Early Life Seizures Differentially Activate c-Fos in Hippocampal CA1 Cell Populations

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Early life seizures (ELS) are quite different from those in adults and can be associated with long-lasting deficits in cognitive and behavioral function. A majority of the epileptic syndromes that occur in childhood indicate that the developing brain has a great capability to generate seizures. The mechanisms of ELS include multiple molecular and cellular processes in the activity-dependent subpopulation of neurons with the expression of immediate-early genes (IEGs, such as *c-Fos*). This present study used a transgenic mouse model, in which CreER and following tdTomato expression was driven by *Fos* promoter (FosTRAP1), to permanently label the ELS-associated cells in the CA1 region of the hippocampus. CreER is Cre recombinase to promote high-level expression. TdTomato is a red fluorescent protein to permanently label CreER recombination. We measured tdTomato expression in the hippocampus in brain sections from FosTRAP1 mice with and without seizures. The chemoconvulsant kainate (KA) induced seizures was associated with a statistically significant increase of *c-Fos* expression shown by permanent tdTomato fluorophore labeling. Video analysis determined that there was a statistically significant correlation between tonic-clonic seizure duration and *c-Fos* expression in FosTRAP1 mice. In immunohistochemistry (IHC) experiments, brain sections were stained with different neuronal markers (NeuN, Iba-2, GFAP, and GAD-67) to confirm cell identity. Image analysis revealed that the vast majority of stained cells were pyramidal neurons, based on colocalization of the NeuN labeled and tdTomato+ cells. IHC staining also determined there was minimal colocalization of tdTomato+ cells and neuronal markers in glial cells, astrocytes, and GABAergic inhibitory interneurons. Given the prevalence of intellectual disability and social deficits following seizures in early life, identification of cells activated by seizures will allow further studies to examine their structure and function after seizures, in order to identify new therapeutic targets for potential clinical use.

Abbreviations: E:I - excitation:inhibition; ELS - early life seizures; FACS - fluorescence-activated cell sorting; IEGs - immediate-early genes; IHC - immunohistochemistry; KA - kainate; LTP - long term potentiation; LLS - later life seizures

Keywords: early life seizures; FosTRAP; immunohistochemistry; hippocampus

Introduction

Early Life Seizures

Early life seizures (ELS) are those that occur in childhood in the rapidly developing brain. ELS are different from those in adults (Hensch, 2004; Lippman-Bell, Zhou et al., 2016; Rakhade and Jensen 2009). It was thought for decades that ELS could be considered less harmful in the brain long term compared to seizures that occur later in life, later life seizures (LLS). The young, immature brain; however, is hyperexcitable compared to the adult brain due to an imbalance in the excitation:inhibition (E:I) signal of neuronal circuits (Leung, 2019; Rakhade and Jensen, 2009; Talos et al., 2012;

Yennawar et al., 2019). This imbalance is considered necessary for the critical period of development correlated with enhanced synaptic plasticity and learning (Hensch, 2004).

Clinically, ELS has been associated with long-lasting deficits in cognitive and behavioral function (Leung, 2019). Many of the epileptic syndromes occurring in childhood indicate that the early brain has an enhanced capacity to generate seizures during a restricted phase of postnatal development (Burnsed et al., 2019; Rakhade and Jensen, 2009; Silverstein and Jensen, 2007). ELS is commonly associated with autism and later life epilepsy (Lippman-Bell, 2013; Talos et al., 2012). Autism and epilepsy evidently display a bidirectional relationship given that up to 40% of children with intellectual disability and autism also suffer from epilepsy (Tuchman, 2015). It is estimated that about 35% of children who suffer from seizures as an infant will develop an intellectual disability (Silverstein and Jensen, 2007; Talos et al., 2012; Tuchman, 2015). It is important to note that while there may be a genetic association between multiple autism-linked genes and epilepsy, the two disorders may also be the result of ELS or early brain injury. There are currently no forms of prevention for early life seizures in susceptible populations (Lippman-Bell et al., 2016; Lippman-Bell et al., 2013; Silverstein and Jensen, 2007; Talos et al., 2012; Yennawar et al., 2019).

