:1000 dilution of Invitrogen Rat anti-GFAP antibody and NeuN in PBS per well and plate was left in the 4 °C refrigerator for 18 hours (overnight). Each set of staining had a negative control. A cortex section was designated as the negative control, and these sections did not receive any primary antibody. The section was left in PBS overnight. The primary antibody was then be removed, and the sections were washed with PBS 4 times for 15 minutes each. The secondary antibody solution was a 1:1000 dilution in PBS of both goat anti-rat (Alexa Fluor 488) secondary antibody and goat anti-rabbit NeuNAb (Alexa Fluor 594) secondary antibody. The samples, including the negative control, were then incubated in the 500 µL per well of secondary antibody for 4 hours at room temperature on the rocker, protected from light. Samples were light sensitive from then on and were protected from direct light. The samples were then washed with PBS for 30 minutes twice. The sections were then mounted on slides and cover slipped with one drop of DAPI mounting medium. The coverslip was sealed with nail polish and slides were stored in the 4°C cold room until viewed. Sections were viewed and imaged using the Nikon C2 confocal microscope in the University of Vermont Microscopy Imaging Center.

Image Analysis

Images were read into MATLAB as a digital RGB image. From there a mask was created around the areas that were not to be included in analysis. For hippocampal section the cortex above CA1 and any ventricles were included in the mask. In PFC sections, the mask included the longitudinal fissure and first cell layer. The “roipoly” tool in MATLAB was used to create the
mask, and masks were made by hand for each image. The masked images were then separated into red, green and blue channels. The mean, median, $80^{th}$, $90^{th}$ and $95^{th}$ percentile of the GFAP pixel intensity was recorded for each image.

**Statistical Analysis**

A One-Way ANOVA was used to compare the pixel intensity of images from ELS, control and negative control groups for both PFC and hippocampus sections. A standard significance value of $p < 0.05$ was used.

**Results**

**Early Life Seizures Affect Behavior**

**Learning and Memory in ELS Animals**

Fear acquisition and extinction was assessed using a two-day protocol. Fear acquisition, where an association between the tone and shock was formed, was performed on day one and fear extinction was performed on day two, where the association between the shock and tone was dissociated. During this acquisition, ELS animals were frozen for a greater amount of time than control animals at all time points ($p < 0.001$). Both groups demonstrated a fear learning curve, with the ELS animals showing more freezing behavior (Figure 1A). During fear extinction ELS animals consistently showed a higher proportion of time freezing at each tone, compared to control animals (Figure 1B). The ELS animals extinguished the fear significantly slower than control animals ($p < 0.001$).
Figure 1: ELS animals show more freezing behavior in both fear acquisition and extinction tasks. A) Fear acquisition was analyzed using time freezing during each tone as well as at baseline (tone 0). ELS animals spent more time freezing at each tone and this was a significant interaction (p < 0.001). B) Fear extinction was hand scored, with 1 = movement and 0 = freezing. An average of timepoint scores was taken for each tone, representing the proportion of time spent moving. This was adjusted to show time freezing. ELS animals exhibited more freezing behavior than controls at all tones. This was a significant interaction (p < 0.001). Controls (N=14), ELS (N=18), Error bars are ±SEM.
Anxiety in ELS Animals Assessed with Elevated Plus Maze

During the elevated plus maze, mice were able to freely explore two open and two closed arms. Mice in the ELS group spent significantly less time in the open arm than controls ($p = 0.002$). In addition, ELS animals were in the closed arm for significantly more time than control animals ($p = 0.047$).

*Figure 2: Elevated Plus Maze time analysis for ELS and control animals.* ELS animals spent less time in the open arm ($p = 0.002$) and more time in the closed arm ($p = 0.047$) than control animals. Controls (N=19), ELS (N=26). Error bars are ±SEM.

Explorative Behavior assessed with Open Field

Mice were freely able to explore an open arena to assess explorative behavior. Both ELS and control animals spent nearly the same percentage of time in both the outer and central zones of the arena, there was no significant difference.
Figure 3: Open Field time analysis for ELS and control animals. Graph shows average percentage of time spent in the central and outer areas of the open field arena for ELS and control animals. No significant differences were found between groups. Controls (N=19), ELS (N=27), Error bars are ±SEM.