Mechanisms of Early life seizures

ELS is found to be responsible for abnormal behavior and altered seizure threshold. The mechanisms of ELS include multiple molecular and cellular processes in the activity-dependent subpopulation of neurons with the expression of immediate-early genes (IEGs, such as *c-Fos*) (Morgan et al., 1987; Sheng and Greenberg, 1990). *C-Fos* expression correlates with tonic-clonic seizure severity, thus aberrant activity and synaptic dysfunctions of ELS-associated nerve cells are important substrates in determining the cognitive deficiency manifested as the brain matures (Hensch, 2004; Morgan et al., 1987; Sheng and Greenberg, 1990). Tonic clonic seizure severity is the extent of the duration and effect of the seizure. The critical period of development is most impacted by ELS.

Induction of ELS in the immature brain leads to the acquisition of abnormal synaptic plasticity of this subpopulation in the early postnatal stage, leading to further dysregulation in adulthood. Based on prior work (Lippman-Bell et al., 2013), this is plausible evidence for contributing molecular alterations that may be novel therapeutic targets specifically in ELS.

ELS mice exhibit synaptic and circuit-level hyperexcitability in various brain regions. Several brain regions, such as the hippocampus and cortex, would be expected to be enriched with TdTomato+ cells. TdTomato is a red fluorescent protein to permanently label CreERT2 recombination. Our research is targeted at the CA1 region in the hippocampus. Specificity within this region is important because prior research has identified that there is a high degree of responsivity in the hippocampus to seizure induction. In the hours to weeks following seizure induction, there are many neuronal changes in the hippocampal CA1 region (Zhou et al., 2011). The CA1 region displays a major role in cognition in the brain. CA1 plasticity is linked to cognition and an ideal location to target for treatments. To study the specific single cell types throughout development is vital to find a target for treatment. Prior research in the Jensen lab discovered seizure-induced persistent attenuation of LTP in the CA1 region of the hippocampus (Zhou et al., 2011). This prolonging of activity demonstrated a clear and novel target for the treatment of ELS in the CA1 region to protect against long-term cognitive deficits.

Seizures in Early vs. Later Life

Immature brains are more prone to seizures than adult brains (Rakhade and Jensen 2009). The immature brain is also more likely to be resistant to the damage induced by ELS due to an increased plasticity. The immature brain is relatively hyperexcitable compared to the adult due to an E:I imbalance in neuronal circuits (Rakhade and Jensen, 2009). A result of an increased E:I imbalance includes cognitive and behavioral deficits. Immature brains tend to be more hyperexcitable than adult brains due to the increased E:I imbalance in neuronal circuits. These neuronal circuits are thought to play a role in synaptic plasticity during the “critical period” of development. Enhanced synaptic plasticity and

learning through development requires this E:I imbalance. The "critical period" of development can be characterized by this brain that is more plastic (Haut et al., 2004).

There is increased awareness of the important differences between age-specific seizures as seizures have been reported to result in structural, neurochemical, and functional changes in the brain. Many changes that are the result of seizures are still unknown due to differences in the duration of seizures, cause of seizures, number of seizures, and genetic effect on seizures throughout development (Haut et al., 2004). The differences between how seizures at different stages of development produce permanent damages in the brain provide target areas for treatment. It is likely that sezires affect young and developing brains significantly different than in developed brains.

Prior Limitations

A limitation of prior work in the field of ELS is that there has been a lack of research tracking the changes in the brain throughout development after ELS (Talos et al., 2012; Yennawar et al., 2019). It is not well known how a single neuron evolves throughout development and into later life. To be able to track cells activated neurons at the single neuron level we can examine how impairments in the brain are determined by specific subsets of cells at a network and *in vivo* level.

The goal for this research project was to determine which type of cell contributes to and causes ELS. An improved understanding of how seizure activity and hyperexcitability networks dysregulate synaptic plasticity at the cell specific level will be required for the development of novel treatment in the clinical space in which no current cure exists. We aim to determine if ELS have persistent, life-long alterations of gluatamate receptor dfunction. We hypothesized that the induction of ELS will significantly increase the amount *c-Fos* expression in the hippocampal CA1 region. We hypothesize this will occur due to an increased AMPAR function. It was also hypothesized that the majority of cells with *c-Fos* expression will specifically be pyramidal neurons instead of surrounding neuronal cells. The discussion of ELS and other epileptic diseases is currently an exciting and evolutionary topic in medical research. Most treatments for ELS are not completely effective and come without side effects. To investigate how ELS affects the brain from a young age and throughout development will be revolutionary in the advancements of understanding how seizures impact the brain (Hensch, 2004). With the use of mouse models, this research and prior research in the Jensen labs have proven that ELS can be associated with behavioral deficits, autistic-like deficits, impaired critical period synaptic plasticity, and spontaneous seizure activity in later life (Drage et al., 2002; Hensch, 2004). The FosTRAP1 mouse line detects Cre-induced permanent tdTomato expression. The FosTRAP1 mouse model helps determine how seizure activity and hyperexcitable networks dysregulate synaptic plasticity, which is required to develop new therapeutic methods for treating or curing ELS, which currently has no known cure.

Material and Methods

Animals

Procedures performed during this lab were conducted in accordance with the guidelines of the National Institute of Health Guide for the care and use of laboratory animals and with the approval of the Institution of Animals Care and Use Committee at the University of Pennsylvania.

FosTRAP1 mice are mice that are heterozygous for Fos^{CreER}. FosTRAP1 Mice are homozygous or heterozygous for FloxtdT. The FosTRAP1 mice were made using techniques from the procedure by Guenthner (Guenthner et al., 2013). It is useful to use FosTRAP1 mice for this project because after seizure induction these mice will experience neuronal alterations (Guenthner et al., 2013; Sheng and Greenberg, 1990). Alterations can be analyzed by the strong *c-Fos* staining (Morgan et al., 1987).

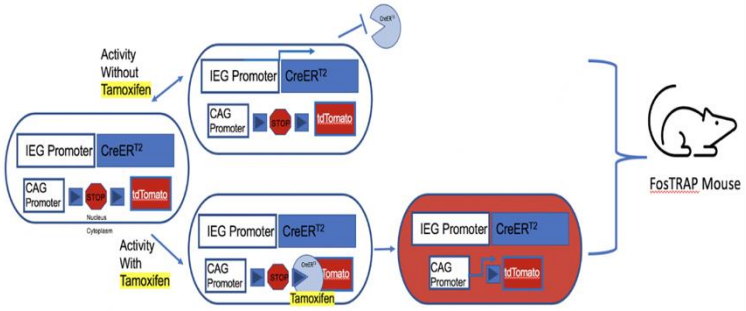

Figure 1: FosTRAP Mice are generated through two transgenes. The first transgene is from an IEG promoter that expresses CreERT2 and the second is a CAG promoter that allows tdTomato expression, or another effector gene. The expression of tdTomato only works in a Cre-dependent manner. Activity in the cell without tamoxifen/4OHT will result in CreER2 remaining in the cytoplasm thus recombination cannot occur. Activity in the cell with tamoxifen will result in the recombination of the cell, so tdTomato expression marking the cell red (Guenthner et al., 2013).

Early Life Seizure (ELS) Experiments:

Mice at age P10 were pre-treated with 4-OHT 1 hour before either a KA (KA 2mg/kg i.p.) or saline vehicle injection. The doses used in this experiment have been tested numerous times to ensure that it is the proper amount to account for a stage 5 seizure with low death rates. Both KA and saline-injected littermates are placed in the chamber for equivalent 90-120 minute periods providing ample time for the induction of tonic clonic seizures and reliable enough duration after injection.

Video Analysis:

After the injection of each mouse, seizure responses are recorded blind to the group to determine seizure severity. The blind investigation was conducted to score the mouse videos. Mice were video recorded for two hours post-injection to monitor the seizures, Seizure severity is ranked upon many factors to determine if the severity is correlated with the number of cells marked after staining. Factors such as the first sign of seizures, freeing, hindlimb clonus, loss of posture, rearing, tonic-clonic seizures, and death were recorded to determine seizure severity for Racine scale (Lüttjohann et al., 2009; Massey et al., 2018). Scores are based on a modified Racine scale, with scores 0 to 5 scale 0: normal behavior, 1: freezing or immobility for at least 5 seconds, 2: myoclonic jerks, 3: forelimb clonus, 4: rearing, forelimb clonus, 5: tonic-clonic, loss of posture.