Social Behaviors unaffected by ELS

Social behaviors were assessed during a two-day protocol. On day one, mice were exposed to an object and a novel mouse and able to explore freely. On day two, mice were exposed to the mouse from day one and a new mouse and were allowed to explore freely. There were no significant differences in the percentage of time that ELS and control mice spent with the novel mouse or object on day one or the familiar mouse or novel mouse on day two.
Figure 4: Social interaction and novelty for ELS and control animals. Graphs indicate the mean percent time spent with each item for both ELS and control groups. No significant differences were found for both A) social interaction and B) social novelty. Controls (N=19), ELS (N=27), Error bars are ±SEM.
Effect of Early Life Seizures on Astrocytes

Astrocytes in PFC unaffected by ELS

Coronal prefrontal cortex sections were taken from mice one day after the final seizure of the repeated flurothyl protocol. Images were taken at 10x and stained for nuclei using DAPI (Figure 5A) and astrocytes using GFAP (Figure 5B). The sections were then analyzed in MATLAB to identify the region of interest and threshold using the 90th percentile of the GFAP channel in the image. We tried multiple reasonable thresholds and achieved the same results, indicating a robust analysis (see Appendix). The ELS group had a significantly higher PFC pixel intensity compared to negative control sections ($p = 0.0138$), but no significant difference was found between ELS and controls.

Early Life Seizures does not affect Astrocytes in Hippocampus

Coronal hippocampal sections were taken from mice one day after the final seizure of the repeated flurothyl protocol. Images were taken at 10x and stained for nuclei using DAPI (Figure 6A) and astrocytes using GFAP (Figure 6B). Using MATLAB, a region of interest and mask of the GFAP channel was created by thresholding the image at the 90th percentile of pixel intensity. There was no significant difference between ELS and control threshold values (Figure 6E). Negative controls were also not significantly different from ELS or controls.
Figure 5: Images from the prefrontal cortex of an ELS mouse, and comparison of pixel intensity across groups. After going through the repeated flurothyl protocol, mice were sacrificed one day following the final seizure. Coronal brain sections were stained and then imaged at 10x using A) DAPI (blue) and B) GFAP (green), as shown in C) a composite of both channels. Scale bar equals 200 µm. D) Images were analyzed in MATLAB to create a region of interest and a mask of the astrocytes. The 90th percentile of the pixel intensity of the green channels was used as the threshold. E) The average of the 90th percentile pixel intensity for each group is graphed. The ELS group was significantly different from the negative control (p = 0.0138). Control (N=4), ELS (N=4), Negative control (N=4) Error bars are ±SEM.
Figure 6: Images from the hippocampus of an ELS mouse, and comparison of pixel intensity across groups. After going through the repeated flurothyl protocol, mice were sacrificed one day following the final seizure. Coronal brain sections were stained and then imaged at 10x using A) DAPI (blue) and B) GFAP (green), as shown in C) a composite of both channels. Scale bar equals 200 µm. D) Images were analyzed in MATLAB to create a region of interest and a mask of the astrocytes. The 90th percentile of the pixel intensity of the green channels was used as the threshold. E) The average of the 90th percentile pixel intensity for each group is graphed. Groups did not differ significantly. Control (N=4), ELS (N=4), Negative control (N=4) Error bars are ±SEM.
**Discussion**

*Behavior Deficits after Early Life Seizures*

Overall the results support the initial hypothesis stating that ELS animals will show deficits in some behaviors. ELS animals demonstrated more freezing behavior in both the fear acquisition and extinction tasks. During fear acquisition, during each tone the ELS mice were frozen for more time on average, therefore learned the association between the shock and tone quicker than controls (Figure 1A). This shows that they were more fearful of the tone, but since both ELS and control animals associated the tone with the shock successfully, ELS does not affect learning behaviors. The ELS animals did acquire significantly quicker than controls, which suggests they are more fearful and anxious, which aligns with the findings of fear extinction (Figure 1B) and elevated plus maze (Figure 2). ELS animals also showed a slower fear extinction than the control animals (Figure 1B). This indicates that ELS animals are more fearful than the control animals, as on average they exhibited more freezing behavior throughout all 30 tones. Since there is greater freezing behavior, a learning deficit is also shown, as the association between the shock and tone did not dissociate as quickly as control animals. ELS animals showed an increase in anxiety-like behavior compared to control animals during the elevated plus maze (Figure 2). By spending more time in the closed arm and less time in the open arm, the ELS animals are showing anxiety-like behaviors and were less likely to explore or spend time in the open arm. The open field test did not present a significant difference between ELS and control mice (Figure 3). Open field has been used to assess anxiety-like behaviors but the factors we measured (i.e. time spent in central vs. outer zones) are a better test of exploration and locomotion in mice (Carola, D’Olimpio, Brunamonti, Mangia, & Renzi, 2002). This could
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indicate that exploratory behavior is not affected by ELS, but that anxiety is, as shown in the elevated plus maze.