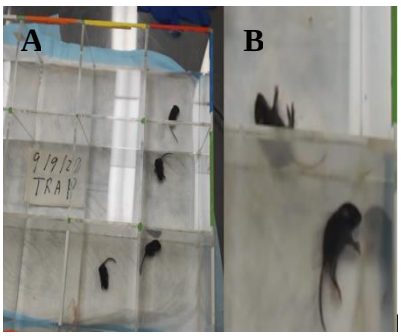

Figure 2: A) 2 hour long videos of FosTRAP1 mice were recorded after ablind saline or KA injection. Seizure severity was the blindly calculated based on Racine scale with a core 0-5 (Lüttjohann, 2009). B) Example of loss of posture and tonic-clonic seizure in KA treated mice.

Immunohistochemistry (IHC)

FosTRAP1 mice were perfused transcardially with 4% PFA at P30. Brains were dissected from the skull and postfixed for 1 hour at room temperature before being transferred to 30% sucrose. Once saturated with sucrose, the brains were embedded in OCT and frozen at -70 degrees Celsius until ready to be sliced. A cryostat procedure was used to slide the slides into 40 μm slices to prepare them for mounting on slides. To stain the cells, we performed on-slide cell staining efficient for IHC. Coronal slices of the brain were selected from the CA1 region (bregma -1.46 to -2.70). After slices were properly plated an antigen retrieval step occurred before IHC staining. The antigen retrieval results in the breakdown of methylene bridges in the

brain and exposes antigenic sites. This alteration results in enhanced binding of antibodies. The antigen retrieval method used was heat-induced epitope retrieval. Where a water bath was set to 90 degrees Celsius and the slices were boiling in the antigen retrieval solution for 15 minutes. Sections were then permeabilized with 0.2% Triton X-100 in PBS for 5 min, then blocked with 10% NGS with 0.1% Triton in PBS for 30 min, and then blocked with 5% AffinPure Fab Fragment – Goat anti Mouse IgG. The use of a blocking buffer decreases the amount of background signal collected under the microscopes. FAB fragments are important to decrease the amount of background staining when primary antibodies are derived from the same species (in this case mouse on mouse). FAB fragments mechanism is to block endogenous Ig epitopes, so secondary antibodies are not able to label. Whole molecules have two binding sites, so they are not able to block endogenous Ig epitopes. The FosTRAP1 model functions to permanently label cells activated by ELS with tdTomato fluorophore, so the cells can be stained with different neuron markers. NeuN, GFAP, GAD67, and Iba-2 are used at concentrations 1:500 to confirm cell identity (Engel et al., 2013; Notenboom et al., 2021). Specifically, staining and comparing colocalized tdTomato+ cells with other cell markers in the CA1 region (bregma -1.46 to -2.70) of P10 KA in FosTRAP1 mice will detect whether ELS induce active subpopulations of cells (Guenthner et al., 2013; Zhou et al., 2011). Fluoromount with DAPI was used to mount the sections with a coverslip. Immunostaining for NeuN identifies pyramidal neurons in the hippocampus. IHC for cell specific markers was conducted with a double labeling methodology for cell counting. Immunostaining for GFAP identifies astrocytes in the hippocampus. Immunostaining for GAD67 identifies GABAergic interneurons in the hippocampus (Engel et al., 2013; Notenboom et al., 2021; Wang et al., 2014). Immunostaining for Iba-2 identifies microglia in the hippocampus.

ImageJ Image Analysis

Analysis of *z* stacks was performed blindly using ImageJ software. Confocal z-projections of TRAPed cells and different neuronal markers were merged to produce overlay images. After extensive images were examined using logistic and linear regressions, GraphPad software was used to produce the figures and an excel spreadsheet was used to record data points.

Results

KA Seizure Increases c-Fos Activation in TRAPed Cells in CA1 Hippocampal Region

This study establishes that KA-induced seizure activity in vivo results in a dramatic and rapid alteration of *c-Fos* expression. We hypothesized based on prior work in the lab the fosTRAP1 mice will display a significant induction of *c-Fos* activation by KA (Falcón-Moya et al., 2018; Guenthner et al., 2013). As seen in Figure 3, baseline levels of *c-Fos* activation in the saline-treated mouse were average of 5.44± 0.7 tdTomato+ cells per mm^2 across six samples with data from at least two averaged sections. *C-Fos* activation in the KA-treated mouse was average of 93.517± 8.9 tdTomato+ cells per mm^2 across six samples with data from at least two averaged sections. The difference between the saline tdTomato+ cells and KA tdTomato+ cells is statistically significant at $p<0.00012$. Data imply that the induction of *c-Fos* by KA is attributable to the seizure-causing activity of the drug (Falcón-Moya et al. 2018). An important consideration is that the *c-Fos* protein may play a part in the normal function of the neuron since tdTomato+ cells are present in untreated FosTRAP1 mouse brain (Barth, Gerkin, and Dean, 2004; Notenboom et al., 2021).

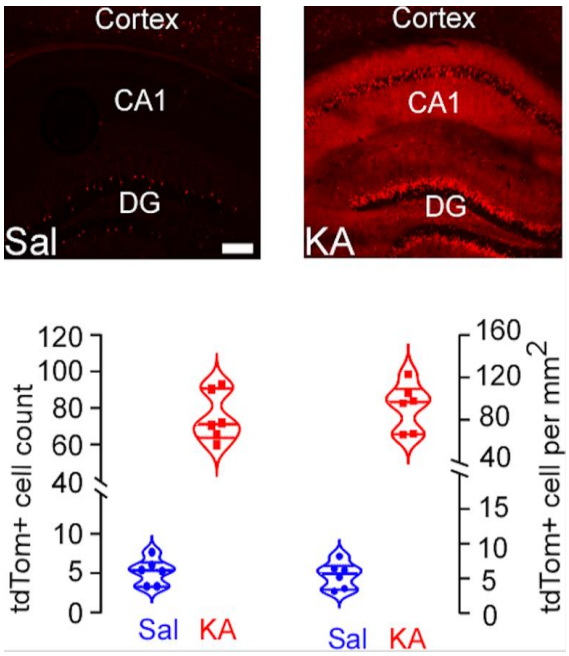

Figure 3: Robust hippocampal staining focusing primarily on the CA1 subfield shows a significant subset of tdTomato positive (tdTomato+) activated neurons (**Figure 3. right top**), which is not seen in the handling saline control, tdTomato negative (tdTomato-) (**Figure 3. left top)**.

Tonic-clonic Seizure Duration and c-Fos Expression are Positively Correlated

We investigated if there was a positive correlation between *c-Fos* expression and seizure severity. Our original hypothesis would have expected a statistically significant positive correlation between *c-Fos* expression and seizure severity. First, we had analyzed this data without using density measurements and did not come to a statistically significant conclusion about our data. However, we decided to apply density measurements to our correlation studies and came up with a statistically significant result.

Tonic-clonic seizure duration was positively correlated with the amount of *c-Fos* expression in fosTRAP1 mice when accounting for density measurements, as seen in Figure 4. There was a correlation coefficient R = 0.8263 between duration and tdTomato+ cell counts. This is a statistically significant result as P<0.05; P = 0.0325.

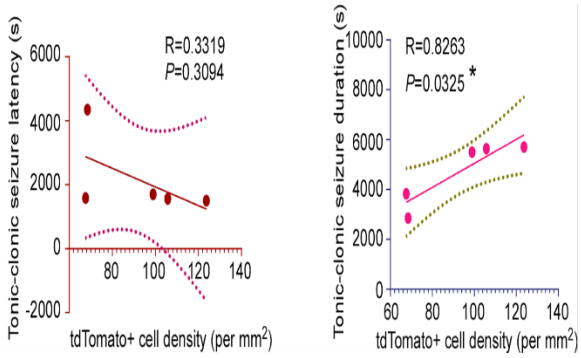

Figure 4: Correlation studies investigated the amount of *c-Fos* expression in relation to tonic clonic seizure latency (left) and tonic clonic seizure duration (right). There was a statistically significant correlation between tonic-clonic seizure duration and *c-Fos* expression. There was a positive correlation for tonic-clonic seizure duration and tdTomato+ cells. Each correlation was produced through the use of blind video analysis and blind cell counting. There were 5 mice that had tonic clonic seizure activity analyzed in each correlational study.

It was evident that tonic-clonic seizure latency was inversely related to tdTomato+ cell count when taking density into account. There was a correlation coefficient of R = 0.3319 between tonic-clonic seizure latency and tdTomato+ cells. There was no statistical significance for this correlation because P > 0.05; P = 0.3094.

This study required video analysis and Image J cell counting of tdTomato+ cells. To improve upon these results increasing N may be a sufficient method to determine if there is a significant correlation between the amount of *c-Fos* expression and seizure severity. Another factor that may have played a role in finding a significant correlation between *c-Fos* and seizure severity is that the Racine scale and video analysis do not have an intensely rigid rating scale. If there was a way to increase the similarity between how mice were scored this would benefit the study.