Here it is interesting that ELS mice were quicker to learn but slower to extinguish the fear of the shock. Since the mice were more anxious and the learning and memory task was learning a fearful association, we believe there is some overlap in behavior outcomes here. With the increased anxiety, the fearful stimulus could be amplified resulting in faster learning and slower extinction. The control mice did not have the increased anxiety, meaning it took longer for them to learn the fearful stimuli, but also allowed them to disassociate the shock and tone association quicker. Anxiety and fear have both been linked to the amygdala, which could explain the connection between the behaviors seen and the ability for ELS mice to learn quicker in fear acquisition but not in fear extinction.

Interestingly, sociability appears to be unaffected by ELS, as there were no significant differences between ELS and control animals for both social interaction (Figure 4A) and social novelty (Figure 4B). This does not align with the comorbidity of ASD and epilepsy seen in human patients. The findings here could suggest that social deficits or autism-related behaviors seen are not directly caused by seizures but instead other biological factors. This is consistent with research on childhood epilepsy, where in most cases of epilepsy and autism there is an underlying factor such as genetics, that causes the comorbidity instead of the seizures (Besag, 2018; Reilly et al., 2014). From this, we can infer that ELS does not impact social behaviors, and seizures may not cause ASD related behaviors.

Due to these differences across multiple different behaviors, the current data shows ELS in mice recapitulate some of the behavioral deficits observed in ELS rats and human patients. These behaviors, specifically those seen in the elevated plus maze and fear acquisition and
extinction, align with what is seen in children with epilepsy, where increased anxiety and learning deficits have been shown. However, ELS in mice only recapitulate some of the cognitive features seen in humans, most notably sociability was not affected, which is used to assess ASD behaviors. This shows that ELS is not a complete model for childhood epilepsy but does demonstrate some of the deficits seen in children.

*Differences in Astrocytosis after Early Life Seizures*

Though the data did not show significant differences between ELS and control images, the predicted trend was shown. In both PFC and hippocampus sections, the ELS group showed the highest average 90th percentile pixel intensity (Figure 5E and Figure 6E). This indicates that ELS animals tend to show a greater intensity of GFAP staining than controls. We did expect to see GFAP+ astrocytes in controls, as they are found in normal physiological conditions (Liddelow & Barres, 2017), however we expected to see significantly more in ELS animals due to the seizures. The higher pixel intensity could indicate higher levels of astrocytosis in the ELS animals; however, this was not a significant result potentially due to low sample size. With this analysis there were lower numbers per group than anticipated, leading to high variability within groups (N=4 controls, N=4 ELS, N=4 negative control). There was also variability between batches, due to changes in antibodies. The slight increase of GFAP staining seen would be consistent with the behavioral deficits seen in these mice, as the PFC and hippocampus are particularly important for the behaviors tested.

Many obstacles arose during the course of analyzing the GFAP images. The original intention was to find a way to count the individual astrocytes in each image; therefore showing if there was an increase in the number of GFAP+ astrocytes after ELS. However due to the complex shape of the astrocytes, we ran into challenges differentiating overlapping astrocytes.
We first tried to identify astrocytes by creating a mask using the DAPI channel. Our intention here was to limit the region of interest to where the nuclei in the image were, where we could then assess if the nuclei was associated with a GFAP+ astrocyte. This was successful for some batches, but not all batches were consistent in the quality of the DAPI stain. This caused GFAP+ astrocytes to be missed in analysis due to poor differentiation in the DAPI staining. After this, we tried to create a mask of the GFAP channel and calculate percent area covered by the mask. This was to try and get a similar score to counting individual astrocytes without trying to differentiate the overlapping astrocytes. However, this did not work as there was not a consistent thresholding mechanism to be able to threshold all images the same without having background influence the score. Due to this, we settled on a comparison of the 90th percentile pixel intensity for each image. We used “roipoly” to create a region of interest which eliminated the influence of ventricles and high fluorescence noise. In this region of interest, we used the 90th percentile of the pixel intensity to create the mask. We compared multiple percentiles for the mask and chose the 90th percentile as this showed the highest specificity of astrocytes without losing the shape of the astrocytes, when assessing a variety of different percentile thresholds (see Appendix). This allowed us to control for the possible confounding variables such as high background fluorescence, while still getting a consistent scoring method. This method also allows for images to be score efficiently in the future as batches can be run through the code, instead of relying on hand counting.