Pyramidal Neurons are Significantly Activated Within the CA1 Region

We next investigated which specific cell types were TRAPed during ELS. With the use of IHC and confocal imaging to quantify colocalization of tdTomato and neuronal markers in postmortem tissue. As shown in Figure 5, not surprisingly, we found that about 94.5% ± 1% of

activated cells were neurons, by colocalization with the marker NeuN. From these results, we reasoned that most TRAPed cells in the CA1 region are excitatory pyramidal neurons. They were CA1 neurons in the pyramidal layer with their apical dendrites extending into stratum radiatum and their basilar dendrites extending into stratum oriens.

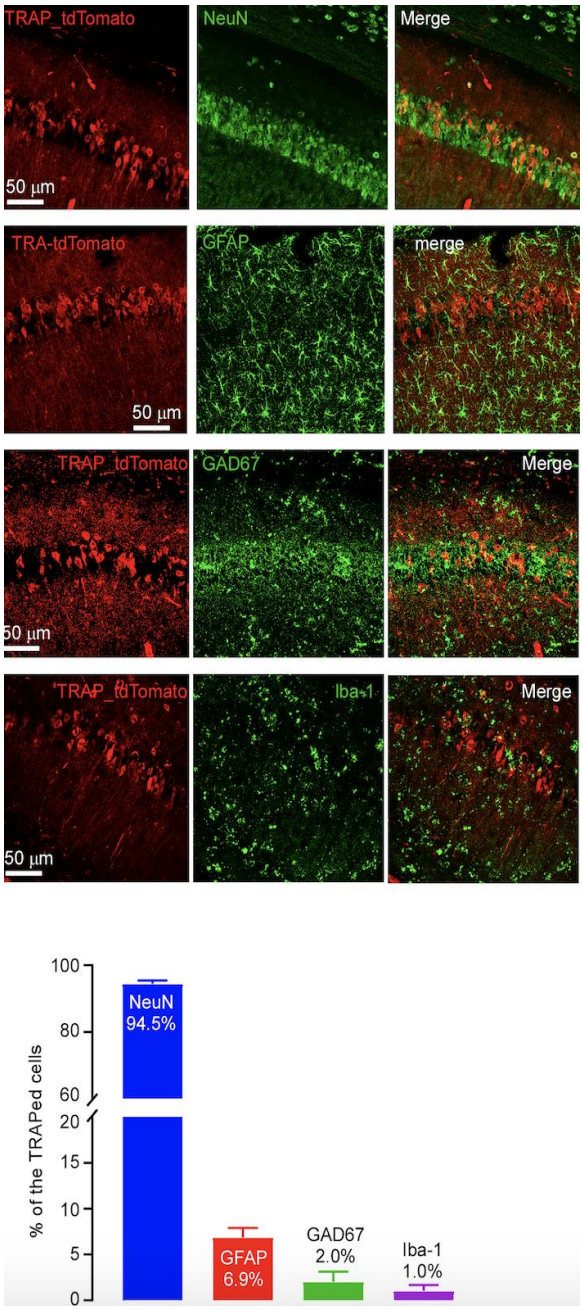

Figure 5: Confocal imaging of the hippocampal CA1 region was used to investigate the specific cell types expressing *c-Fos* after KA seizure induction. From NeuN (top), GFAP (second to top), GAD67 (second from bottom), and Iba-1 (bottom) markers were analzyed for colocalization with tdTomato+ expression.

Furthermore, we used GAD67 to label inhibitory interneurons and found only 2% of tdTomato+ cells overlapped with GAD67, indicating that seizures induced c-Fos primarily in excitatory pyramidal CA1 neurons. With the use of IHC and confocal imaging to quantify colocalization of tdTomato and neuronal markers in postmortem tissue. As shown in Figure 5, as expected, we found that less than 2% ± 1.1% of activated cells were inhibitory GABAergic neurons, by colocalization with the marker GAD67 (glutamic acid decarboxylase 67) (Engel et al., 2013; Notenboom et al., 2021; Wang et al., 2014). Fos^{CreER}-driven tdTomato expression seems to be mostly present in excitatory neurons following seizures, as we only observed very few tdTomato+ neurons co-labeling with the inhibitory GABAergic neuronal marker, GAD67.

TRAPed Cells Are Minimally Expressed as Astrocytes or Microglia Within the CA1 Region

The use of IHC and confocal imaging quantified colocalization of tdTomato and neuronal markers in postmortem tissue. As shown in Figure 5, we found that less than 6.9% ± 1.1% of activated cells were astrocytes, by colocalization with the marker GFAP (glial fibrillary acidic protein) (Engel et al., 2013; Notenboom et al., 2021). In the FosTRAP1 mice, seizure-induced alterations rarely colocalize the astrocytic marker GFAP (glial fibrillary acidic protein). GFAP cells are support cells mainly astrocytes, glial cells, and ependymal cells in the normal brain. It is evident that there is minimal colocalization of GFAP or Iba-1 with the tdTomato staining. KA injection does not activate GFAP expression in the astrocytes (GFAP) or microglia (Iba-1).

As Figure 5 shows, we found that less than 1.0% ± 0.6% of activated cells were microglial, by colocalization with the marker Iba1 (ionized calcium-binding adaptor molecule 1) (Engel et al., 2013; Notenboom et al., 2021).

Discussion

We hypothesized that the induction of ELS will significantly increase the amount *c-Fos* expression in the hippocampal CA1 region. Keeping with our original hypothesis, the induction of ELS in the TRAP mice model led to the acquisition of *c-Fos* expression (Guenthner et al., 2013). It was also hypothesized that the majority of cells with *c-Fos* expression will specifically be pyramidal neurons instead of surrounding neuronal cells. Also, keeping with our hypothesis we saw that the majority of cells activated were pyramidal neurons. Following KA injection, we saw significant differences in the neuronal expression of *c-Fos* compared to mice injected with the saline vehicle. *c-Fos* expression in the CA1 region was significantly higher for KA treat FosTRAP1 mice compared to the saline-treated FosTRAP1 mice. This increase in neuronal expression was due to a hyperexcitable response following KA injection. There was a high colocalization (94.5%) between tdTomato+ and NeuN stained cells, thus *c-Fos* expression is high in pyramidal neurons in the CA1 region of the hippocampus. There was less than 7% colocalization between tdTomato stained cells and GFAP stained cells, thus there is low expression of *c-Fos* in astrocytes in the CA1 region of the hippocampus. There is less than 2% colocalization between tdTomato stained cells and GAD67 stained cells, thus there is low expression of *c-Fos* in GABAergic interneurons in the CA1 region of the hippocampus (Engel et al., 2013; Notenboom et al., 2021; Wang et al., 2014; Zhou et al., 2011). Also, there is less than 1% colocalization between tdTomato and Iba1 thus there is low expression of *c-Fos* in Microglial in the CA1 region following KA induction. It will be important in the future to determine what percent of each cell type was labeled. We will need to determine the number of each cell type within the CA1 region and then quantify what percent of those cells have c-Fos expression. Concluding which cell type has the highest percentage in the CA1 region can provide targets for treatment if the cells are also activated. Also, to determine what percent within those populations of different cells is activated is important, because for example if the most dense cell in the CA1 region it may have a high total population of activated cells, but not a high individual percentage of active cells, thus it may not be the best target.

Neuronal vs Non-neuronal Response

There is more neuronal response to ELS, but LLS causes significantly more permanent cell death which induces non-neuronal responses (glia cell activation). For nuclear RNA analysis, we could use *fluorescence-activated cell sorting* (FACS) and RNA-seq to sort and compare activated from non-activated cells from the hippocampus after ELS. Consistent with prior results, we have the opportunity to delve into FACS sorting to examine activated cell gene expression and protein expression using IHC at both the cell-specific level. This novel FosTRAP1 mouse line enables the tracking of seizure evolution at single-cell/neuronal subpopulation level for *in vitro* electrophysiological investigation RNA transcriptomics by FACs sorting and LT-TISA and detecting subpopulation protein expression by confocal microscopy and IHC (Notenboom et al., 2021). The ability to label cells differentially allows for the use of FACS, which is novel in the setting of examining seizure-induced changes in specific transcriptomes in differentially activated subpopulations of neurons. If this proposal is successful, this technique could be expanded to many other investigations using the FosTRAP1 mice.

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