In addition to the obstacles faced in scoring and analyzing the images, we were not able to collect as much data as we would have liked for the image analysis due to the ongoing COVID-19 pandemic. This put a halt to our data collection, but it was our intention to have more data in the image analysis.
Future Directions

As mentioned previously, the number of subjects used for the image analysis are low. Through an increase in subjects, the variability would decrease to definitively show whether the trend toward higher GFAP staining in ELS is a real effect. In order for this analysis technique to be used in future studies, the effectiveness needs to be assessed. This could be completed through validation testing, increasing the number of subjects and images as well as comparing to hand counting or other quantification methods to see if the effects seen are the same. In addition, we believe the code itself could evolve and be improved upon. Ideally, a universal threshold could be established in order to create the mask, and some sort of area covered score could be found. The obstacle here is finding a threshold that is independent of a percentile pixel intensity of the individual image. Percentiles provided rigorous measure of absolute staining intensity, but thresholding at a percentile cutoff produces the same percent area covered for each image, independent of staining intensity. Ideally, there would be a specific numeric threshold used across all images from which the percent area covered of the mask could be analyzed. To do this, more data needs to be gathered to assess what this pixel threshold would be, so it is not impacted by variables such as background fluorescence. With the current data set we were unable to do this and therefore depended on using a percentile for the threshold, which did not allow us to look at percent area covered.

Once the image analysis technique is established and shown to be consistent and effective through increasing the number of subjects and comparing to other quantification techniques, the possibilities for its use are numerous. In line with the aims of the Epilepsy, Cognition and Development (ECD) group, the tool could be used to compare strains. Preliminary data from the ECD group as well as other research (Kadiyala et al., 2015), indicates a strain difference in the
effects of ELS between C57BL/6J and DBA/2J mice indicating a genetic influence. The strain effect could be analyzed using the image analysis technique to see if there is an underlying biological reason for the phenotypic difference in mortality, seizure susceptibility and behavior. In addition to this, the effect of treatments on astrocytosis could also be studied. For example, ACTH has been shown to be an effective treatment in preventing the deficits seen in fear extinction (Massey, Lerner, Holmes, Scott, & Hernan, 2016) and is the current treatment for infantile spasms (Mytinger, Weber, & Heyer, 2015). The role of ACTH on melanocortin receptors in glial cells, specifically astrocytes, and its anti-inflammatory response in the CNS could be assessed using this analysis method. Work has also been done on different developmental time points, particularly in DBA/2J mice as they tend not to survive seizures at postnatal day 11. A study could be done on astrocytosis in both mice strains at a later, juvenile time point, to see if there would be similar levels of severity of astrocytosis or cell death compared to the younger time point or the other strain.

The current and future studies all work to increase the knowledge surrounding childhood epilepsy and the cognitive deficits associated with the disease. By finding the underlying cause of the cognitive deficits seen post seizure, we can work to find a treatment that not only treats the seizure symptoms but also prevents the damage that leads to these deficits. The novel work here aims to provide a stepping point for further research on underlying genetic factors which could then be translated into human studies. Through this research, we hope to be able to improve the quality of life for those who suffer from childhood epilepsy.
Appendix

When determining the mask threshold for the image analysis, we assessed multiple percentile cutoffs. It was important to maintain the integrity of the astrocyte shape in the mask while still eliminating as much background fluorescence as possible. Based on this, we decided to use the 90th percentile (Figure 7B). To ensure the results would be maintained for each percentile, we found the mean, 80th, 90th and 95th percentile pixel intensity for each image and compared between control and ELS. Each level showed a similar comparison between ELS and controls, indicating that the 90th percentile is a representative threshold.

Figure 7: Comparison of different threshold values used when determining the mask threshold for analysis. To determine the appropriate threshold, the A) 80th, B) 90th, and C) 95th percentile masks were for D) a control hippocampal section. E) The average threshold at each level for each group is shown. These threshold values were compared in all hippocampal sections, and no significant differences were found within each percentile level. Control (N=4), ELS (N=4), Negative control (N=4) Error bars are ±SEM.
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